Murine Norovirus
Manipulation of the Cell Cycle

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Abstract

Human norovirus (HuNoV) causes the majority of acute, non-bacterial endemic gastroenteritis worldwide. Noroviruses belong to the Caliciviridae family and have a positive-sense, single single-stranded, RNA genome of around 7.5 kb. The historic inability to cultivate HuNoV in cell culture and animals has led to the use of closely related models to advance understanding of norovirus replication and pathogenicity. Murine norovirus (MNV) is a routinely researched model for HuNoV and allows for studies of viral replication within cell culture. The MNV genome consists of four open reading frames (ORF), with the non-structural proteins expressed from the polyprotein of ORF1 and a single protein from ORF4. The structural proteins are expressed from ORF2 and ORF3. In order to develop successful antiviral agents against norovirus, an increased knowledge of viral replication and viral-host interactions is needed. Previous microarray results from MNV-1-infected cells identified changes in the levels of transcripts of genes that regulate host cell division. This research aimed to characterise this interaction between MNV-1 and the host cell cycle.

Western blot analysis of MNV-1 infected murine macrophages (RAW-Blue cells) confirmed at a protein level, the down-regulation of cyclins expressed in late phases of the cell cycle. The decrease in cyclin A and cyclin B2 expression is consistent with the effects observed on cell division, with MNV-1 infection causing a decrease in progression through the G1/S checkpoint, leading to an accumulation of cells in the G0/G1 phase. Furthermore, the G1 phase arrest was revealed to be beneficial to viral replication, as cells progressing through the G1 phase supported a two-fold or more increase in viral progeny and VP1 expression over cells synchronised into an alternate cell cycle phase or an unsynchronised population. These findings suggest that MNV-1 infection manipulates the host cell cycle, arresting cell division in a phase favourable to viral replication. This manipulation of the host cell is proposed as a strategy used by MNV-1 to enhance viral replication.
The mechanism by which MNV-1 induces its cell cycle effects was explored. The induction of cell cycle effects was shown to be independent of interferon (IFN) type 1 production as cells non-responsive to IFN, still arrested at the G1/S restriction point during MNV-1 infection. Expression of individual viral proteins identified virus protein genome-linked (VPg or NS5) as the causative agent of the cell cycle arrest. Expression of NS5 from an in vitro transcript induced an arrest at the G1/S checkpoint and increased the G0/G1 population of cells in an analogous manner to MNV-1 infection. Expression of cyclin A was also inhibited, consistent with the observed decrease of this cyclin during MNV-1 infection. Expression of truncated NS5 linked the activity to the first 62 amino acids, indicating a novel mechanism of cell cycle manipulation, independent of the host elongation initiation factor (eIF) binding motif at the C-terminus of NS5. Furthermore, the first 10 amino acids at the N-terminus of NS5 were revealed to be essential in inducing the cell cycle arrest. Bioinformatic analysis of the N-terminus of NS5 suggests this region is involved in nucleotide interactions and proposes a possible mechanism of cell cycle control, due to interactions with host mRNA.

This research is the first documentation of a calicivirus interacting with the host cell cycle, in order to favour viral replication. Moreover, this is the first identification of a VPg protein manipulating the host cell cycle. Due to similarities in NS5 sequences especially at the N-terminal between norovirus genogroups, we propose that the cell cycle manipulation reported here may be conserved in other noroviruses.
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Table of Contents

1 Introduction ........................................................................................................... 1
  1.1 Gastroenteritis ................................................................................................. 1
  1.2 Caliciviruses ..................................................................................................... 1
    1.2.1 Classification ............................................................................................ 1
    1.2.2 Norovirus epidemiology and economic impact ........................................ 3
    1.2.3 Norovirus research models ........................................................................ 4
    1.2.4 Norovirus animal models ........................................................................... 6
  1.3 Murine norovirus (MNV) ................................................................................... 7
    1.3.1 Discovery of MNV and use as a model ..................................................... 7
    1.3.2 Genome .................................................................................................... 8
    1.3.3 Non-Structural proteins ........................................................................... 9
    1.3.4 Structural proteins .................................................................................... 18
  1.4 Norovirus replication ......................................................................................... 19
    1.4.1 Binding and entry ..................................................................................... 19
    1.4.2 Translation ................................................................................................ 20
    1.4.3 RNA replication ........................................................................................ 21
    1.4.4 Viral exit ................................................................................................... 22
    1.4.5 Manipulation of the host environment ..................................................... 22
  1.5 Mammalian cell cycle ....................................................................................... 24
    1.5.1 Regulation .................................................................................................. 24
    1.5.2 Cell cycle progression ............................................................................... 26
  1.6 Viruses and the cell cycle ................................................................................. 29
    1.6.1 DNA viruses and the cell cycle ................................................................ 30
    1.6.2 Retroviruses and the cell cycle ................................................................ 30
    1.6.3 RNA viruses and the cell cycle ................................................................ 31
  1.7 Research techniques for cell cycle analysis ...................................................... 33
    1.7.1 Cell cycle analysis ..................................................................................... 33
    1.7.2 Cell synchronisation ............................................................................... 34
  1.8 Research background ....................................................................................... 37
  1.9 Focus of my research ....................................................................................... 38

2 Methods ............................................................................................................... 40
  2.1 Ethical permits and approvals ................................................................. 40
  2.2 RAW-Blue cells ............................................................................................... 40
  2.3 Storage of RAW-Blue cells ............................................................................. 40
  2.4 Preparation of MNV-1 stock ........................................................................... 41
  2.5 Cell synchronisations ....................................................................................... 41
    2.5.1 G0 – Serum withdrawal .......................................................................... 42
    2.5.2 G1 – N-butyrate ...................................................................................... 42
    2.5.3 G2 – Genistein ......................................................................................... 42
    2.5.4 M - Nocodazole ....................................................................................... 42
    2.5.5 G3> - G1 progressing ............................................................................. 42
  2.6 Infections ......................................................................................................... 43
  2.7 Plaque assay .................................................................................................... 43
  2.8 Analysis of protein expression ......................................................................... 44
    2.8.1 Cell harvest for SDS-PAGE gel ............................................................... 44
    2.8.2 Pierce™ BCA protein assay kit ................................................................. 44
    2.8.3 Making SDS-PAGE gels ......................................................................... 44
2.8.4 Migration of SDS-PAGE gels .................................................................45
2.8.5 Western blotting ..............................................................................45
2.8.6 Nitrocellulose membrane stripping ..................................................46
2.9 Cell cycle analysis ...............................................................................47
  2.9.1 DNA content analysis – Flow Cytometry ............................................47
  2.9.2 MODfit L.T. 3.0 ................................................................................48
2.10 Clone design .......................................................................................48
  2.10.1 Outline of clone construction ..........................................................48
  2.10.2 Design of NS5 plasmids .................................................................49
2.11 Cloning and screening of NS5 constructs .............................................53
  2.11.1 Overview ........................................................................................53
  2.11.2 Reconstitution of synthetic plasmid containing inserts encoding NS5
        constructs .........................................................................................53
  2.11.3 Preparation of competent E. coli cells ..............................................53
  2.11.4 Transformations ............................................................................54
  2.11.5 Plasmid extraction (Miniprep) ..........................................................54
  2.11.6 Double restriction digests ...............................................................55
  2.11.7 DNA agarose gel electrophoresis ....................................................55
2.12 Generation of RNA transcripts ...........................................................55
  2.12.1 Overview ........................................................................................55
  2.12.2 Plasmid extraction (Midiprep) ..........................................................56
  2.12.3 Restriction Digests ..........................................................................56
  2.12.4 Purification from restriction digest ..................................................56
  2.12.5 RNA synthesis from linearised plasmids ...........................................57
2.13 RNA transfections ...............................................................................57
2.14 Validation of NS5 construct expression ...............................................57
2.15 Statistical analysis ...............................................................................58

3 Results .................................................................................................59
  3.1 Characterisation of MNV-1 effects on the host cell cycle .....................59
    3.1.1 Introduction ...................................................................................59
    3.1.2 Synchronisation ............................................................................59
    3.1.3 MNV-1 replication affects host cell cyclin expression .....................66
    3.1.4 MNV-1 infection induces a G0/G1 phase accumulation .....................68
    3.1.5 MNV-1 infection reduces G1/S phase progression .............................70
    3.1.6 MNV-1 infection does not influence late phase cell progression ...........74
    3.1.7 MNV-1 infection inhibits cyclin A expression ..................................76
  3.2 The affect of the host cell cycle on MNV-1 replication ..........................78
    3.2.1 Introduction ...................................................................................78
    3.2.2 Comparison of MNV-1 replication in asynchronous, G0 and M phase
        populations .......................................................................................78
    3.2.3 Cell phase progression of held and released populations ....................83
    3.2.4 MNV-1 protein expression in early cell cycle phases .........................86
    3.2.5 MNV-1 replication is favoured in a G1-> progressing population ...........90
  3.3 The mechanism behind MNV-1-induced cell cycle effects .....................92
    3.3.1 Introduction ...................................................................................92
    3.3.2 IFN-β induces a G0/G1 phase arrest in an asynchronous population .......92
    3.3.3 The IFN response is not the cause of the G0/G1 phase arrest in infected cells.. 94
    3.3.4 MNV-1 protein VF1 and the cell cycle ..............................................98
    3.3.5 Analysis of the effects of NS1-2 and NS5 on the host cell cycle ..........100
    3.3.6 Confirmation of NS5 induced G0/G1 phase arrest ................................103
  3.3.7 NS5 domain construction .................................................................108
List of Tables

Table 1.1. Dilutions of primary and secondary antibodies for Western blot analyses
                                                                                   47
List of Figures

Figure 1.1. Phylogenetic analysis of the *Caliciviridae* family based on nucleotide sequences of the VP1 gene. ................................................................. 2
Figure 1.2. Norovirus genome comparison. ..................................................... 9
Figure 1.3. 3D structure and schematic of MNV NS5 protein. ......................... 14
Figure 1.4. Schematic representation of mammalian cell cycle control, centring on G1/S progression. ................................................................. 28
Figure 1.5. Cell cycle analysis histogram......................................................... 34
Figure 1.6. Changes in the expression of cell cycle-related genes during MNV-1 infection. ......................................................................................... 38
Figure 2.1. Example of gating for FACS analysis of the cell cycle................... 48
Figure 2.2. Design overview for the three synthetic NS5 constructs................. 49
Figure 2.3. Annotated NS5 1-62 sequence insert............................................ 50
Figure 2.4. Annotated NS5 63-124 sequence insert........................................ 51
Figure 2.5. Annotated NS5 11-107 sequence insert........................................ 52
Figure 3.1. Synchronisation of cells into G0 phase......................................... 60
Figure 3.2. Synchronisation of cells into G1 phase......................................... 61
Figure 3.3. Synchronisation of cells into G2 phase......................................... 62
Figure 3.4. Synchronisation of cells into M phase........................................... 63
Figure 3.5. Synchronisation of cells into the G1> phase................................. 64
Figure 3.6. Toxicity of different cell treatments.............................................. 65
Figure 3.7. Effects of MNV infection on host cyclin expression....................... 67
Figure 3.8. MNV-1 infection induces the accumulation of cells in the G0/G1 phase of the cell cycle. ........................................................................ 69
Figure 3.9. MNV-1 infection causes a reduction in G0 to S phase progression..... 71
Figure 3.10. MNV-1 infection inhibits cell cycle progression from G1 into S phase.... 73
Figure 3.11. MNV-1 infection has no affect on late-phase cell cycle progression..... 75
Figure 3.12. Changes to G1 cyclin expression in MNV-1 infected cells released from quiescence. ........................................................................ 77
Figure 3.13. MNV-1 replication compared in different cell cycle phases at 9 hours post-infection................................................................. 80
Figure 3.14. MNV replication compared in different cell cycle phases at 15 hours post-infection................................................................. 82
Figure 3.15. Cell cycle profiles in G0-held and G0-released populations post-infection. ......................................................................................... 84
Figure 3.16. Cell cycle profiles in M-held and M-released populations post-infection. ......................................................................................... 85
Figure 3.17. MNV replication is highest in G1> populations.............................. 87
Figure 3.18. MNV-1 protein expression in late cell cycle phases.................... 89
Figure 3.19. G1 phase progressing cells promote the highest viral replication....... 91
Figure 3.20 IFN-β treatment induces the phosphorylation of STAT1.......................... 93
Figure 3.21. IFN-β treatment of RAW-Blue cells induces a G0/G1 arrest........... 94
Figure 3.22. Treatment of cells with JAKi-1 to knockdown the IFN response....... 95
Figure 3.23. MNV-1 infection induces a $G_0/G_1$ arrest in a cell line non-responsive to IFN type 1. ......................................................................................................................... 97
Figure 3.24. The VF1 protein of MNV-1 is not the cause of the viral induced cell cycle arrest........................................................................................................................................ 99
Figure 3.25. NS1-2 and NS5 RNA expression in RAW-Blue cells.............................................. 101
Figure 3.26. Expression of NS5 induces a $G_0/G_1$ phase arrest............................................. 102
Figure 3.27. MNV-1 protein NS5 induces a $G_1/S$ arrest and inhibits cyclin A expression. ........................................................................................................................................ 107
Figure 3.28. Protean analysis and schematic of NS5 constructs. .............................................. 109
Figure 3.29. Generation of NS5 1-62, NS5 63-124 and NS5 11-107 plasmids for RNA synthesis ........................................................................................................................................ 110
Figure 3.30. Detection of NS5 construct expression................................................................. 111
Figure 3.31. Expression of NS5 1-62 induces a $G_0/G_1$ phase arrest.................................... 114
Figure 3.32. Sequence comparison between murine GV and HuNoV GII.............................. 115
Figure 3.33. Sequence comparisons between calicivirus N-terminus residues.................. 116
Figure 3.34. MNV and HuNoV NS5 predicted binding sites.................................................. 117
Figure 3.35. ELM motif analysis of NS5 from MNV, HuNoV GI and HuNoV GII............... 118
Figure 8.1. pFB MNV-RVmut.................................................................................................... 165
Figure 8.2. pUC8 vector used for RNA construct generation.............................................. 165
Figure 8.3. Plasmid map of pUC57.......................................................................................... 166
Figure 9.1. Changes to genes involved in cell cycle regulation at 12 h.p.i. .................. 167
Figure 9.2. Graphic of DAVID analysis using KEGG pathway mapping of genes involved in cell cycle related processes. .............................................................................. 168
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>APC</td>
<td>anaphase-promoting complex</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CDC</td>
<td>Center for Disease Control and Prevention</td>
</tr>
<tr>
<td>CDI</td>
<td>cyclin-dependent kinase inhibitor protein</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>CIP/KIP</td>
<td>CDK-interacting protein/kinase inhibitor proteins</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dPBS</td>
<td>Dulbecco’s phosphate buffered saline</td>
</tr>
<tr>
<td>DUT</td>
<td>deoxyuridine triphosphatase</td>
</tr>
<tr>
<td>EBHSV</td>
<td>European brown hare syndrome virus</td>
</tr>
<tr>
<td>eIF</td>
<td>elongation initiation factors</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FCV</td>
<td>feline calicivirus</td>
</tr>
<tr>
<td>FMDV</td>
<td>foot and mouth disease virus</td>
</tr>
<tr>
<td>G</td>
<td>genogroup</td>
</tr>
<tr>
<td>G₀</td>
<td>gap 0</td>
</tr>
<tr>
<td>G₁</td>
<td>gap 1</td>
</tr>
<tr>
<td>G₁&gt;</td>
<td>G₁ progressing</td>
</tr>
<tr>
<td>G₂</td>
<td>gap 2</td>
</tr>
<tr>
<td>h.p.i.</td>
<td>hours post-infection</td>
</tr>
<tr>
<td>h.p.r.</td>
<td>hours post-release</td>
</tr>
<tr>
<td>h.p.t.</td>
<td>hour post-transfection</td>
</tr>
<tr>
<td>HBGA</td>
<td>histo-blood group antigen</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylases</td>
</tr>
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HIV  human immunodeficiency virus
HuNoV human norovirus
IDP intrinsically disordered protein
IFN interferon
INK4 inhibitor of CDK4
IPTG isopropyl-β-D-1-thiogalactopyranoside
IRES internal ribosome entry site
JAK Janus kinase
Kb kilobase
kDa kilodaltons
LB Luria broth
M mitosis
mA milliamps
MAV mitochondrial antiviral signalling protein
milliQ ultrapure water
MNV murine norovirus
MNV-4S MNV-1 ORF4 knockout virus
Mock-Trans mock-transfected
MOI multiplicity of infection
mRNA messenger RNA
MVA modified vaccinia Ankara virus
N-butyrante sodium butyrate
NLS nuclear localisation sequence
NS non-structural
OD optical density
ORF open reading frame
PABP poly(A)-binding protein
PAGE polyacrylamide gel electrophoresis
PEC porcine enteric calicivirus
PERK protein kinase RNA-like endoplasmic reticulum kinase
pfu plaque forming units
pRb retinoblastoma protein
PTB  polypyrmidine tract binding protein
qRT-PCR  quantitative real-time polymerase chain reaction
RAG  recombination activation gene
RAW-Blue  murine macrophage cell line with NFκB/AP-1 inducible SEAP gene inserted
RAW264.7  murine macrophage cell line
RdRp  RNA-dependent RNA polymerase
RHDV  rabbit haemorrhagic disease virus
RNA  ribonucleic acid
RRM2  ribonucleotide reductase M2
S  synthesis
SARS-CoV  severe acute respiratory syndrome coronavirus
SD  standard deviation
SDS  sodium dodecyl sulfate
sgRNA  subgenomic RNA
STAT  signal transducers and activators of transcription
TAE  tris-acetate buffer
TK1  thymidine kinase 1
Tyr  tyrosine
Unsync  unsynchronised
UTR  untranslated region
UV  ultra-violet
VF1  virulence factor 1
VLP  virus-like particle
VP  viral protein
VPg  virus protein, genome linked
VPR  viral protein R
WT  wild-type
xg  gravitational force
X-gal  bromo-4-chloro-3-indolyl β-D-galactopyranoside
Yc  common gamma chain
1 Introduction

1.1 Gastroenteritis

Gastroenteritis is an infection or irritation of the stomach or intestines, causing inflammation and an array of symptoms including diarrhoea, vomiting, fever, abdominal cramping and headaches. Enteric infections in the developing world are a major cause of morbidity and mortality, resulting in 1.4 million deaths globally per year (1). In the developed world gastroenteritis is rarely life threatening, but causes a huge financial burden to businesses and health care. Gastroenteritis is caused by a large variety of viruses, bacteria and parasites. Rotavirus is the leading cause of gastroenteritis in children, but immunity to the virus is commonly acquired by adulthood (2). Norovirus from the Caliciviridae family is the second leading cause of gastroenteritis in young children and unlike rotavirus, the protective immunity from infection is only short-term, leading to infections reoccurring into adulthood (3).

1.2 Caliciviruses

1.2.1 Classification

Caliciviruses derive their name from the Latin word for calix, meaning cuplike structure or chalice, due to distinctive cup shaped depressions on their surface. Caliciviruses are a family of small non-enveloped viruses containing a linear single-stranded positive-sense, RNA genome (4). A distinguishing feature of caliciviruses is their genome organisation, with a 5’ non-structural polyprotein preceding the structural proteins at the 3’ end of the genome. The calicivirus family consists of the Norovirus, Vesivirus, Sapovirus, Lagovirus and Nebovirus genera (5) (Figure 1.1). Caliciviruses infect a broad range of hosts, including humans, cattle, pigs, cats, chickens, reptiles, dolphins and amphibians (6-11). The Sapovirus and Norovirus genera both cause gastroenteritis in humans with noroviruses causing more severe complications (12).
Human norovirus (HuNoV) is popularly known as the ‘winter vomiting disease’, due to common outbreaks in the northern hemisphere during the winter months. This pattern of outbreak is observed in Southern hemisphere counties except in Australia and New Zealand (13, 14). HuNoV is highly infectious and very stable in the environment (15). The infectious virions are stable for weeks and resistant to freezing, heating to 60 °C, ultraviolet radiation and disinfection with alcohol, acid or chlorine (16-19). Although the symptoms are short lived, virus shedding can last up to three weeks in healthy adults and over six weeks in infants (20, 21). The high level of viral shedding averages $10^8$ RNA copies per gram of stool in both GI and GII strains, contributing to the spread of disease (22-24).
1.2.2 Norovirus epidemiology and economic impact

The *Norovirus* genus was discovered as the causative agent in a gastrointestinal disease outbreak in Norwalk, Ohio in 1968 (25, 26). It was formally called Norwalk-like virus then later renamed as the *Norovirus* genus from the *Caliciviridae* family. The norovirus family contains five genogroups (GI-GV) that are characterised based on nucleotide sequence analyses of the structural capsid protein (27-29). Members of genogroups GI, GII, and GIV contain noroviruses that can infect humans with members GI and GII responsible for the majority of human disease (30, 31). GIV can also infect lions and dogs while GIII infects ovine and bovine species and the GV genogroup is only known to infect mice (31, 32). The genogroups can be further divided into each isolated viral strain. Since 1995, the strain that is most predominant in the human population is GII.4, which accounts for approximately 80% of all norovirus infections (33). The GII.4 strain undergoes a high degree of antigenic variation, constantly evolving and resulting in new epidemic strains (34). Protective immunity to HuNoV is only short lived as individuals can experience reoccurrence of infection through their lifetime (3). This is due to several factors including large heterogeneity between genogroups and strains as well as high levels of antigenic drift (34, 35). Within a genogroup strains have 69 – 97% nucleotide similarities where between genogroups there is greater variation with 51 – 56% genomic nucleotide similarity (36). Furthermore HuNoV undergoes rapid antigenic evolution of its capsid protein, contributing to short lived immunity to HuNoV (34).

The Centre for Disease Control and Prevention (CDC) USA estimates that HuNoV causes 60% of acute gastroenteritis, or 21 million cases in the United States each year (37) and in New Zealand there are an estimated 400,000 cases per year (38). Although HuNoV can infect individuals at any age, more severe outcomes are observed in both young (<5 years) and elderly (>85 years) (21, 39, 40). HuNoV infections in the developed world are rarely fatal, but in developing countries the disease outcome is particularly severe due to lack of hydration and nutrients, often resulting in fatalities. It is estimated that 200,000 children under the age of five die each year in developing countries from HuNoV infections or associated outcomes (41).
HuNoV is spread predominantly through the faecal oral route, however transmission has also been documented from aerosolised viral particles from vomitus (42). Other mechanisms of spread have been illustrated in water, food and environmental contaminants (43). Outbreaks are often characterised by sporadic, fast spreading gastroenteritis in semi-closed environments; such as cruise ships, hospitals, army camps, teaching facilities and rest homes (44-46). Studies have shown that certain HuNoV genogroups are associated with particular modes of transmission. For example GI.7 and GII.12 are associated more frequently with foodborne outbreaks, while GII.4 is seen more commonly with person to person spread, such as hospital outbreaks (43).

1.2.3 Norovirus research models
Although HuNoV infections are common, their lifecycle is not well understood. This is due to difficulties in replicating HuNoV in cell culture or small animal models (47-52). HuNoV cultivation provides particular difficulties, as until recently there has been no cell line that could support viral propagation (53). Several animal models exist to study the replication of HuNoV. In 2013, Taube et al developed the first mouse model to support HuNoV replication (54). HuNoV replication was supported in BALB/c mice deficient in recombination activation gene (Rag) 1 or 2 and common gamma chain (γc) (Rag-γc). Results showed that Rag<sup>−/−</sup> γc<sup>−/−</sup> mice infected via the intraperitoneal route with HuNoV from the genus GII.4 supported viral replication (54). Although the model doesn’t recapitulate all aspects of HuNoV infection it might allow further mechanistic studies of HuNoV biology, including host and viral factors determining susceptibility. The gnotobioic pig has been used as an animal model for HuNoV due to its ability to propagate HuNoV at low levels (55). The use of non-human primates has also been trialled as models for HuNoV. Infection of Rhesus monkeys with GI norovirus supported viral propagation, indicated by viral shedding and antibody production but there were no clinical symptoms (56). Cotton top tamarin and common marmosets also supported GI norovirus replication, but had no clinical symptoms or antibody response (56). The best non-primate model is currently pigtail macaques, which supports human GII norovirus replication and shows development of diarrhoea during infection (57). A study on chimpanzees showed they supported the replication of HuNoV without producing clinical signs of gastroenteritis. The group went on to test the feasibility of use in vaccines and showed that the virus shedding
and antibodies developed are identical to that observed in humans (58). The use of non-human primates as models for HuNoV is obviously difficult due to the facilities needed and cost per animal, but may be useful in late stage development of vaccines. Experiments on humans were used to study viral transmission and shedding (22, 59), but researchers more commonly use cell culture for norovirus research into the viral life cycle.

In 2007 a group proposed a three-dimensional model of the human intestinal epithelium that could support HuNoV replication. Growing an embryonic intestinal cell line on porous collagen-1 coated micro carrier beads generated a 3D organoid model. Although this study showed the first replication of HuNoV outside a human host, other groups failed to replicate the results (49). In 2014 the first human cell line was developed in B cells using enteric bacteria as stimulatory factor for norovirus infection (53). Although yet to be replicated by other groups this potentially provides a way to accelerate HuNoV understanding.

Other cell-based systems that artificially introduce the viral genome into host cells have been developed to try to support HuNoV replication. In 2005, Asanka et al was able to express HuNoV from a plasmid in human embryonic kidney 293T cells and generate potentially viable virus particles based on a system of reverse genetics (60). The HEK293T cells used are not likely the host of HuNoV infection in vivo, but were used due to high transfection efficiency and kidney cells are known to frequently support the replication of enteric viruses. Viral genomes were introduced into host cells via transfection of plasmids under control of a T7 RNA polymerase promoter, produced by a recombinant modified vaccinia Ankara virus (MVA). The MVA-T7 system successfully expressed viral genomic and subgenomic RNA (sgRNA) that could produce intact viral particles. Recovered virus cannot be tested for infectivity due to lack of a cultivation system but the caesium chloride density of the viral particles is similar to norovirus observed in human faeces. The use of the MVA-T7 expression system to artificially replicate HuNoV has also been successfully used for generation of infectious particles from feline calicivirus (FCV) (61). Chang et al developed a stable RNA replicon system for HuNoV in hamster BHK21 cells and human Huh-7 cells (62). Transfection of full length viral RNA containing a neomycin resistance gene in place of the viral protein (VP1) successfully expressed viral RNA and viral proteins.
Despite developments in HuNoV cultivation in cell culture and animal models, there is still no routine process of propagating HuNoV without artificially introducing the viral genome. More commonly, research has focused on the use of other caliciviral models to study how noroviruses replicate.

1.2.4 Norovirus animal models

Due to the difficulties in cultivating HuNoV, studies into the replication of the Calicivirus genus, has relied on the use of animal models. FCV was the first calicivirus in which a reverse genetics and cell culture was available (63). FCV belongs to the Vesivirus genus and can be propagated in Norden laboratory feline kidney cells or feline kidney cells. FCV shares some similar cellular interactions with HuNoV, such as the recruitment of host eIF to aid in viral protein translation (64). Although FCV is an attractive model due to its propagation and ease of genetic remodelling (63), the virus is decidedly different in terms of cell tropisms and pathogenicity to HuNoV, as FCV causes respiratory or systemic infections and so has had limited use in studying noroviruses (65, 66).

The porcine enteric calicivirus (PEC) is a member of the Sapovirus genus and was first isolated from infected gnotobioic pigs (67). PEC is closely related to human calicivirus, also from the Sapovirus genus and shares similar pathogenesis to human calicivirus and HuNoV, causing a gastrointestinal infection in swine. (68). PEC can be easily propagated in the porcine kidney continuous cell line and is an attractive model for human sapoviruses (69). PEC however is from the Sapovirus genus and has greater nucleotide differences to those in the Norovirus genus (70). Human calicivirus, another member of the Sapovirus genus causes gastroenteritis in young children and would provide a good model for HuNoV due to similarities in pathogenicity (12). However, research on human calicivirus, has been limited due to the inability to propagate the virus in a cell line of tissue culture.

The rabbit haemorrhagic disease virus (RHDV) is a member of the Lagovirus genus and is induces a fatal liver infection characterised by hepatitis and haemorrhage in rabbits (71). Research on RDHV has focused on its use in controlling rabbit populations or its ability to produce virus-like particles (VLP) for vaccine development (72, 73). European brown hare syndrome virus (EBHSV) is another member of the Lagovirus genus and is non-pathogenic
and has yet to be fully characterised (74). Due to the lack of cell culture for RHDV and EBHSV and greatly different pathogenicity to HuNoV they are not generally used as models for analysing norovirus replication.

Viruses from the norovirus genus of the *Caliciviridae* family provide better models for HuNoV due to closer similarities in genome sequences and pathogenicity. Bovine norovirus causes similar symptoms to HuNoV but there is no cell line available and the cost per animal is high (75). Porcine norovirus too would provide a good model but again lacks a cell line permissible to infection. Since is discovery, murine norovirus (MNV) has become the routinely used model of choice for norovirus study and has proved useful in progressing understanding in an area of research that has been limited by the unavailability of an *in vitro* HuNoV system (76). The MNV model is particularly popular due to its ability to propagate in haemopoietic myelocytes, replicating in immortalised microglial cell line BV-2, mouse macrophage immortalised with Abelson Leukemia virus (RAW264.7 cells) and primary dendritic cells (DC) as well as *in vitro* (77). The natural host to MNV is well characterised and easy to genetically modify (78). Although HuNoV is predominantly an enteric pathogen, research has failed to identify infection in intestinal epithelial cells. Analysis from HuNoV infection of chimpanzees identified norovirus in DC-SIGN dendritic cells and B-lymphocytes cells from the lamina propria (58). Although HuNoV was not detected in macrophage cells it shares replication in similar cell lineages, enhancing the benefits of the MNV model.

### 1.3 Murine norovirus (MNV)

#### 1.3.1 Discovery of MNV and use as a model

MNV was initially discovered in 2003 in immunocompromised laboratory mice, first isolated from cerebral tissue (76). Mice lacking factors of their innate immunity (STAT1−/−/RAG2−/−) easily succumbed to infection via intracerebral inoculation, causing a fatal systemic infection. Infections of various mice knockouts lacking different combinations of innate and adaptive immunity gave early insight into important host factors for controlling MNV infection. Mice with impaired innate immunity lacking STAT1−/−, RAG2−/−/STAT1−/−, STAT1−/−/PKR−/− or IFNαβγ−/− succumbed to lethal MNV
infections while RAG1−/− and RAG2−/− knockouts were not associated with lethal infection but lead to establishment of persistent infections (76). These results suggested the importance of the innate immunity in preventing lethal infection and the adaptive immunity role in clearance. Although initially recovered from brain tissue, MNV was later found to be predominantly an enteric pathogen but could cause systemic infection in immunocompromised mice, spreading to the intestine, liver and spleen (76). Immunohistochemical studies of infected immunocompromised mice showed MNV associated with macrophage and dendritic cells in the liver and spleen and later it was shown that these cell types support viral replication (76, 77). Since the initial discovery of MNV there has been multiple strains found, with slightly different characteristics to the original isolate (MNV-1) (79). New strains continue to be discovered with more than 30 already characterised showing greater than 87% genetic identity (80). The use of MNV as a model to HuNoV is supported by multiple similar characteristics, including high infectivity (81), transition through the faecal oral route (76, 82), gastrointestinal pathogenicity and similar cell tropisms (53, 77). Differences to HuNoV include a longer infection length, with MNV-1 detectable in wild type mice one-week post-infection and longer than 6 weeks in other MNV strains (MNV-2, -3 and -4) (83-86). HuNoV can also cause symptoms in both healthy and immunocompromised persons, where MNV shows symptoms only in immunocompromised mice (76).

1.3.2 Genome

MNV has a compact positive-sense single-stranded RNA genome, 7.4 kb in length (78). Like other caliciviruses, the MNV genome is organised with its non-structural proteins (NS) at the 5′ end, which are expressed from a single open reading frame (ORF1), as a single polyprotein and subsequently cleaved by a viral protease and host caspase-3 into 6 proteins (87). Following the non-structural proteins are ORF2 and ORF3, coding for the major structural protein 1 (VP1) and a minor structural protein 2 (VP2) respectively. A fourth ORF is located within ORF2 in a different reading frame and encodes virulence factor 1 (VF1) (Figure 1.2). ORF4 is only seen in MNV and some strains of sapovirus within the Caliciviridae family (88, 89). The 5′ end of the genome is capped by covalent linkage to viral protein NS5, whilst the 3′ end is polyadenylated. The untranslated regions (UTR) located at the 5′ and 3′ end of the MNV genome are short sequences of 5 and 78
nucleotides respectively, while HuNoV has a shorter 3’ UTR, typically 48 nucleotides (76, 90). The UTRs are evolutionarily conserved RNA structures that protrude into the coding regions of norovirus genomes. These structures are important for viral replication, translation and pathogenesis (91, 92).

Figure 1.2. Norovirus genome comparison.

The upper schematic shows the HuNoV genome and the bottom the MNV genome. Green boxes indicate proteins from ORF1 that are expressed as a single polyprotein. Blue boxes are structural genes expressed from ORF2 and ORF3. The red box is virulence factor 1 encoded by ORF4, which is found within ORF2 in an alternate reading frame. The orange hexagon is viral protein NS5 that attaches to the full length and sgRNA at the 5’ end. Alternative gene naming is indicated with arrows under the non-structural genes (green boxes) for both HuNoV and MNV.

1.3.3 Non-Structural proteins

NS1-2 (N-term)

The function of norovirus non-structural protein 1/2 (NS1-2) is not well understood, as it doesn’t share similar sequence homology to proteins with known functions. Initially it was predicted to have a similar function to picornavirus 2A/2B proteins due to a similar genomic location, although sequence analysis revealed otherwise (93). Sequence analysis
of NS1-2 shows that the viral protein contains putative H box/NC sequence motifs and a putative hydrophobic transmembrane domain at the C-terminus (94, 95). The hydrophobic transmembrane suggests anchorage of NS1-2 in a host membrane and the H box/NC motifs are found in cellular proteins (such as H-rev107 and TIG3) that are thought to play a role in the regulation of cell proliferation (94), although this function has not been observed in noroviruses.

The NS1-2 protein is further processed by host cell caspases after being cleaved from the ORF1 polyprotein. Studies have confirmed an additional cleavage site within NS1-2 releasing two mature viral proteins (96-98). The MNV NS1-2 protein can be cleaved by the host caspase-3 protein (87), although not all HuNoV strains have a cleavable NS1-2 and it is possible that this event is lost in some strains. A cleavage site in HuNoV GII Camberwell strain has been reported which is not seen in GI noroviruses or MD-145 GI norovirus (87, 99). The NS1-2 protein in noroviruses is the least conserved non-structural protein, with more nucleotide variability outside of its conserved transmembrane domain (93).

The role of NS1-2 in calicivirus replication is not well understood. Recent studies have shown that the NS1-2 protein in MNV is an inherently disordered protein between the residues 1-142, and that the protein can form a dimer (100). The disordered region in NS1-2 is predicted to be conserved among all noroviruses. The identification of the dimerisation and disordered regions suggests that NS1-2 has a multifunctional role in a viral infection. Analysis of RNAseq data from an NS1-2 transfected RAW264.7 cells showed large changes in genes involved in apoptosis, as well as cholesterol metabolism (101). The effects on apoptosis were expected to be inhibitory and may complement the role that VF1 has on apoptosis inhibition (88). Localisation studies show that the MNV NS1-2 protein co-localises with dsRNA within replication complexes, and associates with the endoplasmic reticulum (ER) (102). This is also seen in the FCV equivalent of NS1-2, in its p32 protein (103). Alternatively, HuNoV NS1-2 localises to the Golgi apparatus, causing disassembly of the organelle (95). Due to the interactions of replication complexes within organelles and the transmembrane domain of NS1-2, it is predicted that the primary function of NS1-2 is to regulate intracellular membrane rearrangements that are associated with viral replication (95). Recently, MNV NS1-2 has been identified to be involved in viral
persistence of disease. A single amino acid change within the 5’ end of NS1-2 significantly increased viral replication within certain regions of the intestines that are implicated in viral persistence (104). This shows that like the capsid protein, NS1-2 can influence viral tissue tropism. The HuNoV NS1-2 protein has also been implicated to affect cellular secretion (93).

NS3 (NTPase)
The norovirus NS3 protein is also called NTPase due to three specific motifs (A, B and C) that appear in a number of NTP-binding proteins and classify the protein in the superfamily 3 of RNA helicases (105, 106). The NS3 protein of noroviruses shares several sequence motifs with other viral NTPases, namely with picornaviruses and flaviviruses. These enzymes bind and hydrolyse host adenosine triphosphase (ATP) at a rate of $1.4s^{-1}$ and use the released energy to unwind viral nucleic acids (106). Studies showed that norovirus NS3 can hydrolyse ATP, but is unable to unwind a synthetic RNA:DNA heteroduplex. This suggests NS3 protein has NTPase, but not helicase activity as the protein was sensitive to the presence of homopolymeric RNA (106). Because norovirus NS3 is sensitive to homopolymeric RNA, it suggests that the activity of NS3 must be down regulated at some point during MNV replication prior to packaging (106).

NS4 (3A-like)
The NS4 protein of noroviruses referred to as p22 in Norwalk virus and 3A like in MNV strains is involved in interactions with host membranes. An early study on the immune response following human infection with HuNoV found an adaptive immune response directed against the NS4 protein (107). The NS4 protein of HuNoV contains a transmembrane motif and an YXΦESDG motif that mimics a di-acidic ER export signal in both sequence and function (108). The di-acidic ER export signal is though to be involved in inhibition of cellular secretion and in Golgi disassembly (108), which is documented to be induced by NS4 expression (103, 109). Like the other MNV non-structural proteins, the NS4 protein from MNV is found localised in replication complexes and has suggested involvement in rearrangement of cellular membranes (110). The HuNoV NS4 protein has been suggested to have similar functions to the MNV NS4. Like MNV NS4, HuNoV NS4 is
predicted to recruit the replication complex to membranes via its transmembrane domain. The NS4 protein, was initially referred to as 3A-like protein due to similarities in genome positioning with the 3A protein in polioviruses (111). The poliovirus 3A protein is involved in localisation of viral replication complexes to cellular membranes but also in immune modulation, decreasing secretion of antiviral chemokine’s and cell surface antigens (112-114). Although the sequence similarity between norovirus NS4 and poliovirus 3A is small, the role in transmembrane rearrangement and membrane localisation of replication complexes suggests similar roles.

**NS5 (VPg)**

The norovirus NS5 named VPg for virus protein, genome linked, is a ~15 kDa protein that is covalently linked to the 5’ end of the genomic and sgRNA of noroviruses. The VPg protein has been extensively characterised from only two families of vertebrate positive-sense RNA viruses, namely members of the *Picornaviridae* and *Caliciviridae*. Computational studies have also indicated a possible VPg protein present in the *Astroviridae* family. Some plant viruses also express VPg, notably members of *Secoviridae* and *Potyviridae* and some members of the *Futeoviridae* and *Sobemovirus* families, all positive-sense RNA viruses (reviewed in (115)). The VPg protein comes in different sizes, ranging from 2-3 kDa in *Secoviridae* viruses to 20-22 kDa in *Potyviridae* viruses. VPg is essential for calicivirus replication but is not required for picornavirus replication (116).

VPg is a multifaceted protein, with several known functions and many predicted functions. The most conserved function of VPg is probably its mechanism for priming the viral RNA genome for viral RNA-dependent RNA polymerase (RdRp) replication. Linkage of VPg to the calicivirus RNA genome occurs through nucleotidylation to the guanine base at the start of the RNA genome sequence via a tyrosine residue (Tyr26) by a viral polymerase (NS7) (117, 118) (Figure 1.3). The tyrosine residue that is nucleotidylated lies in a highly conserved acid rich motif (DEYDE) seen in all caliciviruses (119). The attached VPg protein provides a free hydroxyl that can be extended by the virally encoded RdRp to synthesise a new RNA strand.
The second best understood function for VPg is its ability to act as a protein cap for translation of viral proteins. This is observed in caliciviruses and plant viruses containing VPg but not with picornaviruses. Studies with caliciviruses confirmed that the VPg protein interacts directly with the cap-binding proteins from the elongation initiation complex and that this interaction is essential for viral translation, as caliciviral RNA cleaved of VPg is non-infectious. MNV and HuNoV VPg binds to and recruits the host translation initiation factors to initiate viral translation. MNV VPg pull down assays detected eIF3, eIF4GI, eIF4E, eIF4GI and eIF4E in the protein elution’s (120). Further studies have showed that the binding to eIF is through the C-terminus of the MNV VPg (121). Attachment to eIF in turn recruits ribosomes for preferential translation of viral proteins over host proteins. VPg precursors can also bind eIF4E, this may localise VPg to membranes to anchor VPg and to accumulate initiation factors to the sites of MNV replication, or sequester host eIF to contribute to host protein shut off as seen in plant VPg proteins (122). Mutagenesis studies of amino acid residues in the C-terminus of MNV NS5 inhibited binding to host eIF and consequently reduced MNV replication (121). The final 15-17 residues of the C-terminus of NS5 are highly conserved among noroviruses and are predicted to be involved in binding to host eIF (Figure 1.3B).
Figure 1.3. 3D structure and schematic of MNV NS5 protein.

(A) 3D Structure of MNV NS5 protein between residues 11-85, figure from (123), PDB ID:2M4G. (B) Schematic of full length (1 – 124 residues) NS5 protein from MNV. Helices are shown in purple and the eIF recruitment-binding site in yellow. The nucleotidylation site is shown in green text (Y26), red text indicates interactions between residues involved in hydrophobic interactions and blue text polar interactions. The numbers above the schematic indicate the position of amino acids at key structures.

VPg has been hypothesised to have several other roles, although more research is needed to confirm these ideas. It is possible that one of the benefits of using a protein-primed (i.e. VPG-dependent) mechanism of viral RNA synthesis is the ability to prevent the formation of 5’ triphosphorylated RNA during infection and detection by the cytoplasmic sensors RIG-I and protein kinase R. RIG-I and protein kinase R detect uncapped 5’ triphosphorylated RNA. Capping of the 5’ end with VPg masks viral RNA, thus contributing to extended survival of viral infected cells. Foot-and-mouth disease virus (FMDV) from the Picornaviridae family has three VPg proteins that are involved in virus release and/or
maturation, as deletion of VPg allowed FMDV to replicate but did not result in cytopathic effect or viral release (124). Typically calicivirus VPg proteins contain a charged N-terminus, rich in arginine and lysine (119), these characteristics are often involved in RNA binding activity. VPg from MNV can be easily purified by phosphocellulose P11 or heparin-affinity chromatography, features that many RNA binding proteins share (125). Other RNA interactions with VPg include a Picornavirus 3A-VPg precursor that has RNA chaperone activity (126) and potyvirus VPg can interact with specific RNA in a sequence dependent manner (122). It is not known whether caliciviral VPg can interact with RNA other than its own sequence. Host RNA interactions may serve to regulate host processes that aid in viral replication. Finally RNA bound VPg from FCV can bind to VP1, viral RNA polymerase (NS7) and its genome (127), suggesting a mechanism for specific incorporation of the viral genome during encapsulation, among a high concentration of host mRNA. Locational studies on MNV VPg detected expression in the perinuclear region of the host cell, where viral replication occurs (128). Although protein expression occurred from a tetracycline inducible plasmid in HEK293 cells which do not support MNV replication.

The core of the MNV NS5 protein structure was solved in 2013 by Leen et al, using nuclear magnetic resonance spectroscopy (123). At full length, MNV VPg contains 124 residues with the structure solved for amino acids 11-85, which forms its core (Figure 1.3A). The MNV NS5 protein contains a compact two helical core flanked by flexible N and C termini, stabilised by a network of hydrophobic and salt bridge connections. The first helix in the NS5 core of MNV is a conserved acid rich motif that contains the tyrosine residue that is nucleotidylated by the viral RNA-dependent RNA polymerase (NS7) and covalently attached to the RNA genome. This is thought to act as a form of primer for RNA synthesis of the viral genome. Modelling of NS5 and NS7 from MNV and FCV suggest that the 3D structures would inhibit tyrosine nucleotidylation, as NS7 couldn’t gain access to the active site. It is therefore likely that NS5 undergoes a significant conformational change prior to nucleotidylation in order for NS7 to access the Tyr26 residue in NS5.

A high number of VPg proteins across all virus species are predicted to contain intrinsic disorder (129). An intrinsically disordered protein (IDP) is a protein that lacks a fixed 3D structure and has a flexible shape. Regions of intrinsic disorder allow proteins to have
several functions, altering its structure to interact with multiple partners. Disordered proteins are predominantly linked to activities including; signal transduction, chaperone activity, gene expression and cell-cycle regulation (130, 131). Many IDP are only partly disordered and contain regions of flexibility or inherent structural disorder. Predictions of VPg disorder were found in caliciviruses from RHDV, vesicular exanthema of swine virus, Sapporo virus Manchester, MNV and HuNoV by most disorder prediction engines, sharing conserved protein disorder regions in 25% to 36% of their nucleotide sequences (101, 129). Some plant virus VPg proteins are also documented to be disordered such as several strains from the Potyviridae and Sobemovirus families. (115).

The VPg protein is increasingly being seen as an important, multifaceted protein involved in several aspects of viral replication. Although there are differences in VPg nucleotide sequences between viral species there is significant overlap in protein functions. Calicivirus NS5 has an important role in both viral genome replication and virus translation. Caliciviral NS5 predicted intrinsic disorder is suggestive of alternative functions as seen with disordered proteins. The ability of viral VPg proteins to bind host RNA and cellular proteins is intriguing, further studies may identify new insights into the additional functions of VPg.

**NS6 (Protease)**
The caliciviral NS6 encodes a cysteine protease responsible for cleaving viral proteins from the ORF1 polyprotein (87). The active site of NS6 has been extensively studied and contains a catalytic triad of amino acids composed of cysteine, histidine and an aspartic or glutamic acid residue, and named 3C-like cysteine protease due to its similarities with the picornavirus 3C protease (132-135). The catalytic triad is responsible for determining substrate specificity and is essential for its activity. The viral protease is released initially from the viral ORF1 polyprotein by autocatalytic cleavage in norovirus species and RHDV (87, 96). Following release of the viral protease it functions to cleave the remaining viral proteins from the polyprotein. In several caliciviral strains a protein precursor consisting of the viral NS6 and NS7 exists that is a bifunctional enzyme with both protease and polymerase activities (136-138). Caliciviral proteases from HuNoV and FCV have been implemented in cleavage of host factors involved in translation. HuNoV NS5 can cleave
host poly(A)-binding protein (PABP) and the FCV protease has been shown to cleave host proteins eIF4GI and eIF4GII leading to inhibition of host cellular protein synthesis (139, 140). Shutdown of host protein translation may serve to regulate cellular mRNA translation to more effectively synthesise viral proteins. Alternative functions include manipulation of host apoptosis as NS6 from MNV is shown to co-localise to the host mitochondria (109). Apoptosis regulation as predicted to occur during MNV infection (141) and furthermore, the viral protease from picornavirus has been implicated in downregulation of the innate response to viral infection, suggesting a similar role for the MNV protease. (142).

NS7 (RdRp)
As with other RNA viruses, replication of the norovirus genome occurs via a viral RdRp. Norovirus RdRp, referred to as NS7 is structurally and functionally similar to RdRp found in other positive-sense RNA viruses (143). In vitro biochemical data has shown the norovirus NS7 protein to also be involved in linkage of the viral NS5 (VPg) to the end of the viral RNA. Attachment of the NS5 protein occurs via a nucleotidylation reaction to the 5’ end of the genomic and sgRNA. NS5 then acts as a primer for NS7 transcription of genomic and sgRNA, in which a tyrosine residue from NS5 is used for nucleotide addition (118, 144). While the NS7 protein can transcribe both genomic and sgRNA using NS5 as a primer, the NS5 protein is not required for replication of anti-sgRNA by NS7 (137). Hence, a new de novo model for replication from anti-sgRNA was proposed where NS7 transcription starts on a poly(C) stretch, added at the 3’ terminus of the anti-sgRNA by norovirus NS7 terminal transferase activity (137). The key role the NS7 plays in viral replication and that most eukaryotic hosts lack RdRp makes NS7 an attractive target for antiviral treatments.

ORF4 (VF1)
All caliciviruses contain three ORF with MNV and some human sapoviruses containing a fourth ORF (ORF4), coding for VF1 (89). VF1 is encoded by an alternative reading frame within ORF2 and has been expressed in vitro from sgRNA (88). Although it is possible that expression may also occur from full length genomic RNA by translational termination–reinitiation as the start codon of ORF4 overlaps with the stop codon of ORF1, as seen in expression of bovine norovirus ORF1 and ORF2 proteins (145, 146). Although ORF4 is not
required for MNV infection, mutations to induce premature stop codons for VF1 comes at a fitness cost to MNV (88). During MNV infection VF1 is predominantly found at the mitochondria and is thought to antagonise the innate immune response through suppression of an interferon (IFN) response pathway (88). The IFN regulatory factor IRF54 is down-regulated by MNV VF1, IRF54 responds to IFN type I and III and promotes cellular apoptosis through a mitochondrial pathway (88, 147). In STAT1−/− mice it was shown that the onset of clinical symptoms is significantly delayed by VF1 (88). Together these results show that VF1 contains anti-innate immune activity that delays the onset of apoptosis in infected cells, increasing viral virulence.

1.3.4 Structural proteins

The norovirus icosahedral capsid is composed of 90 dimers of VP1 and one or two copies of VP2 (148). Both proteins are primarily expressed from NS5 linked sgRNA containing both ORF2 and ORF3. Calicivirus virons can assemble in the presence of only VP1, but recent research suggests VP2 may play a role in particle stability (149). A viron containing only a single capsid protein is relatively rare among animal viruses and is more common in plant viruses.

VP1

The VP1 of noroviruses ranges in size from 530 – 555 amino acids, with a molecular weight of ~55 – 60 kDa. Expression of VP1 and VP2 in noroviruses occurs primarily from sgRNA, although expression can occur from genomic RNA through translational termination-reinitiation between ORF1 and ORF2 as documented in RHDV and bovine norovirus (146, 150, 151). The VP1 protein contains a central hypervariable domain that most likely influences antigenic determinants, flanked by two conserved domains (111). If expressed in insect cells, VP1 can self-assemble into VLP that are structurally similar to norovirus virons but without genomic material (152). Because of the self-assembly ability of VP1, it is researched for use in vaccine development and antigen presentation (72, 153-156).

The crystal structure has been solved for the viral capsid of HuNoV showing that VP1 folds into two major domains, the shell domain and a protruding domain (157). The shell domain is located towards the centre of the viron and forms the icosahedral structure and
interacts with genomic RNA. The protruding domain protrudes externally and can be subdivided into two domains, P1 and P2. The protruding domains are a hypervariable region, which have been documented to show importance in binding to the ABO histo-blood group antigens (HBGA), which are associated with differences in susceptibility to norovirus infection (158).

**VP2**

The VP2 protein is smaller than VP1 (~22 – 29 kDa) and has larger sequence variation between strains (159). VP2 is only a minor structural protein and although unnecessary for VLP formation has been shown to be essential for FCV replication (160). The VP2 protein is thought to be involved in stabilisation of the capsid structure and could protect VP1 from protease degradation (160). Expression of VP2 has been documented to occur by translational termination-reinitiation in FCV and MNV where ribosomes tether to the viral RNA after translation VP1 before reinitiating translation at the AUG of VP2 (145, 151, 161). Tethering is thought to occur by an essential termination upstream ribosomal binding sequence (TURBS), which is partly complementary to 18S ribosomal RNA (151, 161). There are several factors though to be important in VP2 regulation, including the ORF2 stop codon, poor initiation coding sequence for ORF3 translation as well as an alternative initiation codon within the sgRNA (150).

### 1.4 Norovirus replication

#### 1.4.1 Binding and entry

Although it is unknown what cells HuNoV replicates in, multiple studies have identified the importance of carbohydrates found on the cell surface for binding and entry. The HBGA are a family of glycans, found on the surface of red blood cells, gut and respiratory epithelia and in biological secretions. The HBGA have been shown to be an important factor used by HuNoV for infection of host cells (162-165). Susceptibility to norovirus infection has been shown to depend on the individual’s HBGA make up, however this varies between norovirus strains. Experiments on norovirus GI.1 showed individuals in blood group O are more susceptible to infection (164). There is a large variability between
viral genogroups, as the GII.2 noroviruses have no correlation between HBGA and susceptibility to infection (166), while reports on GI noroviruses show resistance in type B individuals. While there is not consistency between HBGA and susceptibility to HuNoV infections, it is clear that HBGA binding is an important factor for norovirus infection and that individual norovirus genogroups may only infect a subset of the human population effectively. MNV also uses a carbohydrate receptor for entry with studies identifying sialic acid moieties, glycolipids and glycoproteins important for entry, also in a strain dependent manor (167, 168). MNV entry was shown to be dependent on cholesterol and dynamin levels in the host cell (169, 170). After the binding to carbohydrate receptors on the host cell, the process of viral entry is not understood.

1.4.2 Translation
After attaching to the host cell, norovirus must enter and uncoat in an unknown process. Following release into the cytoplasm the positive-sense RNA genome acts as a template for viral protein translation. The viral RNA does not contain a eukaryotic 5’ cap, but instead encodes its own capping protein, NS5. NS5, otherwise known as VPg is linked to viral genomic and sgRNA, and acts to prime viral transcription and recruit factors for protein translation (171). This function is conserved among caliciviruses and helps to increase the coding capacity of their relatively small genomes (172). NS5 is used as a primer for viral RdRp genomic and sgRNA replication and acts to recruit host eIF to initiate viral protein translation (see section 1.3.3).

The RNA genome has been shown to process protein binding capabilities, binding to host proteins involved in translation. The viral RNA sequence contains conserved regions including hairpins and stem-loops (91), that are able to bind cellular proteins LA, PABP and polypyrimidine tract binding protein (PTB) (90, 173, 174). The role of these proteins in viral replication is not fully understood, although a loss of protein binding to the viral genome results in a fitness cost (91, 92). However the same proteins have been implicated in binding to other viral genomes, and have been shown to enhance viral translation and so it is thought they may function similarly in noroviruses (175-178). Interactions of norovirus RNA with cellular host proteins is also involved in circularisation of the viral genome. Circularisation of the MNV genome is thought to be mediated by binding of host proteins
PCBP2 and huRNPA1 to the 5’ and 3’ extremities (179). Genome circularisation is also documented in HuNoV, with host proteins contributing to stabilisation by contacts between the 5’ and 3’ ends (180). Viral genome circularisation can have several functions in the viral life cycle including aiding in genome replication and in viral protein translation (181-183). As discussed earlier, the translation of structural proteins VP1, VP2 and VF1 occurs primarily from sgRNA. Synthesis of sgRNA occurs from the negative-sense RNA strand via recognition by viral RdRp (NS7). Viral RdRp recognises a conserved promoter sequence found in all caliciviruses at the start of ORF2 that forms a stem loop structure upstream of the sgRNA initiation nucleotide (184). Expression of sgRNA is helps to control the timing of viral protein expression, as structural proteins are needed later in infection, but also to produce higher levels of VP1 for virus assembly. In infected cells, sgRNA is present in much higher levels than genomic RNA, contributing to higher levels of viral structural proteins (148).

1.4.3 RNA replication
Replication of the positive-sense norovirus RNA occurs via virally encoded NS7, a RdRp. Replication occurs in complexes associated with membrane bound organelles within the cytoplasm. Initial replication of the positive-sense strand is primed by NS5, which acts as a primer for NS7 replication of viral RNA (137). Replication of the negative-sense RNA was shown to be independent of NS5 but rather through its interactions with the shell domain of VP1 (185). It was discovered that loop sequences in the shell domain of VP1 interact with NS5 to initiate RNA synthesis. It is proposed that VP1 levels increase due to the production of positive-sense RNA until the capsid protein forms multimeric complexes, preventing interactions with its shell domain, leading to formation of complete viral particles (186). Replication of both positive and negative-sense RNA occurs via formation of a double-stranded replicative form that permits replication of positive-sense genomic and sgRNA (186). Synthesis of sgRNA is proposed to be initiated through two mechanisms. One proposes initiation through detection of a stem-loop structure downstream of VP1 on the negative-sense RNA by viral NS7 (91). This internal binding site is highly conserved among members of the Caliciviridae family. The second model involves premature termination of RNA replication during negative-sense RNA synthesis, arising from a
termination signal. The short negative-sense RNA would then act as a template for positive-sense RNA synthesis and production of the sgRNA (186).

1.4.4 Viral exit

The process of norovirus assembly and exit is not well understood. The well documented self-assembly of calicivirus major capsid protein suggests this is sufficient for driving viron assembly (149), with VP2 promoting structural stability (160). VP2 binds to the VP1 protein during assembly so it is positioned towards the inner face of the capsid (187), suggesting it might play a role in encapsulation via RNA interactions, however no RNA interactions have been documented. The process of release of infectious particles is not known for noroviruses. Several groups have proposed that noroviruses induce apoptosis in order to exit the cell (141, 188, 189). VF1 from MNV has been shown to delay the cellular process of apoptosis, perhaps to allow time for the virus to replicate prior to apoptosis and viral release (88).

1.4.5 Manipulation of the host environment

As obligate intracellular parasites, viruses will frequently manipulate host cellular processes to promote viral replication. This is particularly important for viruses with limited genome sizes, such as RNA viruses that rely on host factors to replicate. Many of these strategies to manipulate the host involve suppression of the immune response to allow time for viral replication and results in a race to replicate before the cell can subdue the infection. Other viral strategies target cellular pathways and processes to change the host environment to benefit viral replication. The replication of all characterised RNA viruses involves formation of a replication complex and so manipulation of the host environment often revolves around aiding this process.

Viruses have evolved to manipulate the host immune response, developing a large variety of evasion strategies. Some RNA viruses are able to disrupt host secretion pathways involved in pathogen detection. MNV has been shown to interact with organelles involved in secretory pathways including the Golgi and ER but doesn’t impede host secretion (102). HuNoV disrupts expression and trafficking of host surface proteins through the action of NS1-2 (93, 95). It is possible that disruption of this pathway and inhibition of surface protein expression interferes with cytokine secretion or antigen presentation. As MNV
infection is relatively short lived, the innate immunity is implicated in controlling infection. The production of IFN in response to norovirus infection has been well documented in being important for controlling replication (76, 77, 190). An interaction of VF1 with viral detection mechanisms has been shown to favour viral replication. MNV VF1 can interfere with the signalling cascade of mitochondrial antiviral signalling protein (MAVS) complex or downstream components to affect IFN production (88).

RNA viruses rely heavily on host factors to facilitate their own replication. Norovirus employs several strategies to target host proteins to accelerate viral replication. Most extensively covered is the recruitment of host eIF by viral NS5 for preferential translation of viral proteins (see section 1.3.3). Norovirus binds multiple host cellular proteins including DDX3, LA and PTB and although the functional significance of these interactions is not well understood, knockdown of the host factors comes at a fitness cost, indicating a role in replication (173). Interactions in host cholesterol synthesis pathways have been noted during norovirus infection. Interactions of MNV with cholesterol has been documented but not completely understood (personal communication) and in HuNoV a down-regulation of cholesterol biosynthesis was found to promote viral replication (191, 192).

Manipulation of the host cell cycle is another strategy used by viruses to aid their own replication. This can function to help in both immune evasion and in aiding viral replication. Unlike cells used in in vitro studies that are constantly undergoing replication, the majority of cells found in vivo are in a quiescent (G₀) or a post-mitotic state and often don’t support viral replication. This causes limitations to viral replication and so viruses have developed strategies to achieve cell cycle progression to aid in viral replication. Other strategies involve arresting cells in a particular phase that contains cellular factors that benefit viral replication. All viral types including DNA viruses, RNA viruses and reverse transcribing viruses have been characterised in manipulating the host cell cycle. Viruses can either delay, arrest, or progress the cell cycle through direct interactions of viral proteins with cellular proteins and pathways or by inducing an innate immune response to viral replication. Manipulation of the host cell cycle can create an environment that is
more favourable to viral replication through the increase of cellular resources or host proteins.

1.5 Mammalian cell cycle

Many viruses are able to alter the host cell cycle to favour their own replication, or have adapted to replicate most effectively in specific cell cycle stages (193-197). Cell division is the process where a cell will duplicate its genome before splitting into two identical daughter cells. The cell cycle is a series of events that describe this process of growth and division of a cell (reviewed in (198)). In the first gap phase (G₁), there is a high rate of translation as components required for DNA synthesis are created. Cells can enter gap 0 (G₀) during G₁ phase, if mitogenic stimulants are not present, where the cell metabolic rate is low. After G₁, cells enter synthesis (S) phase, where the genome of the cell is replicated. Following subsequently is gap 2 (G₂) phase, where protein synthesis and the metabolic rate is high. Finally in mitosis (M) phase, the DNA chromatids and cell contents are split into two daughter cells. Transition through and between each phase is highly controlled by multiple regulators. Each gap phase represents a period of activity where the cell prepares for the progression through the next phase and monitors mitogenic signals, adequate cell size and DNA integrity (199). The M phase can be further divided into five phases; prophase, prometaphase, metaphase, anaphase and telophase prior to cytokinesis where the cell finally divides in two (200).

1.5.1 Regulation

Cell division is one of the most complex processes that takes place within cells and involves interactions of hundreds of proteins. Cell division is orchestrated through multiple levels of regulation, where extracellular and intracellular signals are funnelled down multiple pathways to control the activity of cyclin and cyclin dependent kinases (CDK) complexes that are the primary catalysts of cell cycle progression. The activation and inactivation of cyclin and CDK proteins controls sequential progression through each phase of the cell cycle. Cyclins are a family of proteins that regulate the activity of CDKs through binding and activation of their catalytic subunit, upon formation of a cyclin-CDK complex. There are multiple cyclins that are expressed at different stages in the cell cycle, and show specificity to different types of CDKs. The most characterised cyclins include cyclin A, B, C, D and E
families, that all share a cyclin box region that binds to the N-terminal of specific CDKs (201). Each cyclin is attributed to progression through specific stages of the cell cycle and are expressed and degraded throughout cell division, although there is slight variation between cell lines (202). In contrast CDK levels remain relatively consistent throughout cell division as their activity is controlled by cyclins, inhibitor protein binding and post-translational modifications. The CDKs are a family of serine/threonine kinases that are present in all eukaryotes. Binding of a cyclin to a CDK induces a conformational change, stimulating the kinase activity of the CDK and initiating phosphorylation of their protein substrates. The most characterised CDKs include CDK 1, 2, 3, 4 and 6.

Cell cycle progression is negatively controlled by the cyclin-dependent kinase inhibitor protein family (CDI). The CDI family has two distinct families, the inhibitor of CDK4 (INK4) and the CDK-interacting protein/kinase inhibitor protein (CIP/KIP) (203). The function of the INK4 family revolves around G₁ cyclin/CDK complex inhibition that includes p14, p15, p16, p18 and p19 proteins. The INK4 family of proteins bind to CDKs and cause allosteric changes that inhibit cyclin binding and distort the ATP binding site. The CIP/KIP family has a broader range of inhibition across the cell cycle and includes the proteins p21, p27 and p57. The CIP/KIP family of proteins function through binding to the entire cyclin/CDK complex, inhibiting its activity. There are other mechanisms of cell cycle control including the tumour suppressor protein p53 that induces G₁ and G₂ phase arrests in response to ribonucleotide depletion, oxidative stress, deregulated oncogene expression, and primarily DNA damage (204). Extracellular signals can also play a role in cell cycle regulation with cell growth responding to contact inhibition, extracellular antimitogenic factors and senescence (205).

The pocket protein family plays a critical role in regulating cell cycle progression. There are three members of pocket proteins consisting of the retinoblastoma protein (pRb), p107 and p130 that bind to the cell cycle transcription factor family of E2F proteins (206). Binding of E2F by the pocket proteins inhibits cell cycle progression, as release of E2F and subsequent expression of cell cycle genes is required for cell division to occur. The phosphorylation status of the pocket protein family controls cell cycle progression by releasing E2F proteins upon subsequent phosphorylation. The pRb is the most extensively
characterised pocket protein and arguably the most important cell cycle regulator. When pRb is phosphorylated at low levels it is referred to as in a hypophosphorylated state. When cyclin/CDK complexes phosphorylate pRb it becomes unable to bind E2F, which is released and drives G_{1}/S cell cycle progression. When pRb is phosphorylated at multiple sites it is referred to as hyperphosphorylated. The pRb has several phosphorylation sites that are targeted by specific cyclin/CDK complexes (207).

1.5.2 Cell cycle progression

G_{0}/G_{1} Transition

Cells in the G_{0} phase are considered to be neither dividing nor preparing to divide. Many cell types enter the G_{0} phase once they reach maturity, including heart muscle cells and neurons (208). Not all cells that enter the G_{0} phase remain dormant; entering quiescence is often a consequence of the cell’s lacking any stimulation to re-enter the cell cycle and is reversible. Proteins involved in cell cycle entry from G_{0} include pocket proteins p130 and the relatively unknown cyclin C protein (209-211). Cyclin C mRNA levels peak during G_{0} exit and the cyclin C protein associates with CDK3 to stimulate phosphorylation and activation of cell cycle regulator pRb (212).

G_{1}/S Transition

Transition though G_{1} into S phase is the most highly controlled checkpoint in cell division (Figure 1.4). The main regulator is pRb that binds to the E2F family of transcription factors. If released the E2F transcription factor localises to the nucleus and activates transcription of genes necessary for entry into S phase. After G_{1}/S progression pRb is progressively dephosphorylated by protein phosphatase 1 to return to a hypophosphorylated state, where it can again bind E2F (213). In early G_{1} phase the cyclin D family of proteins are expressed and associate with CDK4 or CDK6 (214-217). The active kinase complex can then phosphorylate pRb pocket proteins and cause dissociation with E2Fs involved in transactivation of the genes needed for late G_{1} phase progression, including cyclin E and cyclin A. After cyclin E is expressed it binds to CDK2 forming an active complex that phosphorylates the G_{1}/S progression inhibitor p21 and regulates its own expression by phosphorylating p27 from the CIP/KIP family of CDI, tagging them for proteasomal degradation (218-222). The p21 protein inhibits CDK1 and CDK2 complexes and the p27
protein is an inhibitor of cyclin D and cyclin E, so depletion of both p21 and p27 promotes cyclin D activity and therefore cyclin E and cyclin A expression (223, 224). Cyclin E/CDK2 complexes further phosphorylate members of the pRb pocket protein complex making them hyperphosphorylated, causing release of a different E2F transcription factor involved in expression of late G1 phase genes involved in DNA synthesis, driving the cell into S phase (225, 226). Cyclin E/CDK2 complexes furthermore regulate the activity of other less known cell cycle proteins including; Smad3, CBP/p300, E2F-5, p220 (NPAT), nucleophosmin and CP110, leading to promotion of S phase transition (227-231). Once a cell passes through the major restriction checkpoint at late G1 phase it moves into S phase where the genomic DNA is replicated. Proteins required for DNA replication are largely expressed during G1 phase and act to unwind the double-stranded DNA, and through the action of DNA polymerases add free nucleotides to generate a copy of the genomic material. Progression through S phase is largely governed by cyclin A expression. Cyclin A associates with two CDKs at different points in the cell cycle. In S phase, cyclin A displaces cyclin E from CDK2 inhibiting the action of cyclin E/CDK2 complexes, and acts to drive DNA synthesis (232-234). Cyclin A/CDK2 complexes have been reported to phosphorylate multiple proteins involved in cell cycle regulation including; pRb, E2F1, B-Myb, cdc6, HSSB, MCM4, BRCA1, Ku70, HIRA, hHR6A, cdc20 p53, p21 and MDM2 (235). In late S phase cyclin A associates with CDK1 to facilitate entry through G2/M phase (236, 237). The cyclin A/CDK1 complex has overlapping targets to the cyclin A/CDK2 complex and its function is not entirely clear in G2 phase progression, but it is thought that cyclin A/CDK1 complex is involved in stabilisation and activation of the cyclin B/CDK1 complex which drives G2/M phase progression (233, 238-240).

G2/M phase
The final stages of cell division involve a period of protein synthesis and cell growth (G2 phase), where the cell readies itself for division of genomic material into two identical daughter cells (M phase) (reviewed in (241)). Progression from G2 to M phase is predominantly governed by the activity of cyclin B in complex with CDK1. Both cyclin B and CDK1 activation and subsequent cell transition into M phase is activated through
phosphorylation of several residues by cdc25 phosphatases, that affect the nuclear import and enzymatic activity of the complex (242-244).

Figure 1.4. Schematic representation of mammalian cell cycle control, centring on G₁/S progression.

Progression through the G₁/S checkpoint is controlled predominantly by the phosphorylation status of pRb by the actions of cyclins (ovals) and CDK (circles). The pRb protein (square) is phosphorylated by cyclin C/CDK3, cyclin D/CDK4/6 and cyclin E/CDK2 complexes. Subsequent phosphorylation of pRb leads to release of E2F transcription factor leading to synthesis of proteins promoting S phase entry. Inhibitors of G₁/S progression include p21 and p27 (triangles).

Cyclin B is found in the cytoplasm until it is phosphorylated, when it is then imported to the nucleus (245). The regulation of cyclin B/CDK1 complexes ultimately controls progression through G₂/M, a DNA damage control checkpoint. There are two mechanisms that control progression through the G₂/M checkpoint. First though the action of p53, which initiates transcription of p21, Gadd45, and 14-3-3σ that sequester cyclin B/CDK1 complexes and displace cyclin B, therefore inhibiting M phase progression (246-248). The second control mechanism is p53-independent and is driven by the actions of chk1 and
chk2. DNA damage is sensed through two kinase proteins ATR and ATM that activate cell cycle suppressor proteins chk1 and chk2 that inactivate cyclin B/CDK1 activator cdc25, leading to a G2 arrest (249, 250). Activation of ATR/ATM furthermore causes p53 activation through phosphorylation and activation of p53 by ATR, ATM, chk1 and chk2, leading to stronger inhibition of M phase progression (251-254). The M phase of the cell cycle is the shortest of all phases and is where the nuclear envelope is broken down and the DNA is divided into two daughter cells. Arguably the most important regulators of M phase completion are the three cdc25 proteins A, B and C. cdc25A has a minor role in G1/S progression while cdc25B and C are key activators of cyclin B/CDK1 complex that functions in progression of G2 phase and initiation of a number of mitotic events. Other regulators include the 14-3-3 proteins and Polo-Like kinase 1 (PLK-1), a serine/threonine protein kinase whose levels increase during S phase and help activate cyclin B/CDK1 complexes through interactions with cdc25C (255). Other targets for PLK-1 include the anaphase-promoting complex (APC) and Mut1 (256). During M phase cyclin B/CDK1 complexes associate with centrosomes and promote separation through phosphorylation of the centrosome-associated motor protein Eg5. (257). Other activities of the cyclin B/CDK1 complex include breakdown of the nuclear lamina and fragmentation of the Golgi network (258, 259). Exit from mitosis requires both cyclin A and cyclin B to be degraded, this occurs through tagging for degradation by the APC ubiquitin ligase (260). The APC is a multi-protein complex that is inactive during the S and G2 phases and becomes active during M phase. Activation is stimulated through phosphorylation by the cyclin B/CDK1 complex once kinetochores are properly attached to the chromatids (261). Phosphorylation of the APC allows for binding of cdc20 that activates the APC ubiquitin ligase and allows for tagging and degradation of cyclin A and cyclin B and finally cytokinesis (Figure 1.4).

1.6 Viruses and the cell cycle

The process of cell division is highly complex, and requires interactions of hundreds of proteins. Each phase of the cell cycle is unique and provides different internal environmental conditions that can aid viral replication. Manipulation of the host cell cycle can cause a significant increase in viral reproduction and is essential for replication of some viruses that require access to particular host proteins and processes.
1.6.1 DNA viruses and the cell cycle

DNA viruses are the most documented in their effects on the host cell cycle. There are two main strategies used by DNA viruses to interfere with the cell cycle. Firstly, viruses with larger genomes often encode their own proteins that facilitate viral DNA replication. A typical example is provided by the herpesvirus family; including Epstein–Barr virus, cytomegalovirus and herpes simplex virus (262-265). These viruses induce cell cycle arrests inhibiting progression into S phase. The purpose is not entirely understood, but it is hypothesised that there will be less demand for deoxyribonucleotides when cellular DNA replication machinery is not active (266). More conventional DNA virus effects on the host cell cycle involve stimulation of cell replication so cells are progressing through S phase of the cell cycle during viral infection, where host DNA machinery is active and deoxyribonucleotides are present in high levels. Therefore the viral genome can be replicated using the host cellular proteins. DNA viruses that infect quiescent cells and do not carry their own DNA polymerase require these cells to enter cell division in order to replicate their genome. There are many mechanisms to induce cell cycle progression, such as papillomavirus that can induce S phase entry through the action of viral E6 and E7 proteins. The E7 protein associates with cell cycle regulator pRb causing release of transcription factor E2F, leading to the expression of proteins necessary for DNA replication, while E6 targets p53 for proteasomal degradation preventing a cell cycle arrest (267-269). The pRb protein is a common target used by viruses to induce cell cycle effects, adenoviruses also target this protein to induce S phase entry (270). Effects of viral proteins that have oncogenic properties can have unfortunate consequences for the host, such as Epstein-Barr virus, human papilloma virus, hepatitis B virus, and human herpes virus-8 that have the ability to cause cancers due to their effects on the cell cycle (271).

1.6.2 Retroviruses and the cell cycle

DNA viruses are not the only viruses that are documented to affect the host cell division. Some retroviruses also possess the ability to influence the host cell cycle. Retroviral replication is dependent on the ability to integrate viral cDNA into the genome of host cells (272). This process is dependent on the permeability of the host nuclear membrane, which is affected by cell division. Retroviral dependency on the nuclear membrane permeability is demonstrated in both murine leukemia virus and spleen necrosis virus that rely on cell
cycle progression into M phase in order for nuclear membrane breakdown and permeabilisation for viral DNA integration (273-275). Other retroviruses can integrate their genome into non-dividing cells but require cell cycle progression for later steps in the virus lifecycle (276, 277). Human T lymphotropic virus is known to induce human cancers in T-cells, due to effects on host cell division. Effects on the host cell cycle is induced through the interactions of the multifaceted, oncogenic, viral Tax protein (278). Another example is human immunodeficiency virus (HIV), whose actions are better characterised. HIV can gain access to the nucleus prior to M phase, but induces cell cycle effects after infection through the action of viral protein R (VPR) (279, 280). There have been several proposed models for cell cycle inhibition by the VPR protein; this includes inhibition of host cell cycle regulatory proteins CDK1 and cdc25 (281, 282). Others mechanisms include activation of the G2 checkpoint protein, ATR (283, 284) or activation of the transcription and expression of p21, a negative regulator of both G1 and G2/M phase transitions (285). The G2 arrest induced by the VPR protein of HIV is thought to aid viral replication in several ways, preventing T-cell clone replication (286) and providing a cellular environment for maximal levels of viral replication (287).

1.6.3 RNA viruses and the cell cycle

Interestingly, RNA viruses that replicate in the cytoplasm can also affect the cell cycle. Both negative-sense and positive-sense RNA viruses have been demonstrated to affect the host cell cycle. Influenza virus has a negative-sense RNA genome and induces a Go/G1 arrest through inhibition of cell cycle progression through the G1/S regulatory checkpoint (193). The cell cycle arrest is induced through the action of viral NS1 protein, inhibiting the RhoA GTPase and decreasing levels of the protein (288). As a consequences pRb phosphorylation is decreased and therefore the E2F transcription factor remains bound to pRb causing a Go/G1 arrest. Furthermore, the Go/G1 arrest incurs a benefit to viral replication as cells arrested in the G0 phase by serum withdrawal supported higher viral replication than an asynchronous population of cells (193). A Go/G1 phase arrest is also induced through infection with negative-sense RNA respiratory syncytial virus (289). Infection with respiratory syncytial virus decreases expression of the cell cycle regulators from the cyclin D family and the corresponding cyclin dependent kinases (CDK4 and CDK6) (195). The cell cycle arrest was likewise shown to benefit viral replication as chemical inhibition of CDK4
and CDK6 allowed for higher viral replication (195). Other negative-sense RNA viruses known to affect the host cell cycle include measles and simian viruses from the *Paramyxoviridae* family (290, 291). Three families of positive-sense viruses have been shown to induce host cycle affects upon infection. The Hepatitis C virus from the *Flaviviridae* family induces a G2/M phase arrest through the activity of viral NS5A protein, by down-regulation of the host Aspm protein (292). Several examples of picornaviruses influencing the host cell cycle have been demonstrated. Most recently the coxsackievirus from the *Picornaviridae* family has been shown to have higher replication in early and late G1 phase arrested populations (293). The persistence of coxsackievirus is thought to come from viral replication regulation by the host cell cycle. It is predicted that infection of G0 phase cells *in vivo* doesn’t support viral replication and when cells re-enter cell division where conditions are optimum for viral replication, this triggers coxsackievirus activation (294).

The most documented positive-sense RNA viruses that affect the host cell cycle are from the *Coronaviridae* family. The coronavirus effects on cell replication are particularly interesting because different viral species cause arrests in various phases of the cell cycle. Murine coronavirus induces a G0/G1 arrest through inhibition of cell cycle transition through the G1/S restriction checkpoint (194). The arrest is caused by the actions of viral protein p28 inducing an increase in host protein p53 levels and activity (295). The p53 protein then consequently stimulates an increase in cell cycle regulator p21, which inhibits G1/S cyclin/CDK complexes to induce a G1/S phase arrest and accumulation of cells in the G0/G1 phase. A G0/G1 phase arrest is also induced by severe acute respiratory syndrome coronavirus (SARS-CoV) through interactions of the viral 3A protein and nucleocapsid proteins (296, 297). Two other coronavirus species break the trend of inducing a G0/G1 arrest and have been discovered to induce cell cycle arrests in both the S and G2/M phases. Transmissible gastroenteritis virus and infectious bronchitis virus induce a late cell cycle arrests, that is favourable to viral replication (197, 298, 299). The reason RNA viruses induce a cell cycle arrest in the G2/M phase is not as clear as a G0/G1 arrest. The Golgi and ER is disrupted during mitosis and so any arrest prior to M phase may benefit viral replication as many viruses including coronaviruses utilise these organelles for protein processing and assembly (300, 301). Another possible advantage might be in optimal IRES-
dependent translation, as some studies showed higher translation from IRES sites when cells are in the G₂/M phase (302-304). IRES sites are commonly found in RNA viruses including hepatitis C virus, transmissible gastroenteritis virus and infectious bronchitis virus that all induce G₂/M arrests and so the cell cycle effects may benefit viral translation (305-309).

Viral effects on the host cell cycle are numerous and the list of recognised effects continues to grow as more viruses are characterised. The host cell cycle and viral replication are highly linked as viral infection influences the host cell cycle and the cell cycle affects viral replication. Replication of viruses is often dependent on cell division and the interactions essential to inducing these effects provide targets for inhibition of viral replication.

1.7 Research techniques for cell cycle analysis

1.7.1 Cell cycle analysis

The ability to distinguish cells in different phases of the cell cycle is an important technique when studying the host cell cycle. The most commonly used methods involve the use of flow cytometry to analyse the DNA content of cells. This can determine what phase a cell is in due to the doubling of DNA during cell division. As described in section 1.5, the DNA content of the cell is doubled as the cell duplicates its genome during S phase. Cells in the G₂ and M phases have twice the DNA content compared to those in the G₀ and G₁ phases, while S phase cells have varying amounts of DNA. The use of a fluorescent dye that stains the DNA of cells can be quantified by flow cytometry to visualise the different populations within a cell culture. Before analysis, the cells are fixed, permeabilised and RNA content degraded by an RNase, as most dyes will stain all nucleic acids and an active cell will have high levels of RNA that affect the fluorescence when detected by flow cytometry. A dye is then added (normally propidium iodide (PI)) that stains the nucleic acid quantitatively. The fluorescence of the dye is then detected by flow cytometry and the intensity measured proportionate to the amount of DNA within the cells. Flow cytometry histograms graphing relative cell count verses relative DNA content will show two peaks within a mixed population of cells, with the lower fluorescence intensity peak indicating the G₀ and G₁.
populations and the second peak with twice the fluorescence intensity indicating the G₂ and M population (Figure 1.5). Between the two peaks are cells with varying quantities of DNA that are the cells in the S phase. The data collected from the flow cytometer can be exported and analysed with programs that fit algorithms to the data to generate the percentages of cells in each phase. Flow cytometry using PI cannot distinguish between the G₀ and G₁ phase populations and the G₂ and M phase populations, as they appear in the same peaks, so they are named the G₀/G₁ and G₂/M populations respectively.

**Figure 1.5. Cell cycle analysis histogram**

Cell cycle analysis of a typical unsynchronised population of RAW-Blue cells. Cells were analysed by Fluorescence-activated cell sorting (FACS) of the DNA content of cells using PI as a nucleic acid dye. Data was exported and histograms generated in MODfit L.T 3.0.

### 1.7.2 Cell synchronisation

The ability to manipulate the stage of the cell phase is an important tool when studying viruses and the cell cycle. It allows investigation of a specific phase or synchronised progression through individual stages. There are numerous mechanisms to induce cell cycle changes to a cell population and each synchronisation protocol has a unique method. Most methods involve the introduction of a chemical that disrupts cellular processes, arresting proliferation of cells at a particular point, increasing the number of cells in a desired stage. The changes in cellular processes due to synchronising treatments using in this project are described below.
Serum Withdrawal – $G_0$ phase arrest

In the absence of growth factors, cells enter a non-dividing state called $G_0$ phase, characterised by low metabolic activity. This can be achieved relatively simply in a cell line by removing serum containing growth factors. Cells are blocked from progressing past the $G_1/S$ checkpoint and accumulate into $G_1$ then to $G_0$ as cell processes slow (310). Serum starvation causes a decrease in expression of Skp2, a cell cycle regulator that has multiple downstream effects. The Skp2 protein interacts with the c-myc protein, co-activating it and increasing expression of CDK2/4, stimulating $G_1/S$ progression. A decrease in Skp2 will cause a decrease in c-myc activation, and in turn causes a decrease in CDK 2/4 expression and an accumulation of cells in the $G_0$ phase (311). Skp2 also directs the degradation of CDI p27, an inhibitor of cyclin D-CDK4/6 and cyclin E-CDK2. So a decrease in Skp2 will lead to an increase in p27 activity and inhibition of cyclin D and E in complex with their corresponding CDK, resulting in an accumulation of cells into the $G_0$ phase.

$N$-butyrate – $G_1$ phase arrest

Sodium Butyrate (N-butyrate) is the sodium salt of butyric acid, a natural compound produced during anaerobic fermentation in mammals. It has various effects on cell culture, including changes to the cell cycle. It has been shown in several cell types to cause a reversible arrest in accumulation of the $G_1$ phase population (312). This is caused by the action of N-butyrate on histone deacetylases (HDAC), a class of enzymes that remove acetyl groups from histones, controlling among other functions, DNA expression. N-butyrate inhibits HDACs, leading to higher levels of acetylated histones. Acetylated histones have a lower affinity for DNA, resulting in an increase in transcription factor binding and an increase in transcription seen during the $G_1$ phase (313). Progression of the cell cycle out of $G_1$ is inhibited as N-butyrate down-regulates cell cycle proteins cyclin D1, cyclin A and c-myc (314). Cyclin D1 activity is needed for phosphorylation of pRb and progression through the $G_1/S$ checkpoint, while cyclin A is involved in S phase progression and c-myc is a transcription factor that leads to expression of several genes involved in proliferation. Expression of CDI p21 that controls G1/S transition is also increased during N-butyrate treatment. This causes inhibition of cyclin D-CDK1/2 and cyclin E-CDK2 complexes by p21, resulting in a $G_1$ phase arrest (314).
Genistein – G₂ phase arrest

Genistein is a naturally produced flavonoid that has reversible effects on the cell cycle, inducing a G₂/M arrest in cell culture (315). How genistein exactly induces its G₂/M arrest is not completely understood, as there are most probably several mechanisms of anti-proliferative activity. Genistein treatment to cell culture causes a decrease in activity of cyclin B1-CDK1 complexes and cdc25C (316). The protein complex cyclin B1-CDK1 helps promote the early events in mitotic entry. Its activity in turn is controlled by protein cdc25C, which dephosphorylates cyclin B1-CDK complex triggering M phase entry. So a decrease of activity in both cyclin B1-CDK1 complexes and cdc25C will cause an accumulation of cells in the G₂ phase. Genistein is proposed to arrest cells in late G₂ phase, this is supported by a decrease in mitotic index after genistein treatment (315, 317). Genistein is also a telomerase II inhibitor, which may contribute to a G₂ phase arrest. Telomerase II is a nuclear enzyme that resolves DNA tangling by forming transient breaks on both strands during DNA processes such as transcription or genome replication. Other similar non-intercalator telomerase inhibitors have been shown to evoke arrests around the G₂ phase. This suggests that genistein might too exert its effects on the cell cycle through telomerase II inhibition (315). Genistein has been shown to depolymerise microtubules during interphase in early M phase (317). Microtubule and tubulin dynamics help control cell cycle progress from G₂ through to the completion of mitotic phase. Normally agents that target microtubules cause an arrest during mitosis so the effect of genistein on microtubules may just contribute to an arrest around and G₂/M phases, with other factors responsible for pushing cells towards the G₂ phase. An increase in p21 is also seen in genistein treated cells. The p21 protein can inhibit cyclin B-CDK1 complexes and proliferating nuclear antigen. Inhibition of either one of these proteins can contribute to G₂ phase arrests (317).

Nocodazole – M phase arrest

Nocodazole is a common chemical used to reversibly arrest cells in early M phase (318). Nocodazole interferes with polymerisation of microtubules preventing chromosome separation and completion of mitosis. Microtubules are involved during cell division, forming a spindle apparatus that attaches to a set of chromosomes and a pole of the cell.
Upon anaphase, depolymerisation of microtubules results in contraction of the spindles and separation of sister chromatids to opposite poles. Blocking polymerisation of microtubules causes an arrest at prometaphase, as spindle fibres do not form and chromatid separation doesn’t occur, leading to accumulation of cells in early M phase (319). Treatment of cells with low levels of nocodazole also causes an increase in cyclin B1 and CDK1, important proteins that form a complex and regulate transition from G2 phase to M phase (320). Cyclin B1/CDK1 complex is involved in breaking the nuclear envelope, chromosome condensation and spindle pole assembly in early M phase. Flow cytometry analysis of nocodazole treated cells shows accumulation in G2/M, however the arrest is in prometaphase during early M phase. Furthermore, nuclear morphology and the mitotic index of nocodazole treated cells show that cells are predominantly arrested in prometaphase, a phase of mitosis (320, 321).

1.8 Research background

There are several ways to discover global changes to virally-infected populations of cells. One such method is through microarray analysis comparing changes in transcript expression between virus-infected and mock-infected populations of cells. Prior to this research project a microarray experiment was performed on MNV-1-infected RAW264.7 cells in order to give an insight into pathways and processes that the virus might be using to replicate. This provides a starting point for further research and is very useful if little is known about the virus replication. In an experiment conducted in the Ward laboratory, RAW264.7 cells were infected with MNV-1 for 18 hours and transcript levels were compared between mock and infected populations. Upon Gene Ontology Biological pathway analysis of microarray data revealed the transcripts that were most significantly down-regulated were those related to the cell cycle (97 genes), with a P-value of 3.1 X 10\(^{-53}\). Of these 97 transcripts coding for genes involved in cell cycle regulation, many cyclins and CDKs were down-regulated >2-fold including; CDK2, CDK4, cyclin A2, cyclin B1, cyclin B2, cyclin E1 and cyclin E2 (Figure 1.6) (published in (322)). Microarrays performed by from Bok et al, 2009 (141) concurred that there were global changes to the cell cycle pathway of MNV infected cells, but changes to individual cell cycle regulators showed some variance (Appendix III) (323, 324). Analysis revealed down-regulation of several transcripts involved
in cell cycle control that overlap with our own published data (322), including transcripts down-regulated >2 fold such as cyclin E1, cyclin E2, cyclin A2, cyclin B2 and CDK4. Although microarray data gave an insight into an interaction with the host cell cycle, it failed to identify any potential mechanisms of cell cycle control, due to the global effects on transcripts involved in all stages of division.

Figure 1.6. Changes in the expression of cell cycle-related genes during MNV-1 infection.

Figure from Davies et al, 2015 (322). Asynchronously growing RAW264.7 cells were mock-infected or MNV-1-infected for 18 hours prior to RNA extraction, and gene expression analysis on Affymetrix Mouse 430 2.0 microarrays. Genes with higher mRNA expression in MNV-1-infected populations compared to mock-infected are shown in orange to red (2-fold), and those with lower levels are in shades of green. Those in darkest green are reduced >5-fold. When multiple Affymetrix probe sets correspond to a gene, more than one bar is shown.

1.9 Focus of my research

Norovirus remains a huge financial burden in the developed world and in the developing world still contributes to high mortality rates. There are no antivirals or vaccines available
for norovirus and knowledge about viral replication is lacking due to difficulties in culturing HuNoV. In order to develop successful antivirals against norovirus, an increased knowledge of viral replication and interactions with host processes is needed. It is important to understand host cellular pathways that are manipulated and viral-host protein interactions that may be targeted in viral therapy. Microarray analysis of MNV-1-infected cells revealed changes to cell cycle transcripts, indicating an association between MNV-1 replication and cell division. The aim of my project was to address this observation and analyse the relationship between norovirus replication and the host cell cycle. Specifically, this was to characterise the effect of MNV-1 infection on the host cell cycle, using flow cytometry to monitor the effects of viral infection on cell cycle progression and Western blot analysis to measure changes to cell cycle regulators at a protein level. Furthermore, changes to the host cell cycle induced by MNV-1 infection was measured for its importance to the viral lifecycle, by comparing viral replication in different phases of the cell cycle. Lastly, the mechanism by which MNV-1 manipulates the cell cycle were explored using cloning of synthetic viral genes and in vitro expression of individual viral gene RNA transcripts. This project aimed to discover important host pathways and interactions that are used by MNV-1 to aid its own replication and provide important leads for treatments to target essential viral-host interactions.
2 Methods

2.1 Ethical permits and approvals
The use of a recombinant murine cell line (RAW-Blue™ cells, InvivoGen) was performed under ERMA approval NOC000707. Analysis of MNV-1 proteins in infected mammalian cells was covered by IBSC approval number GMO07/UO024 (ERMA approval numbers GMD005041, GMD005042, GMD005043). Analysis of MNV-1 proteins in transfected mammalian cells was covered by IBSC approval number GMD05/UO006 (ERMA approval numbers GMD003759, GMD003768).

2.2 RAW-Blue cells
RAW-Blue™ cells (mouse leukaemic monocyte macrophage cell line from BALB/c mice) (InvivoGen) are derived from RAW264.7 cells, with an NFκB/AP-1 inducible secreted embryonic alkaline phosphatase construct integrated into the chromosome. These cells support the replication of MNV-1 and were used for the analysis of the effect of MNV-1 infection on the cell cycle. Cells were maintained in DMEM (GIBCO, Thermo Fisher Scientific) and 10% heat inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific) (RAW med10%). For maintenance the RAW med10% was supplemented with penicillin (100 U/ml) (Roche Diagnostics), streptomycin (0.1 mg/ml) (Roche Diagnostics), normocin (100 µg/ml) (InvivoGen) and zeocin (200 µg/ml) (Thermo Fisher Scientific) and passaged in 75-cm² flasks when reaching 70 – 80% cell confluence (approximately 48 hours). Cells were dislodged using a cell scraper, pelleted (400 g, for 5 minutes), washed with 5 ml RAW med10% and pelleted again (400 g, for 5 minutes). Approximately 2.5 × 10⁶ cells were then resuspended in RAW med10% in 75-cm² flasks. All RAW-Blue cell incubations were performed at 37 °C with 5% CO₂.

2.3 Storage of RAW-Blue cells
RAW-Blue cells were scraped and washed as per normal passage and resuspended in freezing medium (70% DMEM, 20% dimethyl sulfoxide (DMSO), 10% FBS) at 3 × 10⁶
cells/ml. Aliquots of cells (1 ml) were put in cryotube vials, cooled to –80 °C in a Nalgene™ Cryo Freezing container and then transferred to liquid nitrogen.

To recover RAW-Blue cells from liquid nitrogen storage, a vial was thawed at 37 °C in a water bath for 1 minute and added to 4 ml of pre-warmed RAW med10% in a 25-cm² flask. The medium was replaced with RAW med10% the following day once viable cells had adhered to the flask. The RAW-Blue cells were transferred to a 75-cm² flask after approximately 48 hours once they reached 70 – 80% confluence and maintained as above in RAW med10% + antibiotics.

2.4 Preparation of MNV-1 stock
MNV-1 was previously generated through reverse genetics within the lab, with a single base pair mutation creating a silent EcoRV restriction site in the parental virus, MNV CW1.P3 (Appendix II) (325). MNV-1 stocks were propagated in RAW-Blue cells at 80% confluence. MNV-1 was added to 175-cm² flasks at a multiplicity of infection (MOI) of 0.1, and incubated for 48 hours. After infection, flasks were freeze thawed to –80 °C two times to disrupt infected cells. Cell debris was removed through centrifugation at 1000 g, for 20 minutes. In order to remove cytokines such as IFN type 1 from the crude inoculum, a purified MNV-1 stock was generated. The supernatant from the infected cells was further centrifuged at 112,700 g for 4 hours over a 30% w/v sucrose cushion in an L-90K ultracentrifuge, using a SW32 Ti rotor (Beckman Coulter). The supernatant was discarded and the virus pellet resuspended overnight at 4 °C on a rocker in 1X Dulbecco’s phosphate buffered saline (dPBS). The following day the resuspended pellet was filter sterilised through a 0.45 μm filter, divided into 1 ml aliquots and stored at –80 °C awaiting titration by plaque assays.

2.5 Cell synchronisations
Different synchronisation methods were initially screened for their ability to induce cell cycle effects on RAW-Blue cells. Selected synchronisation methods were then optimised to generate high numbers of a single-phase population or synchronised progression through individual stages. Varying dose ranges and treatment times were trialled to minimise any
effects of treatments on cell viability and virus infection. The methodology presented is the final dosage and incubation lengths used in all experiments.

2.5.1 G₀ – Serum withdrawal
Subconfluent cultures of RAW-Blue cells were synchronised into the G₀ phase with serum withdrawal. In 6-well plates, cells were seeded at $5 \times 10^5$ cells/well and at $1.5 \times 10^6$ cells in 25-cm$^2$ flasks. Cells were left to adhere before the medium was removed and the cell monolayer washed three times in FBS-free DMEM. To each well of the 6-well plates, 2 ml of FBS-free DMEM was added and 5 ml to the 25-cm$^2$ flasks. Plates and flasks were incubated for 72 hours to generate a G₀ phase population.

2.5.2 G₁ – N-butyrate
Exponentially growing RAW-Blue cells were seeded in RAW med10% in 6-well plates at $8 \times 10^5$ cells and at $2 \times 10^6$ cells in 25-cm$^2$ flasks. The following day the RAW med10% was replaced and N-butyrate (Sigma-Aldrich) added to a concentration of 3 mM. The cells were incubated for 20 hours to allow synchronisation into the G₁ phase.

2.5.3 G₂ – Genistein
Exponentially growing RAW-Blue cells were seeded in RAW med10% in 6-well plates at $6 \times 10^5$ cells/well and at $1.5 \times 10^6$ cells in 25-cm$^2$ flasks. The following day the RAW med10% was replaced and genistein (Sigma-Aldrich) was added to a concentration of 100 µM, cells were incubated for 48 hours to synchronise cells to the G₂ phase.

2.5.4 M - Nocodazole
Exponentially growing RAW-Blue cells were seeded in RAW med10% in 6-well plates at $1 \times 10^6$ cells/well and at $2.5 \times 10^6$ cells in 25-cm$^2$ flasks. The following day the RAW med10% was replaced and nocodazole (Sigma-Aldrich) was added to a concentration of 50 ng/ml and incubated for 10 hours to synchronise cells to the M phase.

2.5.5 G₁> - G₁ progressing
Approximately $2.5 \times 10^6$ RAW-Blue cells were seeded in 25-cm$^2$ flasks and synchronised to M phase with nocodazole treatment as above. After synchronisation, the monolayer was washed 3 times in RAW med10%, and 5 ml of RAW med10% added. Cells were incubated for 3 hours, in this time the cells progress out of M phase into early G₁ phase. The now G₁
phase cells were scraped, pelleted (400 g for 5 minutes) and plated in RAW med10% at 1 × 10⁶ cells/well.

### 2.6 Infections

RAW-Blue cells were seeded in 6-well plates at various concentrations as stated. After adhesion, 1 ml of medium was removed, discarded and then cells were infected with RAW med10% alone (mock-infected) or with MNV-1 at the stated MOI and incubated for 1 hour with gentle rocking every 15 minutes. Following virus adsorption, the supernatant was removed and the cells were washed with 500 µl of RAW med10%, then 2 ml of RAW med10% was added to each well. The cells were incubated for the times indicated.

### 2.7 Plaque assay

MNV-1 viral titre was measured by crystal violet plaque assays as previously described (326). Briefly, RAW-Blue cells were seeded in 6-well plates at 1.4 × 10⁶ cells/well and grown overnight to approximately 80 – 90% confluence. After the overnight incubation, 1 ml of medium was removed prior to infection with 10-fold virus dilutions prepared in DMEM (200 µl of 10⁻³ – 10⁻⁸) and the plates incubated for 1 hour with regular rocking every 15 minutes. Following adsorption, the inoculum was removed, the cells washed once in 500 µl of RAW med10% and overlaid with 2 ml of overlay mixture per well (0.35% sea plaque agar (Lonza), 3% FBS, penicillin (100 U/ml) and streptomycin (100 U/ml)), allowed to set and then incubated for 48 hours. After plaques formed, cells were fixed by adding 1 ml of 5% formaldehyde directly onto the overlay, followed by a 30 minute incubation at room temperature. The agarose-formaldehyde mixture was removed and the cells rinsed twice with 2 ml MilliQ water to remove the entire overlay before staining in 1 ml 0.2% crystal violet solution (Appendix I) for 5 minutes at room temperature. The stain was removed, the plaques counted and the titre determined accordingly.
2.8 Analysis of protein expression

2.8.1 Cell harvest for SDS-PAGE gel

Cells were scraped, pelleted (400 × g for 5 minutes), the supernatant discarded and the cells resuspended in 1 ml of dPBS. The sample mixture was split in two and pelleted (400 × g for 5 minutes) and the supernatants discarded. One sample mixture was lysed in 200 µl RIPA buffer (Appendix I) for BCA protein assay quantification and the other in 25 µl 1X dPBS and 25 µl 2X sample buffer (Appendix I) for loading to a SDS-PAGE gel. All samples were stored at −80 °C until analysed.

2.8.2 Pierce™ BCA protein assay kit

In order to load equal quantities of protein in each well, protein levels in each sample were first determined by a BCA Protein Assay. Protein concentrations in cell lysate samples were determined using Pierce™ BCA Protein Assay Kit following manufacturer’s instructions. Briefly, cells were collected from 6-well plates and lysed in 200 µl RIPA buffer. BSA standards of 2000, 1000, 750, 500, 250, 125 and 0 µg/ml were made. BSA standards were added in duplicate (25 µl per well) to a 96-well plate along with samples to be quantified (25 µl), in triplicate. If the protein concentration in the samples and did not fall within the BSA standard curve a 1:2 dilution was prepared with RIPA buffer. The BCA working reagent (50:1 Reagent A: Reagent B) (200 µl) (Sigma-Aldrich) was added into each well and the plate incubated at 37 °C for 30 minutes. Absorbance was measured at 595 nm and analysed using Prism software (GraphPad Software) to calculate protein quantities for equal loading of samples in SDS-PAGE gels.

2.8.3 Making SDS-PAGE gels

SDS-PAGE acrylamide gels were used to visualise protein expression by Coomassie staining and Western blot analysis. A resolving gel mix (Appendix I) was prepared, and poured into a gel cast sandwich (Atto Corporation) and overlaid with water-saturated butan-1-ol, and left for 30 minutes to polymerise. The butan-1-ol was then removed and the stacking gel mix (Appendix I) poured on top of the set resolving gel until flush with the top of the plates. A comb was carefully added to prevent bubbles and the overflowing gel mix wiped away. The stacking gel was left to polymerise for 30 minutes.
2.8.4 Migration of SDS-PAGE gels

Whole-cell lysates in 1X dPBS and 2X sample buffer were boiled for 10 minutes prior to separation by SDS-PAGE. Approximately 10 µg of protein was added to each well of a SDS-PAGE gel and proteins separated at 170 V and 250 mA, in 1X electrophoresis buffer (Appendix I), for approximately 90 minutes.

2.8.5 Western blotting

For detection of target proteins, samples were either transferred to nitrocellulose membrane for detection by infrared fluorescence or PDVF membranes for detection by chemiluminescence. Unless stated, protein detection occurred using immunofluorescence detection on nitrocellulose membrane.

Infrared fluorescence detection

For detection of target proteins, the gel proteins were transferred to nitrocellulose membranes using a Trans-Blot SD Semi-Dry transfer cell (Bio-Rad). Briefly, the SDS-PAGE gel was soaked for 20 minutes in Cathode buffer (Appendix I). Meanwhile, six pieces of Whatman™ 3MM Chr Chromatography paper and a nitrocellulose membrane (Amersham Hybond-C extra, GE Healthcare) was cut to the size of the gel. The nitrocellulose paper was wet in milliQ water and soaked for 5 minutes in cathode buffer. Two pieces of filter paper were soaked in anode I buffer and assembled on the bottom of the SD transfer platform followed by one piece of filter paper soaked in anode II, the nitrocellulose membrane, the SDS-PAGE gel and three pieces of cathode buffer soaked filter paper. Air bubbles were removed from the stack using a blotting roller and proteins transferred at 250mA, 20 V for approximately 25 minutes. The length of the transfer time was adjusted slightly depending on the protein size of interest. For detection of smaller proteins (<25kDa), and larger proteins (>90 kDa) membranes were transferred for 20 and 30 minutes respectively. After the transfer, nitrocellulose membranes were washed in 1X dPBS for 5 minutes on a rocker and dried overnight on filter paper.

Following drying, membranes were probed for protein detection using specific antibodies. The dried membranes were wet in 1X dPBS and incubated in blocking solution (Appendix I) for 1 hour. Following blocking, membranes were incubated for 1 hour in primary
antibodies, then washed for 4 × 5 minutes in 10 ml washing solution (Appendix I). After washing the membrane was incubated with corresponding secondary antibodies for 1 hour in the dark, then washed 4 × 5 minutes in 10 ml washing solution before band detection. All antibody incubations were done in blocking solution + 0.1% Tween-20. All incubations were done at room temperature and washes were performed in a tube bottle roller. Band signals were visualised using a LI-COR Biosciences Odyssey Fc Imager. Target signals was analysed in using ImageStudio software version 3.1.4 (LI-COR Biosciences) and normalised to actin fluorescence. Antibodies concentrations used are shown in (Table 1.1).

Chemiluminescence detection

Detection of target proteins by chemiluminescence uses an identical protocol to infrared detection with a few exceptions. Proteins are transferred to a Immobilin-P PDVF (Millipore), which is soaked in methanol, rinsed in milliQ water for 2 min then equilibrated in anode buffer II (Appendix I) for 5 min prior to the transfer. Following transfer, the membrane was blocked in 1% BSA solution (Appendix I) for 1 hour at room temperature, or overnight at 4 °C. The membrane was incubated in primary antibody diluted in 1% casein alanate solution, washed as described previously, incubated with the secondary antibody for 1 hour, prior to another wash step and detected by development using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

2.8.6 Nitrocellulose membrane stripping

If required, the membrane was stripped and re-probed with new antibodies. Stripping was done with Odyssey stripping buffer (LI-COR) as per protocol. Briefly, membranes after imaging were kept wet in 1X dPBS. Membranes were incubated in 20 ml of 1X Odyssey stripping buffer on a rocker for 5 minutes, and then rinsed in 1X dPBS. The membrane was incubated twice more in stripping buffer followed by washing in 1X dPBS. After stripping the membrane was treated as new and went into blocking buffer and probed as described above.
Table 1.1. Dilutions of primary and secondary antibodies for Western blot analyses

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<th>Antibody</th>
<th>Dilution</th>
<th>Source</th>
<th>Catalogue Code</th>
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</thead>
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<td>Ward Laboratory</td>
<td>n/a</td>
</tr>
<tr>
<td>Rabbit α-N54 (3A)</td>
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<td>Ward Laboratory</td>
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</tr>
<tr>
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<td>Ward Laboratory</td>
<td>n/a</td>
</tr>
<tr>
<td>Rabbit α-VP1 (Capsid)</td>
<td>1:2000</td>
<td>Ward Laboratory</td>
<td>n/a</td>
</tr>
<tr>
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<td>1:2000</td>
<td>Santa Cruz</td>
<td>sc-1616</td>
</tr>
<tr>
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<td>abcam</td>
<td>ab166663</td>
</tr>
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<tr>
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<td>1:500</td>
<td>Sigma-Aldrich</td>
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<td>1:4000</td>
<td>Sigma-Aldrich</td>
<td>A9168</td>
</tr>
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</table>

1Antibodies produced by E. Baker with specificity confirmed in (101).
2Monoclonal
3Polyclonal

2.9 Cell cycle analysis

2.9.1 DNA content analysis – Flow Cytometry

The percentage of cells in each phase of the cell cycle was determined through propidium iodide staining and FACS analysis of the DNA content of cells. Upon harvesting, cells were scraped, transferred to 15 ml tubes, pelleted at 400 × g for 5 minutes, the supernatant discarded and cells fixed in 3 ml 70% absolute ethanol (–20 °C) (dropwise resuspension). Ethanol works as both a fixative and permeabilising agent. After >12 hours the cells were pelleted at 800 × g for 5 minutes (a higher centrifuge force is required to pellet fixed cells) and washed twice in 5 ml FACS buffer (Appendix I) to remove the ethanol. The cells were resuspended in 500 µl staining buffer (50 µg/ml propidium iodide [Sigma-Aldrich], 0.1
mg/ml RNase A [Sigma-Aldrich] in FACS buffer) (Appendix I) and incubated for 45 minutes at 37 °C + 5% CO₂. After incubation, cells were transferred to FACS tubes (10 ml) and analysed using FACS (BD FACScalibur or BD Fortessa). Three lasers were used FSC, SSC and YG_610/20 on a linear scale. At least 10,000 cells were counted for each sample, data was analysed with MODfit L.T. 3.0 software (Verity Software House).

2.9.2 MODfit L.T. 3.0

MODfit is a program with a specific purpose for analysing DNA content of cells. FSC 3.0 files from FACS machine program BD FACSDiva Software 6.0 were opened with MODfit and gated to remove doublets and debris. Parameter YG_610/20-A is used to analyse the data as this detects the emission wavelengths of propidium iodide. The gates used are SSC-A v.s. FSC-A for debris exclusion and SSC-A v.s. SSC-H, and FSC-A v.s. FSC-H for doublet exclusion. Linearity was set to 1.97 G₂/G₁ ratio, with one cycle and auto debris removal. A CV value of <5 was targeted, but values <10 were accepted. An example of gating is shown in Figure 2.1.

Figure 2.1. Example of gating for FACS analysis of the cell cycle.

Gating was used to include only single, viable cells in the analysis and eliminate any debris, dead cells and clumps or doublets.

2.10 Clone design

2.10.1 Outline of clone construction

Cloning experiments aimed to generate RNA transcripts encoding a single viral gene or segment of gene for transfection into RAW-Blue cells. Constructs needed to contain flanking restriction sites for cloning as well as the appropriate sequences for RNA synthesis and protein expression. Constructs used for cloning and protein expression had either previously been generated within the laboratory (101), or were designed and ordered
though GenScript as a synthetic plasmid. Previously, pUC8 plasmids containing viral protein sequences for full length NS1-2 and NS5 were transfected into calcium competent *E. coli* cells and stored at –80 °C, awaiting purification of plasmids and generation of RNA transcripts (101). New constructs were generated expressing three different partial sections of NS5 within a plasmid and were used to generate RNA for transfection and expression of protein.

### 2.10.2 Design of NS5 plasmids

Constructs were designed using SeqBuilder and Protean from the Lasergene suite of sequence analysis software (DNASTAR). Three plasmids were designed to contain an NS5 insert sequence flanked by restriction sites BamHI (5’) and HindIII (3’), a T7 promoter sequence, a Kozak sequence for optimal RNA transcription upstream of the NS5 sequence, followed by a stop codon (Figure 2.2). Plasmid sequences for NS5 were from MNV-1 clone CW1 (GenBank: EF014462.1). The NS5 protein from MNV is 124 amino acids long. Three NS5 constructs were designed expressing NS5 amino acids segments 1-62 (NS5 1-62), 63-124 (NS5 63-124) and 11-107 (NS5 11-107). Three nucleotide sequences were inserted into the synthetic plasmid pUC57-simple as shown in Figure 2.3, Figure 2.4 and Figure 2.5.

![Figure 2.2. Design overview for the three synthetic NS5 constructs.](image)

Each construct contained the consensus sequences of BamHI, T7 promoter, Kozak sequence, methionine (Met), stop codon (Stop) and HindIII. Three different NS5 sequences were ordered, expressing different sections of the NS5 gene.
Sequences were obtained from MNV-1 clone CW1 and modified in SeqBuilder, the NS5 1-62 sequence is shown in green. Other annotated features include the nucleotidylation site (red), hydrophobic interactions (yellow) and polar interactions (pink). The three structural helices are indicated in orange as well as added restriction enzyme sites flanking the sequence, the T7 promoter sequence, a Kozak sequence, a methionine and a stop codon.

Figure 2.3. Annotated NS5 1-62 sequence insert.
Figure 2.4. Annotated NS5 63-124 sequence insert.

Sequences were obtained from MNV-1 clone CW1 and modified in SeqBuilder, the NS5 63-124 sequence is shown in green. The third structural helix and eIF recruitment site is indicated in orange as well as added restriction enzyme sites flanking the sequence, the T7 promoter sequence, a Kozak sequence, a methionine and a stop codon.
Figure 2.5. Annotated NS5 11-107 sequence insert.

Sequences were obtained from MNV-1 clone CW1 and modified in, the NS5 11-107 sequence is shown in green. Other annotated features include the nucleotidylation site (red), hydrophobic interactions (yellow) and polar interactions (pink). The three structural helices are indicated in orange as well as added restriction enzyme sites flanking the sequence, the T7 promoter sequence, a Kozak sequence, a methionine and a stop codon.
2.11 Cloning and screening of NS5 constructs

2.11.1 Overview

The three synthetic plasmids obtained from GenScript (NS5 1-62, NS5 63-124 and NS5 11-107) were cloned into *E. coli* and screened for their correct insert sizes. Plasmids were received in lyophilized form and reconstituted before transformation and blue-white screening in calcium competent XL1-Blue *E. coli* cells. Following transformation, single colonies displaying positive transformation was were screened by plasmid extraction, restriction digest and visualisation of the digested plasmids on DNA agarose gels. The *E. coli* clones containing the NS5 1-62, NS5 63-124 and NS5 11-107 synthetic plasmids were stored at –80 °C in 20% glycerol in Cryotube vials (Nalge Nunc).

2.11.2 Reconstitution of synthetic plasmid containing inserts encoding NS5 constructs

Each engineered genome segment was synthesised and cloned into a pUC57-simple plasmid vector and delivered in a lyophilised powder. The pUC57-simple plasmid contains an ampicillin resistance gene, an origin of replication and a disrupted LacZ gene (Appendix II). Following the recommendations of GenScript, the vials containing each of the NS5 constructs in the pUC57-simple plasmid were centrifuged to avoid loss of material during opening. Reconstitution was achieved by resuspension in 20 µl of sterilised milliQ water.

2.11.3 Preparation of competent *E. coli* cells

XL1-Blue MRF *E. coli* cells were streaked from –80 °C stocks onto an LB agar plate containing 10 mM magnesium chloride and 12.5 µg/ml tetracycline. The plate was incubated at 37 °C overnight and a single colony used to inoculate 5 ml of LB broth containing 50 µg/ml ampicillin. After 14 – 16 hours of growth at 37 °C (agitation at 200 rpm), 2 ml of the culture was transferred to 120 ml of pre-warmed LB broth and growth was continued at 37 °C with agitation (200 rpm) until reaching an OD$_{600}$ (optical density) of between 0.35 and 0.4. The culture was then cooled on ice for 15 minutes, divided into four pre-chilled JA-20 tubes (Beckman Coulter) and centrifuged at 3000 × g for 10 minutes at 4 °C. The supernatant was discarded and each aliquot of cells resuspended in 10 ml of Transformation Buffer 1 (Appendix I) and combined into two tubes. The cells were pelleted again at 3000 × g for 10 minutes at 4 °C, the supernatant discarded and the two cell pellets each resuspended in 2 ml of Transformation Buffer 2 (Appendix I). The resuspended cells
were divided into 100 μl aliquots in microfuge tubes, snap frozen in dry ice and ethanol, and stored at −80 °C.

2.11.4 Transformations
An aliquot (100 μl) of calcium competent *E. coli* cells from the −80 °C stocks was thawed on ice for 30 minutes, before addition of 1 – 3 μl of reconstituted plasmid. A further 15 – 30 minute incubation period (on ice) was followed by 5 minutes at 37 °C, before addition of 900 μl of pre-warmed LB broth and a 45 – 60 minute shaking incubation at 37 °C. The transformed cells (200 μl) were spread onto LB agar containing 50 μg/ml ampicillin. To allow for blue/white selection, 50 μg/ml of 5-Bromo-4-chloro-3-indoly1 β-D-galactopyranoside (X-gal) (Progen Pharmaceuticals Limited) and 12 μg/ml of isopropyl-β-D-1-thiogalactopyranoside (IPTG) (Progen) were added. The plates were left to stand for ten minutes, before inversion and overnight incubation at 37 °C.

2.11.5 Plasmid extraction (Miniprep)
Transformed colonies were screened for correct insert size using Nucleospin Miniprep plasmid extraction (Macherey-Nagel). White colonies were picked from transformed overnight plate cultures and used to inoculate 5 ml of LB broth containing 50 μg/ml ampicillin. Incubation was performed at 37 °C with agitation (200 rpm) for approximately 16 hours, until reaching an OD₆₀₀ of 0.6 – 1.0. Plasmids were extracted as per manufacturer’s protocol. Briefly, 3 ml of overnight *E. coli* culture was pelleted at 12,000 × g for 1 minute and the supernatant discarded. Cells were lysed through resuspension in 150 μl buffer A1 and vortexing to completely resuspend cells, then addition of 250 μl buffer A2 and incubation for 2 minutes at room temperature. Directly following, 350 μl buffer A3 is added and the microcentrifuge tube inverted until the solution cleared. After lysis, cell debris was removed through centrifuging at 12,000 × g for 3 minutes. The supernatant was loaded into a NucleoSpin plasmid column in a collection tube and centrifuged for 30 seconds at 1,500 × g. The flow-through was discarded the pellet was dried with a dry spin of 1,500 × g for 30 seconds. The column was placed in a new 1.5 ml microcentrifuge tube and the DNA was eluted with a 1 minute room temperature incubation in 30 μl milliQ water and centrifuging at 12,000 × g for 1 minute.
2.11.6 Double restriction digests
To confirm the correct insert size of the NS5 constructs, restriction digests were preformed to digest the viral insert at flanking restriction sites. For the double digest on plasmids NS5 1-62, NS5 63-124 and NS5 11-107, approximately 2 μg DNA, 2 μl 10X Buffer B (Roche), 5 Units of BamHI, 5 Units of HindIII and milliQ water to a final volume of 20 μl was incubated at 37 °C for 2 hours. Following the digest the DNA was analysed on a DNA agarose gel for correct plasmid and insert sizes.

2.11.7 DNA agarose gel electrophoresis
Agarose gel electrophoresis was performed on digested DNA using Agarose LE (Roche) in 1X TAE (Tris-acetate) buffer (Appendix I) in a mini gel system (Bio-Rad). Loading dye (3 μl, Appendix I) was added to each sample prior to loading and the gel was run in 1X TAE Buffer at 100 volts, 400 mA for 1 hour. The gel was then stained in ethidium bromide solution (1 μg/ml) for 1 hour and DNA bands were visualised and photographed using ultraviolet (UV) light in a ChemiDoc™ gel documentation system (Bio-Rad).

2.12 Generation of RNA transcripts

2.12.1 Overview
RNA transcripts were generated from five E. coli cultures containing plasmids expressing viral NS5 1-62, NS5 63-124, NS5 11-107, full length NS1-2 and full length NS5. As stated earlier, E. coli cultures expressing full length NS1-2 and full length NS5 within pUC8 plasmids were generated previously within the laboratory (101), while E. coli cultures expressing NS5 1-62, NS5 63-124, NS5 11-107 within pUC57-simple were generated from synthetic plasmids, obtained from GenScript. To generate plasmids for RNA transcription, E. coli containing plasmids with viral sequences were amplified, extracted, linearised with restriction sites at the 3’ end of their viral sequences and purified by ethanol precipitation. The plasmids were checked for linearisation on a DNA agarose gel, quantified on a NanoDrop 1000 Spectrophotometer (Version 3.7.1, Thermo Scientific) and used to generate capped mRNA transcripts.
2.12.2 Plasmid extraction (Midiprep)

Plasmids were extracted for linearisation and subsequent RNA transcription using HiPure plasmid Midiprep plasmid extraction kit (Invitrogen) as per manufacturer’s instructions. Briefly, 50 ml of E. coli cultures containing plasmids were grown overnight until reaching an OD$_{600}$ of 0.6 – 1.0, decanted into a 50 ml falcon tube and the cells pelleted at 4,000 × g for 10 minutes. The supernatant was discarded and the cells resuspended in buffer R3. Following, 4 ml of lysis buffer L7 was added and the cells incubated for 5 minutes at room temperature. Succeeding the incubation, 4 ml of precipitation buffer N3 was added and the cells were pelleted at 12,000 × g for 10 minutes. The supernatant was drained through a filter that had been pre-equilibrated with 2 × 10 ml of wash buffer W8. The filter was placed on a new column and the DNA eluted with 5 ml buffer E4. To the flow through, 3.5 ml of isopropanol was added before centrifuging at 12,000 × g for 30 minutes at 4 °C. Following the spin, the DNA was washed in ethanol by discarding the supernatant and adding 3 ml of ice cold 70% ethanol. The DNA was aliquoted into microcentrifuge tubes and centrifuged at 12,000 × g for 5 minutes at 4 °C. The supernatant was then removed and the pellet air-dried then resuspended in 35 μl sterile milliQ water and stored at −80 °C.

2.12.3 Restriction Digests

Linearisation of plasmid constructs were all achieved in a 50 μl reaction mixture. Each digest contained 16 - 19 μg of DNA, 20 Units of the required restriction enzyme, 5 μl of the appropriate buffer (supplied by Roche or New England Biolabs (NEB) Inc.) and milliQ water was added to a final volume of 50 μl. The reaction mix was incubated at 37 °C for 3 hours then DNA agarose gels performed to check for linearisation.

2.12.4 Purification from restriction digest

Digested DNA was purified after restriction digests by ethanol precipitation. To each reaction the following was added; 1/20 volume of 0.5 M EDTA, 1/10 volume of 3 M sodium acetate and a 2X reaction volume of ~20 °C ethanol and the reaction mix incubated at ~20 °C for 45 minutes before pelleting the DNA at 13,000 × g for 15 minutes at 4 °C. The supernatant was removed and the pellet air-dried before resuspending in 20 μl of 0.1X TE buffer (Appendix I). A 1/10 dilution was prepared for analysis using a NanoDrop Spectrophotometer to measure the DNA concentration and purity. The remainder was stored at −20 °C.
2.12.5 RNA synthesis from linearised plasmids

Linearised plasmids were used to generate RNA transcripts using the mMessage mMachine T7 Ultra kit (Ambion, Life Technologies) following manufacturer’s instructions. Briefly, to generate RNA transcripts, to a RNase-free microcentrifuge tube; 10 µl T7 2X NTP/ARCA, 2 µl 10X T7 Reaction Buffer, 1 µg linearised DNA template, 2 µl T7 enzyme mix and nuclease-free water to 20 µl were added. The reaction mix was incubated at 37 °C for 2 hours after which 1 µl TURBO DNase reagent was added to degrade the DNA plasmid and the reaction was incubated for a further 15 min. To add the poly(A) tail, 20 µl mMessage mMachine T7 Ultra reaction, 36 µl nuclease-free water, 20 µl 5X E-PAL buffer, 10 µl 25 mM MnCl₂, 10 µl 10 mM ATP solution and 4 µl E-PAL was added. The reaction mix was incubated at 37 °C for 45 minutes and transferred immediately to ice. Recovery of the RNA was performed using MEGAclear kit (Ambion) following manufacturer’s instructions. RNA was diluted 1:10 in milliQ water and the concentration and purity was measured using a NanoDrop Spectrophotometer. RNA was stored at –80 °C in 4 – 5 µg aliquots.

2.13 RNA transfections

Transfection of RNA transcripts into RAW-Blue cells was performed using a Neon Transfection system (Life Technologies), following manufacturer’s instructions. Briefly, RAW-Blue cells were scraped and centrifuged at 400 × g for 5 minutes and resuspended in 10 ml 1X dPBS. The cells were counted and the cells centrifuged at 400 × g for 5 minutes. Cells were resuspended at 1 × 10⁷ cells/ml in resuspension buffer (Appendix I). RNA (4 – 5 µg) was combined with 100 µl of resuspended cells (1 × 10⁵). Cells were drawn into a Neon pipette tip, placed in the transfection system and transfected using 1 pulse of 1730 V and 20 mA. Transfected cells were added to 2 ml of pre-warmed RAW med10% in a 6-well plate and incubated for the times indicated.

2.14 Validation of NS5 construct expression

To validate expression of NS5 constructs, samples were processed for Western blot detection, as discussed previously, or mass spectrometry. For mass spectrometry, samples were separated by SDS-PAGE gel electrophoresis on 12.5% SDS-PAGE acrylamide gels, with solutions and gels made and filter sterilised through a 22 µm filter in a class-2 biological
hood. Proteins were separated on a 12.5% SDS-PAGE gel and stained overnight with colloidal Coomassie (Appendix I). The following day, the gel was washed in destain solution (Appendix I) for 24 hours and detected by mass spectrometry at the Centre of Protein Research (University of Otago).

2.15 Statistical analysis
Statistical analysis was determined using Prism software (Version 6.0, GraphPad Software, Inc). Statistical significance was determined using the appropriate test as stated. Error bars in graphs represent means and standard deviation (SD).
3 Results

3.1 Characterisation of MNV-1 effects on the host cell cycle

3.1.1 Introduction
The host cell cycle has been shown to have significant effects on viral replication. Analysis of two independently performed microarrays revealed changes to cell cycle transcripts during MNV infection (accession numbers GSE12518 and GSE61562) (141, 322). Multiple pathways involved in regulation of the host cell cycle were down-regulated, with significant overlap between microarrays. Viral manipulation of the cell cycle is becoming a more commonly recognised occurrence, as research groups understand how viruses create conditions to favour their own replication. The relationship between the Caliciviridae family and the host cell cycle has yet to be explored and has not been studied with noroviruses. The aim of this research was to test the effect of MNV-1 infection on the host cell cycle. Expression of key regulators of cell replication were examined post MNV-1 infection. Additionally, the host cell was analysed for cell cycle changes post MNV infection and transition through cell cycle restriction points was analysed.

3.1.2 Synchronisation
An essential task in studying cell cycle related processes is synchronisation of cells into specific cell phases. Protocols were developed for synchronisation of populations of RAW-Blue cells into G₀, G₁, G₁ progressing, G₂ and M phases. Chemical inhibition as well as serum starvation was used to generate each population. Different concentration ranges, cell densities and incubation times were tested to get a high percentage of cells in the desired phase, while minimising the toxicity to the cells. Results presented are the final results after a series of optimisation experiments.
\textit{G}_0 \textit{phase synchronisation}

Cells were synchronised into the \textit{G}_0 phase with serum withdrawal. Cells were incubated in serum-free medium (DMEM) devoid of FBS for 48 and 72 hours. Cells accumulated into the \textit{G}_0/\textit{G}_1 phase post-serum withdrawal. At 48 and 72 hours post-serum withdrawal approximately 65\% and 80\% of cells were accumulated into \textit{G}_0/\textit{G}_1 phase respectively (Figure 3.1). The \textit{S} phase population decreased proportionally, as the \textit{G}_0/\textit{G}_1 phase population increased. Treatment of cells with serum withdrawal for 72 hours was used from here on to generate a \textit{G}_0 population.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{diagram.png}
\caption{Synchronisation of cells into \textit{G}_0 phase.}
\end{figure}

Asynchronously growing RAW-Blue cells were seeded in 6-well plates at approximately $6 \times 10^5$ cells/well and the following day washed three times in serum-free medium. Cells were incubated in serum-free medium for the indicated times. (A) Cells were harvested and analysed for FACS analysis of the cell cycle. (B) The histograms from (A) were analysed with MODfit LT 3.0 and the percentage of cells in each phase of the cell cycle is shown. The results are shown as means (+SD) for three independent experiments.
**G**<sub>1</sub> **phase synchronisation**

RAW-Blue cells were synchronised into the G<sub>1</sub> phase by treatment with N-butyrate for 20 hours (Figure 3.2). Cells accumulated into the G<sub>1</sub> phase after N-butyrate treatment, increasing the G<sub>0</sub>/G<sub>1</sub> phase cells to approximately 80% of the population. Concentrations over 10 mM were detrimental to the cells, as a high apoptotic region (sub-G<sub>0</sub>/G<sub>1</sub>) was seen in ungated populations. Treatment with 3 mM of N-butyrate increased the G<sub>1</sub> population to 79% with no identifiably apoptotic cells (Figure 3.2). Cell treatment with 3 mM N-butyrate for 20 hours was used to generate the G<sub>1</sub> phase population used in experiments.

![Diagram](image)

**Figure 3.2. Synchronisation of cells into G<sub>1</sub> phase**

Asynchronously growing RAW-Blue cells were seeded in 6-well plates at approximately 8 × 10<sup>5</sup> cells/well and the following day the RAW med10% was replaced and the cells treated with the indicated concentration of N-butyrate for 20 hours. (A) Cells were harvested at the indicated times for FACS analysis of the cell cycle. (B) The histograms from (A) were analysed with MODfit LT 3.0 and the percentage of cells in each phase of the cell cycle is shown. The results are shown as means (+SD) for three independent experiments.
G2 phase synchronisation

RAW-Blue cells were synchronised into the G2 phase by treatment with genistein for 48 hours (Figure 3.3). Cells accumulated in the G2/M phase with increasing concentrations of genistein. With 100 µM of genistein, approximately 66% of cells accumulated into the G2/M phase. Concentrations above 100 µM were toxic to the cells as the sub-G0/G1 population increased (data not shown). Treatment of cells with 100 µM of genistein for 48 hours was used to generate the G2 population used in later experiments.

Figure 3.3. Synchronisation of cells into G2 phase.
Asynchronously growing RAW-Blue cells were seeded in 6-well plates at approximately 6 × 10^5 cells/well and the following day the RAW med10% replaced and the cells treated with the indicated concentration of genistein for 48 hours. (A) Cells were harvested at the indicated times for FACS analysis of the cell cycle. (B) The histograms from (A) were analysed with MODfit LT 3.0 and the percentage of cells in each phase of the cell cycle is shown. The results are shown as means (+SD) of three independent experiments.
M phase synchronisation

RAW-Blue cells were synchronised into M phase by treatment with 37.5 and 50 ng/ml of nocodazole for 10 hours (Figure 3.4). With 50 ng/ml of nocodazole, approximately 91% of cells accumulated into G₂/M phase with less than 5% of cells in G₀/G₁ and S phases. For the rest of the experiments 50 ng/ml of nocodazole was used to treat cells for 10 hours.

Figure 3.4. Synchronisation of cells into M phase.

Asynchronously growing RAW-Blue cells were seeded at 1.6 x 10⁶ cells/well in 6-well plates and the following day the RAW med10% replaced and cells treated with the indicated concentrations of nocodazole for 10 hours. (A) Cells were harvested at the indicated times for FACS analysis of the cell cycle. (B) The histograms from (A) were analysed with MODfit LT 3.0 and the percentage of cells in each phase of the cell cycle is shown. The results are shown as means (+SD) of three independent experiments.
**G₁ progressing phase (G₁>) synchronisation**

RAW-Blue cells were synchronised into a G₁ progressing phase (G₁>) by release from an M phase arrested population. Cells were synchronised to M phase with nocodazole treatment then released from the arrest through replacement of the nocodazole medium with RAW med10%. Cells successfully accumulated into the G₀/G₁ phase after nocodazole removal. At 3 hours post-medium addition, 69% of cells had progressed into the G₀/G₁ phase (Figure 3.5). For the remaining experiments cells were synchronised into G₁> by treatment with 50 ng/ml nocodazole for 10 hours, followed by monolayer washing and RAW med10% addition for 3 hours.

**Figure 3.5. Synchronisation of cells into the G₁> phase.**

To generate a G₁ phase progressing population (designated as G₁>), approximately 3.5 × 10⁶ cells were seeded into 25-cm² flasks and synchronised to M phase with nocodazole treatment. The monolayer was washed 3 times in 3 ml of RAW med10% to remove the nocodazole inhibition and the cells incubated. (A) Cells were harvested at the indicated times post-nocodazole release for FACS analysis of the cell cycle. (B) The histograms from (A) were analysed with MODfit LT 3.0 and the percentage of cells in each phase of the cell cycle is shown. The results are shown as means (+SD) for three experiments.
Cell treatments - toxicity

The toxicity of chemicals used to treat cells were analysed for their effects on the cells through flow cytometry analysis of the DNA content using a DNA stain. Cells in the sub $G_1$ phase have reduced DNA content due to DNA fragmentation because of apoptotic or necrotic cells. After treatment of cells with a specified dosage and time, cells were harvested and the sub $G_1$ population calculated. All cell treatments increased the sub $G_1$ population compared to a mock-population. Treatments used in synchronising cells had less than 10% of cells in the sub $G_1$ phase (Figure 3.6). Treatment with IFN-β and JAKi-1 were more toxic and increased in the sub $G_1$ population by 45.4% and 37.1% respectively.

![Graph A](image)

**Figure 3.6. Toxicity of different cell treatments.**

RAW-Blue cells were seeded in 6-well plates as previously stated and treated with N-butyrate at 3 mM for 20 hours ($G_1$), treated with 50 ng/ml nocodazole and released for 3 hours ($G_{1>}^r$), treated with 100 µM genistein for 48 hours (M), treated with 1000 U/ml IFN-β for 12 hours, treated with 10 µM JAKi-1 for 12 hours or washed and seeded in serum-free medium for 72 hours. Following incubations cells were harvested for FACS analysis of the DNA content of cells. (A) Data was analysed in FlowJo v8 for the sub $G_1$ population. The results shown representative of three experiments. (B) Data from (A) was graphed using Prism software and the percentage of sub $G_1$ cells shown. The results are shown as means (+SD) of three independent experiments.
3.1.3 MNV-1 replication affects host cell cyclin expression

Microarray analysis of MNV-1-infected RAW264.7 cells from several groups showed large fluctuations in multiple transcripts involved in regulation and control of the cell cycle (141, 322, 327). This led us to ask whether MNV-1 infection affects the host cell replication. Cyclins are key regulators of cell division, whose expression and activity control transition between each phase of the cell cycle. As MNV infection of RAW264.7 cells affected host cell cycle regulators at a transcript level, it was addressed if this was occurring at a protein level. RAW-Blue cells were infected with MNV-1 and harvested post-infection at the indicated times for Western blot analysis. Proteins were detected by their corresponding antibodies. After immunofluorescence band analysis, each protein signal was normalised to actin, and the change was calculated (n-fold) for each time point between MNV-1-infected and mock-infected cells. MNV-1 infection caused a 41% decrease in cyclin A expression at 12 hours post-infection (Figure 3.7). Cyclin B2 expression was decreased 44% at 9 hours post-infection, while cyclins D1, E and B1 showed no differences between asynchronously growing mock-infected and MNV-infected cells. This correlates with microarray transcriptomic data from Davies et al (322) and Bok et al (141) (Appendix III), where cyclin A2 and B2 decreased at an mRNA level. Although there is some variation in protein expression among triplicates (indicated by the error bars in Figure 3.7B), the results show a significant decrease in both cyclin A and B2 expression despite fluctuations in cyclin expression in mock-infected populations. The decrease in A and B cyclins could indicate that cells were accumulating in the G0/G1 phase of the cell cycle, as expression of these proteins would be low in a G0/G1 population.
Figure 3.7. Effects of MNV infection on host cyclin expression.

(A) Asynchronously growing RAW-Blue cells were seeded at $1 \times 10^6$ cells/well in 6-well plates and mock-infected (Mock) or infected with MNV-1 at an MOI of 5. (A) At the indicated times post-infection, whole-cell lysates were collected and subjected to Western blot analysis with cyclin antibodies. The data are from one of three experiments. Actin was used as a loading control. Cyclin D1 and cyclin E bands migrate as doublets around 36 kDa and 50 kDa, respectively. Cyclin B1 migrates at 60 kDa, cyclin B2 at 45 kDa, and cyclin A at 54 kDa. (B) Cyclin levels were quantified with Image Studio Lite software (LI-COR) and normalised against actin, and results are presented as means and SD from three experiments. Statistical significance was compared to the corresponding value at 0 hours post-infection for each cyclin using a one-sample t-test *, $P \leq 0.05$. 

Fold change

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Actin

Mock

MNV

0 9 12 h p.i.
3.1.4 MNV-1 infection induces a $G_0/G_1$ phase accumulation

Based on the observation that MNV-1 infection dysregulates regulatory cell cycle mRNA transcription and expression of host cell cyclin proteins, it is likely that this is having an effect on host cell division. Therefore it was asked if MNV-1 is affecting the cell cycle during an infection. RAW-Blue cells were mock-infected or infected with MNV-1 and at selected times post-infection, cells were collected and their DNA contents analysed by flow cytometry. At 9 hours post-infection there was a 24% increase in the fraction of population in the $G_0/G_1$ phase of the cell cycle and a 26% reduction in the proportion of cells in S phase when compared to mock-infected cells (Figure 3.8). These results correlate with changes in cyclin expression post MNV-1 infection. The increase in the $G_0/G_1$ population and proportional decrease in the S phase population is consistent with an arrest at the highly regulated $G_1/S$ restriction point.
Figure 3.8. MNV-1 infection induces the accumulation of cells in the G₀/G₁ phase of the cell cycle.

(A) Asynchronously growing RAW-Blue cells were seeded at 1 × 10⁶ cells/well in 6-well plates and mock-infected (Mock) or infected with MNV-1 at an MOI of 5. Cells were collected post-infection at the times indicated for FACS analysis of the cell cycle. The data presented are representative of 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 is shown. The results are means and SD from three experiments. Statistical significance was determined for comparisons between mock-infected and MNV-1-infected cells for each time point using a one-way ANOVA with Tukey’s post-test. **, P ≤ 0.01; ***, P ≤ 0.001.
3.1.5 MNV-1 infection reduces G\textsubscript{1}/S phase progression

*MNV-1 affect on G\textsubscript{0} to S phase progression*

It was hypothesised that the increase in the G\textsubscript{0}/G\textsubscript{1} phase was due to an arrest at the G\textsubscript{1}/S checkpoint. To better characterise the G\textsubscript{0}/G\textsubscript{1} arrest, synchronised cells were monitored for cell cycle progression through early cell cycle phases G\textsubscript{1} and S of the cell cycle. Cells were synchronised to the G\textsubscript{0} phase of the cell cycle with serum withdrawal, then stimulated to re-enter cell division through addition of RAW med10%. Cells were infected with MNV-1 at the same time as RAW med10% addition and at selected times post G\textsubscript{0} release, cells were collected and analysed by flow cytometry for analysis of the DNA content of cells. MNV-1 infection caused a reduction in S phase entry, compared to mock-infected cells (Figure 3.9). At 8 hours post-infection, mock-infected cells re-entered cell division, indicated by a reduction in the G\textsubscript{0}/G\textsubscript{1} phase and a proportional increase in the S phase population. Progression through the G\textsubscript{1}/S restriction point is reduced in MNV-1-infected cells compared to the mock-infected population. At 16 hours post G\textsubscript{0} release in mock-infected cells there was a 36.8% decrease in the G\textsubscript{0}/G\textsubscript{1} population, while in MNV-1-infected cells there was a 23.9% decrease in the G\textsubscript{0}/G\textsubscript{1} population. The S phase population increased proportionally to the G\textsubscript{0}/G\textsubscript{1} decrease, while the G\textsubscript{2}/M population remained relatively unchanged. Later time points showed an increase in G\textsubscript{2}/M phase as cells from S phase move into G\textsubscript{2}/M phase (data not shown). These results indicate a reduction in S phase entry but not a complete arrest, for a population of cells infected in the G\textsubscript{0} phase and stimulated to re-enter cell division.
Asynchronously growing RAW-Blue cells were seeded in 6-well plates at $6 \times 10^5$ cells/well and synchronised to the $G_0$ phase with removal of FBS from the culture medium. Synchronised cells were mock-infected (Mock) or infected with MNV-1 at a MOI of 5 and after absorption, cells were washed 3x in 1 ml RAW med10% then incubated in 2 ml RAW med10%. (A) Cells were harvested at the indicated times post-infection and analysed for FACS analysis of the cell cycle. The data presented is representative of 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 is shown. The results are means and SD from three experiments. Statistical significance was determined for comparisons between mock-infected and MNV-1-infected cells for each time point using a one-way ANOVA with Tukey’s post-test. *, $P \leq 0.05$; **, $P \leq 0.01$.
MNV-1 inhibits G1/S progression

In a similar experiment, transition from G1/S phase was analysed in cells released from a G1 population during infection with MNV-1. Cells were synchronised with N-butyrate treatment to the G1 phase, then stimulated to re-enter cell division through removal of the N-butyrate medium and addition of RAW med10%. Cells were incubated for 3 hours in RAW med10% prior to infection with MNV-1, and at selected times post-infection harvested and analysed by flow cytometry. MNV-1 infection inhibited S phase entry, compared to mock-infected cells (Figure 3.10). In mock-infected cells the G1 phase population decreased at 7 hours post-infection and the S phase population increased proportionally as cells re-entered cell replication. In contrast MNV-1-infected cells remained predominantly in G0/G1 phase post G1 release. At 13 hours post-infection 63.5% of MNV-1-infected cells remained in the G0/G1 phase, while in the mock-infected population only 36.4% remained in G0/G1 phase. The S phase population increased proportionally in the mock-infected population, as cells re-entered cell division. However, MNV-1-infected cells showed only a very slight increase in the S phase population post-release. These results indicate that MNV-1 infection induces a G0/G1 phase accumulation through either a decrease in progression through the G1/S phases or a prolonging of the G0 or G1 phases.
Figure 3.10. MNV-1 infection inhibits cell cycle progression from G₁ into S phase.

RAW-Blue cells were seeded at $8 \times 10^5$ cells/ml in 6-well plates and synchronised to the G₁ phase by treatment with 3 mM of N-butyrate for 20 hours. Following synchronisation, cells were released from the arrest by 3 washes with RAW med10% and the addition of RAW med10% for a 3 hour incubation. The cells were mock-infected (Mock) or infected with MNV-1 at an MOI of 5. (A) After 1 hour of virus absorption, RAW med10% was added and cell cycle profiles were taken at the indicated times post-infection for FACS analysis of the cell cycle. The data are from one of three experiments. (B) The histograms were analysed using MODfit LT 3.0, and the percentage of cells in each phase of the cell cycle is shown. The results are means and SD from three experiments. Statistical significance was determined for comparisons between mock-infected and MNV-1-infected cells for each time point using a one-way ANOVA with Tukey’s post-test. **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$. 

![Graph showing cell cycle profiles and phase percentages](image-url)
3.1.6 **MNV-1 infection does not influence late phase cell progression**

Another possible explanation for the $G_0/G_1$ phase accumulation seen during MNV-1 infection is faster progression through the later phases of the cell cycle through to the $G_0/G_1$ phase. To examine this, progression of cells through the later stages of the cell cycle were compared between mock-infected and MNV-1-infected cells. RAW-Blue cells were arrested in early M phase with nocodazole treatment. The M phase population of cells were infected with MNV-1 and the nocodazole-containing medium was removed and fresh RAW med10% was added to induce cell cycle progression. In both mock-infected and MNV-1-infected cell populations, the $G_2/M$ population progressed through the cell cycle into the $G_0/G_1$ phase by 2 hours post release. This is shown by a 40% decrease in the $G_2/M$ population and a simultaneous 40% increase in the $G_0/G_1$ population in both mock-infected and MNV-1-infected populations (Figure 3.11). These results indicate that MNV-1 infection has no influence on progression of the cell cycle through the M phase to the $G_0/G_1$ phase.
Figure 3.11. MNV-1 infection has no affect on late-phase cell cycle progression.

RAW-Blue cells were seeded at $1 \times 10^6$ cells/ml in 6-well plates and synchronised to the M phase by treatment with 50 ng/ml of nocodazole for 10 hours. Synchronised cells (M phase) were mock-infected (Mock) or MNV-1-infected at an MOI of 5. After 1 hour of viral absorption, the nocodazole-containing medium was added back to the cells, and the virus was given 3 hours of replication time. (A) At 3 hours post-infection, the cell monolayer was washed three times with RAW med10%, and cell cycle profiles were taken at the indicated hours post-release (h.p.r.) for FACS analysis. The data are from one of three experiments. (B) The histograms were analysed by MODfit LT 3.0, and the percentage of cells in each phase of the cell cycle is shown. The results are means and SD from three experiments.
3.1.7 MNV-1 infection inhibits cyclin A expression

Changes to cyclin expression in RNA transcripts and protein levels as well as cell cycle profiles of infected cell populations suggest that MNV-1 impacts the G1/S restriction point. Other RNA viruses are also known to target the G1/S transition as a means of manipulating the cell into a favourable phase of the cell cycle for viral replication (288, 295). Based on these observations, G1/S cyclin expression was examined in infected cells passing through the G1/S restriction point. Cells were synchronised to the G0 phase, infected, and simultaneously given serum (FBS) to stimulate cell cycle progression. Cells re-entered the cell cycle after 9 to 12 hours of serum stimulation (Figure 3.12A). Cyclin D1 levels were not significantly effected post-infection compared to mock-infected cells. Cyclin E protein levels did not change after serum stimulation or post-infection. Cyclin A is not expressed in the quiescent G0 population; however, its levels gradually increased in mock-infected cells post-release. After 12 hours post-infection, cyclin A expression increased in mock-infected cells, while MNV-1 infection limited the accumulation of cyclin A. At 15 hours post-infection, there was a 0.28-fold decrease in cyclin A expression between mock-infected and MNV-1-infected cells (Figure 3.12C). These results combined with the lack of effect upon progression from G2/M confirm that MNV-1 infection inhibits progression through the G1/S restriction point, possibly through inhibition of cyclin A expression.
Figure 3.12. Changes to G₁ cyclin expression in MNV-1 infected cells released from quiescence.

Asynchronously growing RAW-Blue cells were seeded in 6-well plates at 6 × 10⁵ cells/well and synchronised to the G₀ phase with removal of FBS from the culture medium for 72 hours. Serum-starved (G₀) RAW-Blue cells were either mock-infected (Mock) or MNV-1-infected at an MOI of 5. After 1 hour of virus absorption the cells were washed three times and incubated in RAW med10%.

(A) At the indicated times post-infection, mock-infected cells were harvested for FACS analysis of the cell cycle. MODfit LT 3.0 was used to calculate the percentage of cells in each cell cycle phase.

(B) Mock-infected and MNV-infected cells were collected post-infection for Western blot analysis of cyclins D1, E, and A. The data shown are from one of three experiments. (C) Cyclin expression results from panel B were quantified by Image Studio Lite software (LI-COR) and normalised against actin loading control. Data are means and SD from three experiments. Statistical significance was determined for comparisons between 0 h.p.i. and MNV-1-infected cells for each time point using a one-way ANOVA with Dunnett’s post-test. *, P ≤ 0.05.
3.2 The affect of the host cell cycle on MNV-1 replication

3.2.1 Introduction

Results in the previous section showed that the host cell cycle is affected during MNV-1 infection, resulting in an increase in the number of cells in the G₀/G₁ phase population. The arrest in the cell cycle may be just a consequence of MNV-1 infection or a purposeful tactic to aid MNV-1 replication. Several RNA viruses cause G₀/G₁ phase arrests and it has been shown that this is often beneficial to viral replication (193, 297). The G₀/G₁ phase may support better RNA virus replication for many reasons, including abundance of ribonucleotides, prevention of membrane disassembly and higher transcription and translation rates. This section examines the relationship between the host cell cycle and MNV-1 replication, specifically comparing MNV-1 replication in various populations of synchronised cells to an asynchronous population.

3.2.2 Comparison of MNV-1 replication in asynchronous, G₀ and M phase populations

MNV-1 replication was compared in populations of cells synchronised into various phases of the cell cycle. Initial experiments assessed only serum withdrawn cells (G₀ phase) verses nocodazole synchronised cells (M phase). Cell populations in the G₀ and M phases were compared with an unsynchronised control, in which cells were in a mixture of cell cycle stages during division. The hypothesis was that if MNV-1 replication was favoured in a certain phase it would be seen by higher replication compared to the unsynchronised control. The full effects of prolonged synchronisation on the cell health wasn’t known, so for each population (G₀ and M) an additional well was seeded in which the cells at the time of infection were washed and incubated in RAW med10% to preserve cell health during MNV-1 infection. These populations are referred to as G₀-released and M-released, as they have been released from the cell cycle arrest. The G₀ population incubated in serum-free DMEM and the M phase population incubated in nocodazole treated medium are referred to as G₀-held and M-held respectively. It was hypothesised that MNV-1 would have greater replication in the G₀-held population, as MNV-1 increased the population of cells in the G₀/G₁ phase of the cell cycle during an infection. The G₀ and M populations of cells were synchronised in flasks prior to seeding in 6-well plates at equal cell densities. For synchronisation the G₀ population of cells was incubated in DMEM free of FBS while the M phase cells were resuspended in RAW med10% containing nocodazole. After
synchronisation, cells were either held or released from their arrest, infected with MNV-1, harvested immediately for analysis of the cell cycle and harvested at 9 hours post-infection for quantification of viral protein and progeny. Synchronisation of cells was successful in generating three distinct populations at the time of infection. The G$_0$ population had >80% of cells in the G$_0$ phase and the M phase population was >60% (Figure 3.13A). Western blot analysis showed the highest levels of viral VP1 and NS5 protein expression in the M-released population (Figure 3.13B). Unsynchronised, G$_0$-released and M-held populations had similar levels of viral protein expression while the G$_0$-held supported extremely low replication of MNV-1. The progeny quantification showed similar replication of MNV-1 in unsynchronised, M-held and M-released populations with much lower replication observed in the G$_0$-released population (Figure 3.13C).
Figure 3.13. MNV-1 replication compared in different cell cycle phases at 9 hours post-infection.

Cells were synchronised in flasks to the G₀ phase with serum withdrawal for 72 hours and M phase with treatment of nocodazole at 50 ng/ml for 10 hours. An additional unsynchronised (unsync) flask was seeded the day prior to infection. After synchronisation, all cell populations were recovered and counted before seeding at 1 × 10⁶ cells/well in 6-well plates. The G₀ population was incubated in either serum-free medium (G₀-held) or FBS was added to 10% within the well (G₀-released). M phase synchronised cells were incubated in RAW med10% either with nocodazole (M-held) or were washed and seeded in RAW med10% free of nocodazole (M-released). (A) Remaining cells from the flasks synchronised were harvested for flow cytometry analysis of the cell cycle. Histograms generated were analysed with MODfit LT 3.0 program and the percentage of cells in each phase shown. (B) Cells were infected with MNV-1 at a MOI of 1 for 1 hour, and then harvested at 9 hours post-infection for chemiluminescence Western blot analysis of VP1 and NS5 protein expression. Actin is shown as a loading control. (C) MNV-1 progeny in the supernatant was quantified by plaque assay and analysis shown as PFU as a % of MNV-1-infected unsynchronised control. A single experiment was performed.
The experiment was repeated, allowing viral replication for a longer time period of 15 hours. Synchronisation of cells was successful in generating three distinct populations. The G0 population had >80% of cells in the G0 phase and the M phase population was >70% (Figure 3.14A). Western blot analysis showed equal expression of VP1 in unsynchronised, G0 released and M-held populations, with higher expression in the M-released population. Viral NS3 protein levels were highest in unsynchronised and M-released populations, with lower expression in G0-released and M-held, while G0-held had below detectable levels (Figure 3.14B). The G0-held population was again very poor at supporting MNV-1 replication, as both protein levels and progeny replication were very low. MNV-1 progeny was highest in the M-released population, 2-fold higher than the unsynchronised control (Figure 3.14C).

Results comparing MNV-1 replication in the G0 and M phases to an unsynchronised population were not as expected. The G0-held population did not support viral replication as viral protein expression and progeny virus was virtually undetectable at 9 and 15 hours post-infection. The G0-released population supported higher MNV-1 replication than G0-held, but still supported similar viral replication to the unsynchronised control. The M-held population supported similar VP1 expression to the unsynchronised control at both 9 and 15 hours post-infection, while the M-released population supported higher VP1 expression at 9 hours post-infection, while at 15 hours post-infection showed similar protein expression but higher MNV-1 progeny titres. NS3 expression was highest at 15 hours post-infection in unsynchronised and M-released populations, while expression in G0-released and M-held was reduced comparably and the G0-held had below detectable levels. Although the experiment was only conducted once, the differences in replication between held and released population suggest that there are notable changes to the cell as a result of removing the arresting agent. Of particular interest is the M-released population that supported increased viral replication over the unsynchronised population.
Figure 3.14. MNV replication compared in different cell cycle phases at 15 hours post-infection.

Cells were synchronised in flasks to the G\textsubscript{0} phase with serum withdrawal for 72 hours and M phase with treatment of nocodazole at 50 ng/ml for 10 hours. An additional unsynchronised (unsync) flask was seeded the day prior to infection. After synchronisation, cells were recovered and counted before seeding at \(1 \times 10^6\) cells/well in 6-well plates. The G\textsubscript{0} population was incubated in either serum-free medium (G\textsubscript{0}-held) or FBS was added to 10% within the well (G\textsubscript{0}-released). The M phase synchronised cells were incubated in RAW med10% either with nocodazole (M-held) or were washed and seeded in RAW med10% free of nocodazole (M-released). (A) Cells were harvested at the time of infection for flow cytometry analysis of the cell cycle. Histograms generated were analysed with MODfit LT 3.0 program and the percentage of cells in each phase shown. (B) Cells were then infected with MNV-1 at a MOI of 1 for 1 hour, and then harvested at 15 hours post-infection for chemiluminescence Western blot analysis of VP1 and NS3 protein expression. Actin is shown as a loading control. (C) MNV-1 progeny in the supernatant was quantified by plaque assay and analysis shown as PFU as a % of MNV-1-infected unsynchronised control. A single experiment was performed.
3.2.3 Cell phase progression of held and released populations

The previous results showed that MNV-1 replication varies depending on what phase of the cell cycle the host population is in. Interestingly, the effect of retaining the synchronising agent (held) or removing the agent (released) had large differences in the replication of MNV-1. It was hypothesised that the differences seen in MNV-1 replication were due to differences in the cell cycle phase between held and released populations. In order to address this question, cells were analysed for changes to their cell cycle after being held or released from their synchronisation. To address what happens to the cell cycle in cells held or released post G0, cells were synchronised to the G0 phase with serum withdrawal in flasks and then transferred to 6-well plates. Populations of cells were either held in the G0 phase through incubation in serum-free medium or released with addition of serum (10% FBS). Cells were harvested post-seeding for analysis of the cell cycle. RAW-Blue cells held through incubation in serum-free medium remained in the G0/G1 phase. At 0, 9, and 15 hours >75% of cells remained in G0/G1 phase (Figure 3.15). The populations of cells released through addition of serum remained in the G0/G1 phase at 9 hours but cells progressed out of G0 by 15 hours, indicated by a 39% decrease in the G0/G1 population and an 32% increase in the S phase population. These results are in agreement with data from Figure 3.12, showing cells released from G0 will enter cell division after 12 to 15 hours, indicated again by an increase in S phase and a simultaneous decrease in the G0/G1 phase.
Figure 3.15. Cell cycle profiles in G₀-held and G₀-released populations post-infection.

Cells were synchronised in flasks to the G₀ phase with serum withdrawal for 72 hours. After synchronisation, cells were recovered and counted before seeding at 1 × 10⁶ cells/well in 6-well plates. The cells were incubated in either serum-free medium (G₀-held) or FBS was added to 10% (G₀-released). (A) Cells were harvested at 9 and 15 hours post-seeding for flow cytometry analysis of the cell cycle. (B) Histograms generated were analysed with MODfit LT 3.0 program and the percentage of cells in each phase shown. A single experiment was performed.

To address what happens in cells released from an M phase synchronised population, cells were synchronised to M phase in flasks then transferred to 6-well plates. Cells were either incubated with nocodazole (M-held) or without (M-released), and harvested post-seeding for analysis of the cell cycle. Results showed that populations treated with nocodazole after seeding (held), remained in the G₂/M phase, as at both 9 and 15 hours post-seeding >75% of cells remained in G₂/M phase (Figure 3.16). M-held populations showed high levels of apoptotic cells, indicated by the high sub G₁ population. Although nocodazole treatment at shorter time points had minimal toxicity effects, longer incubation periods...
appeared more detrimental to cell health. The released populations progressed into G₀/G₁ phase by 9 hours post-release with >60% of cells in the G₀/G₁ phase. This phenomenon may explain why MNV-1 replication is favoured in an M-released population. The M-released population was in fact a population of cells progressing through the G₁ phase during the majority of MNV-1 infection. This suggests that MNV-1 replication is favoured in a G₁ population and that the G₀/G₁ arrest induced by infection is beneficial to MNV-1 replication.

**Figure 3.16. Cell cycle profiles in M-held and M-released populations post-infection.**

Cells were synchronised in flasks to the M phase with treatment of 50 ng/ml of nocodazole for 10 hours. After synchronisation, cells were recovered and counted before seeding at 1 × 10⁶ cells/well in 6-well plates. The M-held and M-released populations of cells were incubated with or without new nocodazole treatment respectively. (A) Cells were harvested at 9 and 15 hours post-seeding for flow cytometry analysis of the cell cycle. (B) Histograms generated were analysed with MODfit LT 3.0 program and the percentage of cells in each phase shown. A single experiment was performed.
3.2.4 MNV-1 protein expression in early cell cycle phases

MNV-1 replication is indicated to be favoured in a population of cells released from an M phase population, progressing through the G\(_1\) phase of the cell cycle. To further investigate this model, MNV-1 replication was compared in populations of cells synchronised to early phases of the cell cycle to an unsynchronised control population. Cells were synchronised into the G\(_0\) phase with serum withdrawal, G\(_1\) with N-butyrate treatment and a population of G\(_1\) phase progressing cells (G\(_1\)>) generated by removal of nocodazole from a M phase synchronised population to produce a population of cells synchronised into early G\(_1\) phase prior to infection. This latter population was designed to mimic the population of M-released cells that had the greatest MNV-1 protein and progeny expression in the previous experiment. The G\(_1\)> population was included because MNV-1 may not cause a cell cycle arrest in G\(_1\), but rather induce a prolonged G\(_1\) phase, and hence this population of cells could simulate the cell state during an infection.

RAW-Blue cells synchronised into G\(_0\), G\(_1\) and G\(_1\)> populations were compared to an unsynchronised population for their ability to replicate MNV-1. MNV-1 VP1 protein expression was used to quantify MNV-1 replication through quantitative Western blot analysis. The M phase released population that supported the greatest MNV-1 replication in the previous experiment would have had a doubling in cell number due to cells dividing during mitosis. In order to have equal cell number throughout infection, M phase cells (nocodazole treated) were released in flasks prior to seeding, through washing the cell monolayer with RAW med10% and allowing time for cells to progress out of M phase before they were harvested and re-seeded in early G\(_1\) phase. These experiments were first optimised to generate early G\(_1\) phase cells prior to seeding (Figure 3.5). At the time of infection, cells were synchronised to their intended phases. At 9 hours post-infection, cells had remained synchronised in their target cell phase during the course of infection, with a characteristic increase in the percentage of G\(_0\)/G\(_1\) phase cells in MNV-1-infected asynchronous populations (Figure 3.17A and B). The G\(_1\) population had no difference in MNV-1 VP1 expression compared to an unsynchronised population, while the G\(_0\) arrested cells had below detectable levels of MNV-1 protein expression (Figure 3.17C and D). VP1 expression was highest in G\(_1\)> cells at approximately 1.5-fold that of expression levels in unsynchronised cells.
Figure 3.17. MNV replication is highest in G₁> populations.

RAW-Blue cells were synchronised in flasks treated by serum starvation (G₀) for 72 hours, treated with 3 mM N-butyrate (G₁) for 20 hours or treated with 50 ng/ml nocodazole for 10 hours and then released for 3 hours (G₁>). The synchronised and unsynchronised cells were then harvested and centrifuged, and the supernatant was discarded. Unsynchronised, G₁ and G₁> synchronised cells were resuspended in RAW med10% and G₀ cells in medium with no FBS. Cells were then seeded at 1 × 10⁶ cells per well into 6-well plates and infected with MNV-1 at an MOI of 1 for 1 hour. After absorption, N-butyrate was added to the G₁ population at the same concentration that was used originally. At 9 hours post-infection, cells were harvested and analysed for DNA analysis by flow cytometry and Western blot analysis of protein levels. (A and B) Cell cycle profiles pre- and post-infection were determined by FACS analysis. The histograms generated were analysed with MODfit LT 3.0, and the percentage of cells in each phase of the cell cycle is shown. (C) MNV-1 VP1 expression was determined by Western blot analysis (top); actin was included as a loading control. (D) VP1 levels from three experiments were quantified with Image Studio Lite (LI-COR) and normalised against actin loading. All results are presented as a percentage of the MNV-1-infected unsynchronised control population (bottom). Statistical significance was determined for comparison to the value for the MNV-1-infected unsynchronised population using a one-sample t-test. *, P ≤ 0.05; ****, P ≤ 0.0001. The results are means and SD from three experiments.
3.2.5 MNV-1 protein expression in late cell cycle phases.

MNV-1 protein expression was compared in late phases of the cell cycle. RAW-Blue cells were synchronised with genistein treatment (G2 phase) and with nocodazole treatment (M phase) and compared to an unsynchronised population for their ability to support viral VP1 expression. Cells were infected and harvested pre- and post-infection for analysis of the cell cycle and viral protein expression. All cell populations were synchronised to their desired phases pre-infection (Figure 3.18A). At 9 hours post-infection, cells had remained synchronised in their target cell phase during the course of infection (Figure 3.18B). Cells synchronised into the G2 phase had reduced viral VP1 expression compared to the unsynchronised control population. The combination of prolonged nocodazole treatment during MNV-1 infection created a large sub G1 population post-infection in the M phase cells. It is interesting to note that this population (M phase) supported similar MNV-1 VP1 expression to the unsynchronised control. This is consistent with preliminary experiments that showed an M-held population supported similar MNV-1 replication as measured by viral protein expression and progeny titre. It is possible that apoptosis occurs late in infection of the M phase population, where MNV-1 has already had time to replicate. It is also of interest that the G2 population had a significantly lower expression of VP1 than the unsynchronised control and M phase cells.
Figure 3.18. MNV-1 protein expression in late cell cycle phases.

RAW-Blue cells were synchronised in flasks, treated with 100 µM genistein (G2) for 48 hours or treated with 50 ng/ml nocodazole (M) for 10 hours. An unsynchronised (unsync) population was included as a control population. The synchronised and unsynchronised cells were then harvested, centrifuged and the supernatant discarded. Cells were then seeded at 1 × 10^6 cells/well in 6-well plates and infected with MNV-1 at a MOI of 1 for 1 hour. After absorption, fresh synchronising agent was added to synchronised cells at the same concentration that was used originally. At 9 hours post-infection, cells were harvested and analysed for DNA analysis by flow cytometry and Western bolt analysis of protein levels. (A and B) Cell cycle profiles pre- and post-infection were determined by FACS analysis. The histograms generated were analysed with MODfit LT 3.0 program and the percentage of cells in each phase of the cell cycle shown. (C) MNV-1 VP1 expression was determined by Western Blot analysis, actin is shown as a loading control. (D) VP1 levels from (C) was quantified with Image Studio Lite program (LI-COR) and normalised against actin. All results are presented as a percentage of the MNV-1-infected unsynchronised control population (bottom). Statistical significance was determined for comparison to the value for the MNV-1-infected unsynchronised population using a one-sample t-test. *, P ≤ 0.05; ****, P ≤ 0.0001. The results are means and SD from three experiments.
3.2.5 MNV-1 replication is favoured in a G₁> progressing population

To further confirm that MNV-1 replication is favoured in a G₁> progressing population, MNV-1 progeny titres were compared in cell populations synchronised into various phases of the cell cycle and viral replication quantified by plaque assays. All five different cell populations were synchronised as before, G₀, G₁, G₁>, G₂ and M phases and were compared to an unsynchronised MNV-1-infected control. Synchronised cell populations were infected and harvested pre- and post-infection for flow cytometry analysis of the cell cycle and supernatants were collected for plaque assays. All cell populations were synchronised to their desired phases pre-infection (Figure 3.19A). At 9 hours post-infection, cells had remained synchronised in their target cell phase during the course of infection (Figure 3.19B). MNV-1 virus titres were highest in cell populations progressing through G₁>, with an approximately 2-fold increase in virus titre compared to an unsynchronised population. Synchronised populations in G₁, G₂ and M phase had no difference in MNV-1 replication compared to an unsynchronised population. The results showing an increase in MNV-1 progeny in a G₁> population are consistent with the increase in MNV-1 protein expression in a G₁> population. This strongly supports the observation that MNV-1 replicates better in a G₁> population of cells and correlates with the increase in the G₀/G₁ population of cells during MNV-1 infection.
Figure 3.19. G₁ phase progressing cells promote the highest viral replication.

RAW-Blue cells were synchronised in flasks treated by serum starvation (G₀) for 72 hours, treated with 3 mM N-butyraté (G₁) for 20 hours, treated with 50 ng/ml nocodazole for 10 hours and then released for 2 hours (G₁>), treated with 100 μM genistein (G₂) for 48 hours, or treated with 50 ng/ml nocodazole (M) for 10 hours. The synchronised and unsynchronised cells were then harvested and centrifuged, and the supernatant was discarded. Unsynchronised and G₁, G₁>, G₂, and M-synchronised cells were resuspended in RAW med10% and G₀ cells in medium with no FBS. Cells were then seeded at 1 × 10⁶ cells per well into 6-well plates and infected with MNV-1 at an MOI of 1 for 1 hour. After absorption, fresh synchronising agent was added to synchronised cells at the same concentration that was used originally. At 9 hours post-infection, cells were harvested and analysed. (A and B) Cell cycle profiles pre- and post-infection were determined by FACS analysis. The histograms generated were analysed with MODfit LT 3.0, and the percentage of cells in each phase of the cell cycle is shown. (C) MNV progeny in the supernatant were titrated by plaque assay, and quantitative analysis data, in PFU, are shown as a percentage of the value for the unsynchronised MNV-1-infected control. The results are means and SD from three experiments. Statistical significance was determined for comparison to the value for the MNV-1-infected unsynchronised population using a one-sample t-test. ***, P ≤ 0.001.
3.3 The mechanism behind MNV-1-induced cell cycle effects

3.3.1 Introduction

Analysis of the relationship between MNV-1 and the host cell cycle revealed that MNV-1 infection induces cell cycle changes that are beneficial to viral replication. This section aims to expand on these results, addressing the mechanism behind the effect of MNV-1 on the host cell cycle. More specifically, this section analyses the possible causes of cell cycle arrest, including indirect cellular responses and direct viral protein induction of the cell cycle arrest.

3.3.2 IFN-β induces a G₀/G₁ phase arrest in an asynchronous population

*Optimisation of IFN-β stimulation*

It is possible that the host cell cycle arrest observed during MNV-1 infection is due to an indirect response to host IFN production. IFN is known to induce cell cycle arrests at several phases of the cell cycle including a G₁ arrest, but is often cell type dependent (328). It was hypothesised that IFN might be induced in response to MNV-1 infection, which acts on neighbouring cells to induce the G₀/G₁ phase arrest and that MNV-1 has adapted to replicate more efficiently in this phase. Analysis of microarray data of MNV-1-infected RAW264.7 cells from three independent research groups showed that IFN-β is the most upregulated cytokine post MNV-1 infection (141, 322, 327). IFN-β was first assessed for its activity by evaluating its ability to stimulate the phosphorylation of STAT1. Cells were treated with various concentrations of IFN-β and harvested for Western blot analysis of STAT1 phosphorylation at Y701. Both 100 and 1000 Units/ml of IFN-β induced the phosphorylation of STAT1 after 2 hours of treatment (Figure 3.20.). The toxicity of IFN-β on RAW-Blue cells was also tested through analysis of the sub G₁ population (Figure 3.6).
RAW-Blue cells were seeded in 24-well plates at $2 \times 10^5$ cells/well and treated with IFN at 100 and 1000 Units/ml for 2 hours. Cells were collected post-treatment for Western blot analysis of STAT1 phosphorylation (top) and actin (bottom).

**Treatment of cells with IFN-β increases the G0/G1 phase population**

IFN was analysed for its effects on the host cell cycle. RAW-Blue cells were treated with various concentrations of IFN-β and harvested post-infection for flow cytometric analysis of the cell cycle. As previously discussed (Figure 3.6), IFN-β treatment induces high levels of apoptosis, indicated by a high <G1 population compared to untreated populations. These results were again mimicked with all concentrations of IFN-β increasing the <G1 population. At 9 and 12 hours post-treatment all concentrations of IFN-β increased the relative number of cells in the G0/G1 phase of the cell cycle in comparison to mock-treated cells (Figure 3.21). All concentrations of IFN-β increased the G0/G1 population by approximately 18% and decreased the S phase proportionally. The G2/M phase population was unaffected by IFN-β treatment.
Figure 3.21. IFN-β treatment of RAW-Blue cells induces a G₀/G₁ arrest.

Asynchronous RAW-Blue cells were seeded at in 6-well plates at $8 \times 10^5$ cells/well and treated with the indicated concentration of IFN-β for 9 and 12 hours. (A) At the indicated times pre- and post-infection, cell cycle profiles were determined by FACS analysis. (B) The histograms from (A) were analysed with MODfit LT 3.0 and the percentage of cells in each phase of the cell cycle is shown. The results are means and SD from three experiments. Statistical significance was determined for comparison to the value for the untreated control population at the corresponding time point using a one-way ANOVA with Tukey’s post-test. ****, $P \leq 0.0001$.

3.3.3 The IFN response is not the cause of the G₀/G₁ phase arrest in infected cells

As IFN-β, the most upregulated cytokine during MNV-1 infection, induced a G₀/G₁ arrest in RAW-Blue cells, it was hypothesised that this was the cause of the viral-induced cell cycle
arrest. In order to study the effects of IFN on the cell cycle during MNV-1 infection, a cell population unresponsive to IFN was developed. This population of cells was then used to measure the effects of MNV-1 infection on the host cell cycle. If MNV-1 could not induce a cell cycle arrest in a cell line non-responsive to IFN then it would be likely that IFN is the cause of the cell cycle arrest.

_Inhibition of the IFN type 1 pathway_

In order to block the IFN response in RAW-Blue cells, a Janus kinase (JAK) inhibitor was used to inhibit JAK1, 2 and 3 prior to infection, rendering the IFN pathway unresponsive to IFN autocrine signalling. STAT1 is phosphorylated in the presence of IFN and starts the signalling cascade leading to expression of IFN-stimulated genes (329). Cells were treated with a range of concentrations of JAK inhibitor 1 (JAKi-1) (Merck Millipore) and treated with IFN-β prior to cell harvesting for Western blot analysis of STAT1 phosphorylation. Treatment with JAKi-1 for 12 hours resulted in a dose-dependent reduction of STAT1 phosphorylation following IFN treatment, with undetectable STAT1 phosphorylation after treatment of 10 μM of JAKi-1 (Figure 3.22). Treatment of JAKi-1 for 24 hours resulted in complete inhibition of STAT1 phosphorylation for all concentrations tested.

![Figure 3.22. Treatment of cells with JAKi-1 to knockdown the IFN response.](image)

RAW-Blue cells were seeded in 6-well plates and 1 x 10^5 cells/well and treated with JAKi-1 at the indicated concentrations and incubated for 12 and 24 hours. Following incubation, cells were treated with IFN-β at 100 pg/ml for 2 hours prior to harvesting for Western blot analysis. Samples were probed for actin and a STAT1 phosphorylation site (PY701). Experiments were performed once.
The MNV-1 induced cell cycle arrest is independent of IFN signalling

Following development of a cell culture system with an inhibited IFN response, MNV-1-infected cells were analysed for cell cycle changes in the presence of IFN inhibitor JAKi-1. Cells were treated with JAKi-1 to knockdown their response to IFN, then infected with MNV-1 and analysed post-infection for changes to their cell cycle. In both MNV-1-infected and JAKi-1 treated MNV-1-infected populations the G0/G1 population increased significantly at 12 hours post-infection (Figure 3.23). This was accompanied by a comparable decrease in the S phase population in MNV-1-infected and JAKi-1 treated MNV-1-infected cells. The G2/M population remained relatively unchanged. Knockdown of the IFN pathway had no effect on the ability of MNV-1 infection to induce cell cycle effects. These results indicate that although IFN-β can induce a G0/G1 phase arrest in RAW-Blue cells, the IFN response is not the cause of the cell cycle arrest induced through MNV-1 infection.
Figure 3.23. MNV-1 infection induces a G₀/G₁ arrest in a cell line non-responsive to IFN type 1.

RAW-Blue cells were seeded in 6-well plates at 4 × 10⁶ cells/well and treated with 10 µM of JAKi-1 for 12 hours prior to infection with MNV-1 at a MOI of 5. (A) Cells were collected post-infection at the indicated times for FACS analysis of the cell cycle. The data 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. The results are means and SD from three experiments. Statistical significance was determined for comparisons between the indicated columns using a one-way ANOVA with Tukey’s post-test. *, P ≤ 0.05; **, P ≤ 0.01.
3.3.4 MNV-1 protein VF1 and the cell cycle

The previous experiment suggested the effects of MNV-1 on the host cell cycle were independent of the host type 1 IFN response, leading us to ask if MNV-1 might induce cell cycle effects through direct viral protein interactions within the host cells. Due to the small genome size of MNV-1 it was plausible to analyse the effects of individual genes on the host cell cycle. MNV-1 can replicate without the presence of a functional VF1 protein, but at a fitness cost to the virus (88). To determine if VF1 was responsible for cell cycle changes, cells were infected with a mutant MNV-1 ORF4 knockout virus (MNV-4S), available in the Ward laboratory, containing a stop codon near the beginning of the ORF4 gene, thus generating a truncated VF1 protein product during virus replication (330). Cells infected with MNV-4S were compared for their ability to induce a cell cycle arrest compared to mock-infected and MNV-1-infected cells. Cells were also analysed post-infection by Western blot analysis to confirm viral replication. Expression of viral NS1-2 protein was detected in both MNV-1 and MNV-4S post-infection at similar levels (Figure 3.24A). The knockout MNV-4S virus was able to induce accumulation of cells in the G₀/G₁ phase post-infection in an identical manner to MNV-1-infected cells. In both MNV-1 and MNV-4S infected cells the G₀/G₁ population increased significantly by approximately 20% at 9 and 12 hours compared to the mock-infected cells (Figure 3.24C). The S phase population decreased proportionally in both MNV-1 and MNV-4S infected population. These results conclusively show that the VF1 protein from MNV is not responsible for viral effects on the host cell cycle.
Figure 3.24. The VF1 protein of MNV-1 is not the cause of the viral induced cell cycle arrest.

RAW-Blue cells were seeded in 6-well plates at 3 x 10^6 cells/well and infected with MNV-1 and MNV-4S at a MOI of 5. (A) Following infection, cells were incubated and harvested at the indicated times post-infection for analysis of protein expression of viral NS1-2 by Western blot analysis. (B) Cells were collected post-infection at the indicated times for FACS analysis of the cell cycle. The data presented is of one of three experiments. (C) The histograms from (B) were analysed 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 experiments. Statistical significance was determined for comparisons between infected populations and the corresponding mock-infected time point using a one-way ANOVA with Tukey’s post-test. *, P ≤ 0.05; **, P ≤ 0.01, ***, P ≤ 0.001, ****, P ≤ 0.0001.
3.3.5 Analysis of the effects of NS1-2 and NS5 on the host cell cycle

Introduction

Analysis of the effects of MNV-1 non-structural genes on the host cell cycle was continued. As MNV has a limited genome, the non-structural genes play an essential role in MNV replication and cannot be deleted without significant effects on the virus lifecycle. In order to determine the cause of cell cycle effects, analysis was done on the effects of expression of single viral proteins within cells. NS1-2 and NS5 were chosen as potential candidates for inducing cell cycle effects. NS1-2 is a largely disordered protein, and its role in viral infection is not well documented (100). The role of NS5 in viral replication is better documented, but it is also a disordered protein and its interaction with host translation factors might affect the cell cycle. Work by previous members of the Ward laboratory generated plasmids that contain sequences for NS1-2 and NS5 proteins that can be expressed in cell culture. Expression of proteins from plasmids transfected into the RAW264.7 cell line was previously shown to give poor expression due to the nature of macrophages being stimulated by the introduction of plasmid. However, transfection of RNA transcripts gave more success in expressing functional viral proteins within the RAW264.7 cell line in the absence of macrophage activation. In these experiments RNA coding for NS1-2 and NS5 were generated and transfected into RAW-Blue cells for analysis of effects on the host cell cycle.

Expression of NS1-2 and NS5

In order to generate RNA transcripts for transfection, linearised DNA plasmids were first generated from stocks of E. coli containing plasmids with NS1-2 and NS5 protein sequences. The plasmids were purified, linearised-using restriction sites flanking the viral gene insert and RNA generated using in vitro synthesis. This generated capped and poly(A)-tailed RNA sequences encoding NS1-2 and NS5, ready for transfection and subsequent protein expression. Following generation of RNA, expression of individual viral proteins was analysed in RAW-Blue cells through transfection of RNA and Western blot analysis of viral protein levels. Both viral NS1-2 and NS5 proteins were detected by their corresponding antibodies at 12 and 24 hours post-transfection at their expected molecular
weights (Figure 3.25). Viral proteins were easily detected, suggesting high expression, although detection of NS5 was reduced at 24 hours post-transfection.

![Image](image_url)

**Figure 3.25. NS1-2 and NS5 RNA expression in RAW-Blue cells.**

Approximately $1 \times 10^6$ RAW-Blue cells were transfected with 4 – 6 μg of NS1-2 and NS5 RNA transcripts. Cells were collected post-transfection for Western blot analysis of NS1-2 (A) and NS5 (B) protein expression.

*Expression of NS5 induces a $G_0/G_1$ cell cycle arrest in an asynchronous population of cells*

Following confirmation of expression of NS1-2 and NS5 within cell culture, virus proteins were analysed for their effects on the host cell cycle. RNA coding for NS1-2 or NS5 were transfected into RAW-Blue cells and the cells harvested at a range of times post-transfection for Western blotting and FACS analysis of the cell cycle. Both NS1-2 and NS5 showed good expression post-transfection, with protein levels detectable through to 24 hours (Figure 3.26A and B), although there was a reduction seen in expression of both proteins over 24 hours. The mock-transfected cell cycle profile remained unchanged over the 24-hour period. Expression of NS1-2 caused small cell cycle profile fluctuations but no change was significant. At 12 hours post-transfection, expression of NS5 caused a 19.4% increase in the $G_0/G_1$ population and a 19.4% decrease in the S phase population compared to the mock-transfected population. At 18 hours post-transfection there was an even greater change with the $G_0/G_1$ population increasing by 29.3% and the S phase population decreasing by 26.7% compared to mock-transfected cells (Figure 3.26D). At 24 hours post NS5 transfection the cell cycle profile returned to that of an untreated population. Changes to the cell cycle induced by expression of NS5 at 12 and 18 hours were similar to that seen following MNV-1 infection of an asynchronous population. The return to an untreated cell cycle profile in NS5 transfected cells at 24 hours may be a
consequence of this assay system, as NS5 protein expression had decreased and in an MNV-1-infected population by 24 hours post-infection you would observe near 100% cell lysis. These results strongly suggest that the NS5 protein is the causative agent of MNV-1 effects on the host cell cycle.

Approximately $1 \times 10^6$ RAW-Blue cells were transfected with 4 – 6 μg of NS1-2 and NS5 RNA transcripts. (A and B) Cells were collected at the indicated times post-transfection for Western blot analysis of NS1-2 (A) and NS5 (B) protein expression. The blot presented is a representative blot from three experiments. (C) Cells were collected at the indicated hours post-transfection (h.p.t.) for FACS analysis of the cell cycle. The data presented is of one of three experiments. (D) The histograms from (C) were analysed 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 experiments. Statistical significance was determined for comparisons between transfected populations and the corresponding mock-transfected (Mock-Trans) time point using a one-way ANOVA with Tukey’s post-test. **, $P \leq 0.01$; ***, $P \leq 0.001$; **** $P \leq 0.0001$. 

Figure 3.26. Expression of NS5 induces a $G_0/G_1$ phase arrest.
3.3.6 Confirmation of NS5 induced G₀/G₁ phase arrest

If the MNV-1 protein NS5 is the causative agent for the G₀/G₁ phase arrest, then it should induce its effects through inhibition of cell cycle transition through the G₁/S restriction point as observed in MNV-1 infections. To test this, NS5 was expressed in a G₁ phase population transitioning into S phase. This experiment was designed to mimic previous experiments looking at the effect of MNV-1 on G₁/S transition (Figure 3.10), but examining the effects of NS5 expression instead of viral infection. Cells were synchronised 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 was used as a non-viral RNA negative control. N-butyrate was also added to released populations and used as a positive control. Cells were then analysed for their transition from G₁ into S phase by FACS analysis of the host cell cycle and Western blot analysis of cyclin A expression. All three RNA transcripts were translated into protein and expressed from 15 to 24 hours post G₁ release (Figure 3.27A). The N-butyrate treated cells remained predominantly in G₁ phase post-release while the mock cells re-entered cell division quickly, indicated by a drop in the G₀/G₁ phase and a consequent increase in the S phase population from 15 to 24 hours post-release (Figure 3.27C). 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. The release from the G₁ arrest was slower in mock-transfected than mock treated cells, as the G₀/G₁ population in mock treated cells decreased considerably by 15 hours, while the mock-transfected population only decreased significantly by 21 hours. This indicates a delay in cell cycle progression induced by the transfection process. Transfection of GFP and NS1-2 RNA had no affect on the transition of cells from G₁ into S phase compared to the mock-transfected population. Both GFP and NS1-2 transfected populations progressed into S phase, with a substantial decrease in the G₀/G₁ population observed at 21 hours post-release, similar to the mock-transfected population. In contrast, transfection of NS5 RNA induced a G₁/S phase arrest as indicated by cells remaining in the G₁ phase post-release. In the NS5 transfected population the S phase does not increase post-release and the G₀/G₁ population remains above 70% at all time points. At 24 hours post-release in NS5 transfected populations, 73.4% of cells remained in the G₀/G₁ phase, while in the mock-transfected population only 46.2% remained in the G₀/G₁ phase. In the S phase population, the mock-transfected
population had risen to 46.5% while in the NS5 transfected cells only 19.4% of cells had entered S phase (Figure 3.27C).

As shown previously (Figure 3.12), cyclin A expression is inhibited by MNV-1 infection in cells transitioning through the G\textsubscript{1}/S restriction point. Cells were harvested post-G\textsubscript{2} release for Western blot analysis of cyclin A. This aimed to investigate the effects of NS5 expression on cyclin A expression. Expression of NS5 inhibited the accumulation of cyclin A in populations progressing from G\textsubscript{1} to S phase. In a G\textsubscript{1} arrested population expression of cyclin A is low, indicated by N-butyrate treated cells not expressing cyclin A. In mock, mock-transfected, GFP and NS1-2 transfected populations, cyclin A expression increased at 18 to 21 hours post-release as cells entered S phase (Figure 3.27D). In NS5 transfected cells, cyclin A levels remained non-detectable post-release, as transfected cells remained in G\textsubscript{0}/G\textsubscript{1} phase and did not progress into S phase. Whether inhibition of cyclin A is induced directly by NS5 expression or is a consequence of the G\textsubscript{0}/G\textsubscript{1} phase arrest is unknown. These results strongly confirm NS5 as the causative agent of the MNV-1 induced cell cycle arrest. Not only does NS5 expression increase the G\textsubscript{0}/G\textsubscript{1} population, but also observed is an arrest at the G\textsubscript{1}/S restriction point and inhibition of cyclin A expression.
A

B

Relative cell number

Relative DNA content

5  15  18  21  24 (h.p.r.)

N-butyrate

Mock

Mock-Trans

GFP

NS1-2

NS5

105
C

G₀/G₁ phase

% of cells in each phase

N-butyrate  Mock  Mock-Trans  GFP  NS1-2  NS5

S phase

% of cells in each phase

G₂/M phase

% of cells in each phase

N-butyrate  Mock  Mock-Trans  GFP  NS1-2  NS5
Figure 3.27. MNV-1 protein NS5 induces a G1/S arrest and inhibits cyclin A expression.

Approximately $7.5 \times 10^6$ RAW-Blue cells were seeded in 75 cm$^2$ flasks and treated with 3 mM N-butyrate for 20 hours to synchronise cells into the G1 phase. Following synchronisation, cells were washed 3 times in 5 ml of RAW med10% and incubated for 5 hours. After incubation cells were recovered, counted and approximately $1 \times 10^6$ 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). Mock (non-transfected) and mock-transfected (Mock-T) controls were seeded at the time of transfection (negative controls). (A) At the indicated hours 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 data presented is of one of three experiments. (C) The histograms from (B) were analysed 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 experiments. Statistical significance was determined for comparisons between RNA transfected populations and the corresponding mock-transfected time point using a one-way ANOVA with Tukey’s post-test. **, $P \leq 0.01$; ****, $P \leq 0.0001$. (D) Cells were harvested at the indicated hours post-release for Western blot analysis of cyclin A and actin expression. The blot presented is a representative blot from three experiments.
3.3.7 NS5 domain construction

Expression of viral NS5 protein increased the G₀/G₁ population in an analogous manner to MNV-1 infection, and the arrest was induced through inhibition of G₁/S phase progression. To further narrow down the cause of the cell cycle arrest, three different regions of NS5 were expressed and analysed for their effects on the cell cycle to determine which NS5 motif or known protein activities might be responsible. NS5 regions were ordered from GenScript in pUC57-simple plasmids, transcribed into RNA and transfected into an asynchronous population of cells and screened for their effects on the host cell cycle.

**NS5 construct design**

The NS5 protein is 124 amino acids long and its structure has been solved between residues 11-85 by nuclear magnetic resonance spectroscopy (123). It is a largely disordered protein with two large helices towards its N-terminus and a third smaller one in the mid section of the protein (Figure 3.28B). The two larger helices interact with each other through polar and hydrophobic interactions and contain the site of nucleotidylation (Y26) to the viral genome. The C-terminus is relatively disordered and is involved in interactions with host eIF. The initial hypothesis was that the C-terminus of NS5, which is known to be involved in translation of viral proteins (119), could be responsible for the cell cycle arrest due to global translation inhibition.

Three NS5 domains were expressed that encompassed various regions of NS5 known to be involved in different functions. Constructs designed were NS5 1-62, NS5 63-124 and NS5 11-107 containing the indicated amino acids (Figure 3.28B). The NS5 1-62 construct contained the disordered regions at the N-terminus, the first two helices and the nucleotidylation site at Y26. The NS5 11-107 construct contained the three helices and the middle disordered region up to but not including the C-terminus involved in binding eIF. The NS5 63-124 construct contains the third helix, a region of disorder and the C-terminus region involved in eIF binding. Sites of truncation were chosen to have minimal effects on the hydrophobicity plots and antigenic index (Figure 3.28A). To each NS5 construct, flanking restriction sites were added, BamHI at the 5’ terminus and HindIII at the 3’ terminus. To facilitate protein expression a T7 promoter sequence, a Kozak consensus
sequence (suggested by Ambion) and an initiating methionine were added to each construct (Figure 2.2).

![Figure 2.2](image)

**Figure 3.28. Protean analysis and schematic of NS5 constructs.**

(A) The amino acid sequence for MNV-1 NS5 was analysed using Protean from the Lasergene suite of sequence analysis software (DNASTAR). Structural predictions, hydrophobicity, amphipathicity, antigenic index, surface probability and truncation sites are shown as indicated. (B) Schematic of full length NS5 (1 – 124 residues) and synthetic constructs NS5 1-62, NS5 63-124 and NS5 11-107. The nucleotidylation site is shown in green text (Y26), red and blue text indicates interactions between residues involved in hydrophobic and polar interactions respectively. The numbers above the schematics indicate the position of amino acids at key structures.
**Generation of NS5 1-62, NS5 63-124 and NS5 11-107 RNA transcripts**

The three NS5 constructs were first screened for there correct insert sizes. The NS5 constructs in pUC57-simple plasmid were transformed into calcium competent *E. coli* cells. Following transformation, *E. coli* cells containing the plasmids with NS5 construct inserts were amplified and the plasmids extracted with a MiniPrep kit. The purified plasmids were digested with the flanking restriction enzymes BamHI and HindIII. Cleavage products were visualised on a DNA agarose gel to determine the insert sizes. As predicted both NS5 1-62 and NS5 63-124 showed insert sizes of approximately 232 bp and NS5 11-107 showed an insert size of approximately 337 bp (Figure 3.29). Undigested NS5 1-62 and NS5 63-124 migrated as a single supercoil while NS5 11-107 migrated as an apparent double supercoil.

**Figure 3.29. Generation of NS5 1-62, NS5 63-124 and NS5 11-107 plasmids for RNA synthesis.**

Approximately 1 μg amounts of undigested plasmid DNA and double digested DNA from NS5 1-62, NS5 63-124 and NS5 11-107 were separated on a 0.8% DNA agarose gel. The 1-kb plus molecular marker bands are shown on the left, while plasmid and insert sizes are indicated on the right.

**Expression of NS5 1-62, NS5 63-124 and NS5 11-107 constructs**

Following conformation of the correct insert sizes, *E. coli* cells containing the plasmids with NS5 constructs were amplified, plasmids extracted using an endotoxin free kit, linearised and RNA generated using *in vitro* synthesis, creating capped and poly(A)-tailed RNA sequences encoding NS5 1-62, NS5 63-124 and NS5 11-107. Cells were transfected with
either NS5 1-62, NS5 63-124 and NS5 11-107 RNA and harvested 18 hours post-transfection for Western blot detection with anti-NS5 antibody. The NS5 antibody was able to detect a protein band for NS5 1-62 at approximately 12 kDa and for NS5 11-107 at approximately 13 kDa (Figure 3.30). The NS5 63-124 transcript was unable to be detected by Western blot analysis using the α-NS5 antibody. Whether or not NS5 63-124 is expressed but unable to be detected by α-NS5 antibody or it is not expressed is unknown. The NS5 1-62 protein is detected at a higher molecular weight than predicted, this is likely due to the nature of disordered proteins routinely migrating larger than their predicted sizes, with the N-terminus of the NS5 protein predicted to be highly disordered (123) (Figure 3.35). The NS5 1-62 and NS5 11-107 protein bands migrated lower than the full length NS5 that migrates at approximately 16 kDa (87). Given that the synthetic NS5 bands migrate lower than the full length NS5 (1-124), are detected in transfected NS5 construct cells but not mock cells (data not shown) and that the NS5 1-62 protein appears slightly smaller than the larger NS5 11-107 protein supports the conclusion that NS5 constructs 1-62 and 11-107 can be expressed and detected in cell culture.

Figure 3.30. Detection of NS5 construct expression.
Approximately $1 \times 10^6$ RAW-Blue cells were transfected with 4 – 6 μg of NS5 1-124, 1-62, NS5 63-124 and NS5 11-107 RNA for 18 hours. Following transfection, cells were harvested and samples separated on 12.5% SDS-PAGE gels before transferring to nitrocellulose membrane. Proteins were detected with α-NS5 antibody and actin used as a loading control. A PageRuler Plus prestained protein ladder was used to estimate protein sizes.
3.3.8 NS5 construct effects on the host cell cycle

With positive protein expression detected for NS5 1-62 and NS5 11-107 transcripts and unknown expression of NS5 63-124, research was continued to investigate the effects on the host cell cycle of expressing the NS5 constructs in an asynchronous cell population. Asynchronous cells were transfected with RNA encoding the NS5 constructs and harvested post-transfection for protein detection with α-NS5 antibody and for FACS analysis of the cell cycle. Both NS5 1-62 and NS5 11-107 could be detected post-transfection, with protein levels peaking at 12 hours but decreasing to undetectable levels by 24 hours post-transfection (Figure 3.31A). NS5 63-124 again was not detectable using the α-NS5 antibody. At 12 hours post-transfection, expression of NS5 1-62 induced a 20% increase in the G0/G1 population and a 21% decrease in the S phase population compared to mock-transfected cells (Figure 3.31C). The effects on the host cell cycle were reduced by 18 hours and by 24 hours cell cycle profiles were back to that of mock-transfected. The effects on the cell cycle by NS5 1-62 matches its protein expression, as at 12 hours post-transfection where the effects are most predominant, protein expression is the highest. Band density analysis of NS5 construct expression shows an 8.8 fold decrease in protein expression from 12 to 18 hours, which may explain the drop in cell cycle effects at 18 hours. NS5 63-124 and NS5 11-107 had no effect on the host cell cycle, as their cell profiles closely resembled that of a mock-transfected population. These results show that the cell cycle affects caused by NS5 are induced by the first 1-62 amino acids and is independent of the host eIF binding domain. Furthermore the NS5 11-107 construct had no effect on the host cell cycle suggesting the first 10 amino acids play an essential role.
Figure A: Representative images of Actin expression in different time points (0, 12, 18, 24 h.p.t) for Mock-Trans, 1-62, 63-124, and 11-107

Figure B: Graphs showing the relative fluorescent units for NS5 expression in different time points (0, 12, 18, 24 h.p.t) for Mock-Trans, 1-62, 63-124, and 11-107

Figure C: Histograms showing the relative cell number and DNA content for Mock-Trans, 1-62, 63-124, and 11-107

Figure D: Bar graphs showing the percentage of cells in G0/G1, S, and G2/M phases for Mock-Trans, 1-62, 63-124, and 11-107
**Figure 3.31. Expression of NS5 1-62 induces a G₀/G₁ phase arrest.**

Approximately $1 \times 10^6$ RAW-Blue cells were transfected with 4 – 6 μg of NS5 1-62, NS5 63-124 and NS5 11-107 RNA transcripts. A mock-transfected (Mock-Trans) control was seeded at the time of transfection (negative control). (A) At the indicated hours post-transfection (h.p.t.) cells were harvested for Western blot analysis of NS5 construct expression. The blot presented is a representative blot from three experiments. (B) Cells were collected post-transfection at the indicated times for FACS analysis of the cell cycle. The data presented is of one of three experiments. (C) The histograms from (B) were analysed 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 experiments. Statistical significance was determined for comparisons between transfected populations and the corresponding mock-transfected time point using a one-way ANOVA with Tukey’s post-test. ***, P ≤ 0.001. ***, P ≤ 0.001.

### 3.4 Bioinformatic analysis of MNV-1 NS5

#### 3.4.1 Introduction

The NS5 protein from MNV-1 induces a G₀/G₁ phase arrest when expressed in cell culture. Furthermore, expression of the first 1-62 amino acids of NS5 induces similar cell cycle effects to full length NS5. Expression of NS5 11-107 had no measurable effect on the cell cycle suggesting that residues 1-10 must play an essential role. Bioinformatic analyses of proteins can give insights into function and interactions based on protein sequences. This section aimed to postulate possible mechanisms of cell cycle manipulation by NS5 through analysis of functional motifs, sequence alignments and hydropathy, focusing on the essential N-terminus sequences.

#### 3.4.2 Sequence comparison to other caliciviruses

*Comparison between MNV and HuNoV NS5 sequences*

Sequences of VPg proteins from a range of caliciviruses were compared in order to determine conserved structures and functions. The NS5 sequence of MNV-1 CW1 was first compared to the HuNoV, GII.4, Sydney, 2012 strain. The comparison showed similarities at conserved functional sites such as the elF binding region at the C-terminus and around the nucleotidylolation site at Y26/27 (Figure 3.32). Both nucleotidylation and recruitment of host
eIF has been documented in MNV and HuNoV (118, 120, 123, 331). The N-terminal sequences show close similarities, as 10 out of the first 11 residues are identical. Due to the importance of the first 10 amino acids of MNV-1 NS5 to its cell cycle related activity, the similarities with HuNoV NS5 through this region suggest these properties may be conserved across norovirus genogroups.

![Sequence comparison between murine GV and HuNoV GII](image)

**Figure 3.32. Sequence comparison between murine GV and HuNoV GII.**

Sequences from MNV-1 GV (GenBank accession number DQ285629.1) and HuNoV GII (GenBank accession number JX459908.1) were aligned using Clustal Omega analysis. Colours of amino acids indicate those with similar properties. Red amino acids (AVFPMILW) indicate small positive hydrophobic residues, blue (DE) indicate acidic, pink (RK) indicate basic and green (STYHCNGQ) indicate hydroxyl, sulfhydryl and amines. Amino acid positions are indicated above the sequence.

**Comparison of NS5 N-terminal sequences between caliciviruses**

Sequences upstream of the nucleotidylation site were compared between MNV-1, HuNoV GI and GII, RDHV, bovine norovirus and FCV. Several residues were conserved among caliciviral strains including the sequence KGKXX (Figure 3.33). The bovine norovirus NS5 shows more variance at the N-terminus however the others display close similarities. The amino acids at the N-terminus include several with positively charged side chains such as lysine (K) and arginine (R). Positively charged amino acids are often involved in nucleic acid binding due to charge attraction (332).
Murine  -GKKGKNKKGGRPGVFTRG
Human-GI  GKNKKTGGGRGKNNYFA-
Human-GII -GKKGKNKSRGKKHATSSK
Rabbit  -GVKGTGKRRGARVNLGNDE
Bovine  -PVLKKSRRKTNAFSRG
Feline  -EAKGKTLKIG----------

**Figure 3.33. Sequence comparisons between calicivirus N-terminus residues.**

Amino acid sequences from the N-terminus were compared between MNV-1 (Murine) (GenBank accession number DQ285629.1), HuNoV GI (GenBank accession number KF039737.1), HuNoV GII (GenBank accession number JX459908.1), RHDV (Rabbit) (GenBank accession number AB300693.2) and bovine norovirus (Bovine) (GenBank accession number EU794907.1) and FCV (Feline) (GenBank accession number M86379.1) using Clustal Omega analysis. Colours of amino acids indicate those with similar properties. Red amino acids (AVFPMILW) indicate small positive hydrophobic residues, blue (DE) indicates acidic, pink (RK) indicates basic and green (STYHCNGQ) indicates hydroxyl, sulfhydryl and amines.

### 3.4.3 Functional analysis of MNV and HuNoV NS5 proteins

MNV-1 and HuNoV NS5 sequences were examined for protein interactions using PredictProtein analyses (333). Results showed several sites of predicted polynucleotide binding regions clustered at the N-terminus in both MNV-1 and HuNoV NS5 sequences (Figure 3.34). There are 10 predicted sites of potential nucleic acid binding in the first 20 amino acids of the MNV-1 NS5 protein and 8 in the HuNoV GII NS5. The charged amino acids found at the N-terminus of MNV-1 and HuNoV are predicted to bind nucleic acids. The binding of nucleic acids by norovirus NS5 might be involved in nucleotidylation to the viral genome or binding of host mRNA or DNA. The first 10 amino acids of MNV-1 NS5 are shown to be essential in inducing cell cycle effects. It is possible that the predicted polynucleotide-binding sites may play a role in this process. Several sites of protein-protein interactions are predicted throughout both NS5 sequences, including several around the site of NS7 protein binding involved in nucleotidylation to the viral genome.
Figure 3.34. MNV and HuNoV NS5 predicted binding sites.
MNV-1 (A) and HuNoV (B) NS5 sequences were analysed with ProteinPredict for detection of predicted binding sites. Sites of polynucleotide-binding are indicated by circles and protein binding regions indicated by diamonds. Amino acid positions are shown above the graphics.

3.4.4 NS5 motif prediction

NS5 sequences from MNV-1 and HuNoV were analysed for functional sites using the ELM server. The ELM server predicts protein function based on sequence analysis and provides a list of potential motifs and their functions. The NS5 analyses for MNV-1, HuNoV GI and HuNoV GII revealed several predicted function motifs across the entire sequence. Motifs at the N-terminus of MNV-1 NS5 that may play a role in cell cycle manipulation include a di-Arg ER retrieval and retention motif (TRG_ER_diArg_1) and a classical Nuclear Localisation Signal (NLS) (TRG-NLS_MonoExn_4) at positions 10 – 13 and 5 – 11 respectively (Figure 3.35). The di-Arg ER retrieval and retention motif is a sequence recognised for ER localisation and is involved in monitoring correct folding and complex assembly (334). The sequence has also been documented in interactions with several proteins including cell cycle regulator protein 14-3-3 (335). The classical monopartite NLS sequence is recognised by the importer protein importin-alpha, which transports proteins with this sequence to the nucleus (336). This suggests that MNV-1 NS5 may be able to gain access to the nucleus, although its known functions take place within the cytoplasm. The classical monopartite NLS sequence is found in HuNoV GI but is not seen in HuNoV GII genogroups. The di-Arg ER retrieval and retention motifs are found in the HuNoV NS5 sequence but not at the N-terminus that is essential in cell cycle effects induced by MNV-1 NS5. Predictions of disorder show all three sequences are disordered at the N-terminus (Figure 3.35). It has
been shown previously that the N-terminus of the HuNoV GII NS5 is essential in nucleotidylation, an event that has been linked to Y27, downstream of the N-terminus. This data suggests that disruption of the N-terminus may impact other protein functions downstream. ELM server analysis revealed a STAT5 binding motif (LIG_SH2_STAT5) in NS5 from MNV-1, HuNoV GI and HuNoV GII downstream of the N-terminus. Although the STAT5 binding motif is a promiscuous motif found in ELM predictions, it provides a potential mechanism of a cell cycle arrest the G1 phase. Binding and inhibition of host STAT5 would lead a decrease in cellular proliferation and a G1 phase arrest (337).

Figure 3.35. ELM motif analysis of NS5 from MNV, HuNoV GI and HuNoV GII. Amino acid sequences from MNV-1 and HuNoV GI and GII were analysed with the ELM prediction motif server (338). Predicted disorder and functional motifs are shown next to their position within the protein sequence.
4 Discussion

4.1 Characterisation of MNV-1 effects on the host cell cycle

This study explored the relationship between the host cell cycle and MNV-1 replication. Analysis of microarray data initially provided clues as to an interaction between MNV-1 and the host cell cycle with several key cell cycle regulatory proteins dysregulated in response to infection. This lead to research investigating the effects of MNV-1 infection on the host cell cycle through analysis of cell cycle regulatory proteins and cell cycle profiles.

MNV-1 infection affects the host cell cycle

The effects of dysregulation of cell cycle related transcripts first highlighted by microarrays on MNV-1-infected RAW264.7 cells, correlates with effects seen at the protein level in MNV-1-infected RAW-Blue cells. An alternative cell line was used to confirm cell cycle effects in a related but independent cell line. RAW-Blue cells show similar MNV-1 growth kinetics to the RAW264.7 cells and frozen stocks with lower passage numbers were available. This allowed faster cell replication with significantly lower doubling times. Furthermore the RAW-Blue cell line is more tolerant to treatments, overcrowding, nutrient depletion and exhibits more consistency between replicates. Effects on cell cycle regulators shown at a transcript level in RAW264.7 cells, were subsequently shown to be commensurate with the level of cyclin proteins in infected RAW-Blue cells. Cyclins are key regulators of cell division that help govern transition between each phase of the cell cycle. Protein levels and activity of host cyclins will oscillate during cell division, with specific cyclins increasing in quantity during a certain phase where it is most active and decreasing in others. Analysis of host cyclins showed that cyclin A and cyclin B2 decreased post-MNV-1 infection. Cyclin A is a S-phase cyclin while cyclin B2 is a G2/M phase cyclin. A decrease in both S and G2/M phase cyclins suggests that there are fewer cells within these cell cycle phases, due to an increase in early cell phases (G0/G1). Furthermore, the effect seen during MNV-1 infection on cell cycle regulators at a protein level was shown to affect host cell DNA replication. Infection of an asynchronous population of cells lead to an increase in the proportion of cells in the G0/G1 phase and a decrease in the percentage of S phase cells.
An increase in the number of cells in a particular phase of the cell cycle can be caused by several mechanisms including cell cycle arrests, the prolonging of a phase or an accelerated phase transition. Progression through later stages of the cell cycle was studied to see if MNV-1 was pushing progression of the host cell through the later stages of the cell cycle, causing an increase in the percentage of cells in the $G_0/G_1$ phase. Infected RAW-Blue cells released from a nocodazole induced M phase arrest showed identical progression into $G_0/G_1$ phase, showing MNV-1 has no affect on cell cycle progression through these phases. The M phase arrest induced by nocodazole treatment is rapidly reversed after the chemical is removed through media replacement. For this reason, cells were infected prior to release, so as to allow time for the virus to replicate and have an effect on the host cell cycle prior to release from the arrest. Nocodazole arrests cells relatively late in the cell cycle (early M phase) and it was possible that MNV-1 causes faster progression through the later stages of the cell cycle ($G_2$-M phase), before the nocodazole arrest. To address this question, the transition of infected cells from $G_2$ phase through to $G_1$ phase was analysed. Synchronisation of cells with genistein was used in later experiments as it causes an arrest late in cell division during the $G_2$ phase. Several attempts were made to release cells from a genistein $G_2$ phase arrested population, however cells remained in $G_2$ phase (data not shown). It was considered if an alternative method to explore the effect of MNV-1 on late phase cell cycle progression was needed, such as using the double thymidine block method (339), however due to later results showing a strong $G_1/S$ arrest it was concluded such an approach was not necessary.

Progression though the $G_1/S$ phase of the cell cycle was analysed through two similar experiments. Cells were synchronised to the $G_0$ or $G_1$ phase, infected and monitored for progression into $S$ phase. In cells released from a $G_0$ arrest, progression into $S$ phase was only partially inhibited, while in cells released from a $G_1$ arrest there was a significant arrest in $G_1/S$ phase progression. As MNV-1 infection had no affect on late phase cell cycle progression, it was concluded that the increase in the $G_0/G_1$ phase population was due to inhibition of $G_1/S$ progression. The reasoning why progression of infected cells released from a $G_0$ population did not have a large effect on $G_1/S$ progression was explained by later results comparing MNV-1 replication in various cell populations. These results showed that
a G₀ arrested population was unable to support replication of MNV-1, illustrated by near undetectable viral protein levels and minimal progeny virus replication. This may explain why MNV-1 infection did not considerably reduce progression from G₀ to S phase. If MNV-1 was unable to replicate efficiently in a G₀ arrested population then viral proteins would not have been expressed at sufficient levels to induce effects on the host cell cycle, leading to no inhibition of G₁/S progression.

The effect of MNV-1 infection on cyclin expression matches what is expected in a population of cells progressing through the G₁ phase. The B family of cyclins are highly expressed during late S phase until late mitosis (340, 341). A decrease in cyclin B2, as seen during MNV-1 infection from transcriptome data and protein quantification would suggest a decrease of cells in the G₂/M phases. This is consistent with the effect of MNV-1 on the cell cycle, as cyclin B2 expression would be low in a G₁ phase arrested population due to decreased S phase and G₂/M phase cells. Levels of the cyclin D family are high during the G₂ phase through to the end of the G₁ phase (342). MNV-1 infection had no significant effect on cyclin D1 expression during an infection of asynchronous cells, or in cells released from a G₀ phase arrest progressing into S phase. Cyclin E is involved in the transition between the G₁ and S phases of the cell cycle. Although MNV-1 infection caused changes in transcript levels of cyclin E (322), MNV-1 had no observed effect on cyclin E protein levels during infection of asynchronous cells or on cells released from quiescence. Changes in mRNA transcripts don’t always correlate with changes in protein levels, as there are multiple layers regulating protein translation and clearance. Lastly, A cyclins are involved in several phases of the cell cycle, but predominantly S phase, where they are involved in entry and progression through S phase and into the G₂ phase (343). MNV-1 infection caused a reduction in cyclin A expression in an asynchronous cell culture and prevented expression in cells released from quiescence. The down-regulation of cyclin A strongly supports the G₁ phase accumulation seen during infection. We propose that the inhibition of cyclin A expression is either a consequence of a G₁/S arrest or a possible cause of the cell cycle phase arrest. A range of factors regulates cell replication including; transcriptional and post-translational modifications to cell cycle regulators, protein localisation, CDK activity and CDI (344). The mechanism by which MNV-1 is inducing a G₁/S phase cell cycle arrest and consequent cyclin A protein decrease is of particular interest.
This section has characterised a novel finding about the relationship between MNV-1 replication and the host cell cycle. Notably the increase in the G_0/G_1 phase population due to decreased progression through the G_1/S checkpoint. Host cyclin expression matches that of a population of cells in G_1 phase with considerable interest in cyclin A expression, the expression of which was strongly inhibited by MNV-1 infection. What contributes to the decrease in cyclin A expression in MNV-1-infected cells may unlock the mechanism of the G_1/S arrest.

4.2 The effect of the host cell cycle on MNV-1 replication

*MNV-1 manipulation of the host cell cycle creates favourable conditions for viral replication*

It was hypothesised that an arrest in the G_0/G_1 phase induced by MNV-1 infection could create a more favourable environment for MNV-1 replication. Initial experiments hinted that a population of cells released from an M phase arrest supported the highest viral replication. The M phase released population was later found to have quickly progressed into G_1 phase, where the population remained for the majority of viral infection. Initially the increase in viral replication in a M-released population was thought to be due to doubling of the cell population as cells completed mitosis and divided into two daughter cells, effectively doubling the initial plated cell population. However, later experiments used a G_1 progressing population that allowed M phase cells to divide and enter G_1 phase prior to seeding. When MNV-1 replication in the G_1 progressing population was compared with replication in other phases of the cell cycle, it was revealed that MNV-1 VP1 expression and progeny replication is highest in a G_1 progressing population. Viral VP1 was chosen as a measure of viral replication as it is highly expressed from sgRNA later in the infection cycle. Furthermore the VP1 protein is easily detected through Western blot analysis. The increase in viral replication in a G_1 > population correlates with the effect that MNV-1 has on the cell cycle, as we propose that MNV-1 infection prolongs the G_1 phase of the cell cycle by inhibiting G_1/S transition, thus providing a more beneficial environment for MNV-1 replication.
DNA viruses have been studied extensively in regard to effects on cell cycle control. Traditionally DNA viruses manipulate the cell cycle to push cells into S phase, where DNA replication machinery of the host is active. This is an important strategy for DNA viruses that infect non-dividing cells; since if the virus does not contain proteins of its own to undertake DNA replication, they must rely on the host machinery, which is only active during S phase. RNA viruses that effect the cell cycle are less characterised, although several have been shown to cause a G₀/G₁ phase arrest including; influenza A virus, respiratory syncytial virus, measles virus, coxsackievirus, murine coronavirus and severe acute respiratory syndrome-coronavirus (193, 194, 288, 290, 297, 345). MNV-1 similarly influences the host cell cycle, causing a G₀/G₁ phase arrest that benefits its own replication. However it cannot be excluded that the effects on the cell cycle are host driven, as a consequence of MNV-1 infection and that the virus has adapted to better replicate in these conditions.

**Biological significance of favoured viral replication in a G₁ population**

The biological significance of MNV-1 induced cell cycle arrest or prolonging of the G₁ phase is supported by more efficient MNV-1 capsid (VP1) production and progeny replication. The benefit to the virus may be explained by several factors. Ribonucleotides are precursors for deoxyribonucleotides with levels oscillating throughout cell division. Levels of ribonucleotides drop as cells enter S phase due to the increased demand for deoxyribonucleotides (346). A cell cycle arrest in the G₁ phase would provide increased amounts of ribonucleotide pools for MNV-1 genome synthesis. Expression of enzymes involved in the processing of ribonucleotides to deoxyribonucleotides, are inhibited during MNV-1 infection. Thymidine kinase 1 (TK1), ribonucleotide reductase M2 (RRM2) and deoxyuridine triphosphatase (DUT) expression was decreased at a transcript level as shown by microarray analysis (322). These changes were confirmed by qRT-PCR showing decreases in TK1, RRM2 and DUT (J. Ward, personal communication). An induced arrest at the G₁/S restriction point by MNV-1 would prevent a decrease in ribonucleotide levels, favouring the replication of MNV-1 RNA genome.
Different phases of the cell cycle have varying metabolic rates, with the G₁ phase having the highest translation efficiency (347, 348). The increased translation efficiency in the G₁ phase is exploited for recombinant protein expression by industries, and may also be beneficial for MNV (349, 350). Translation efficiency of Hepatitis C virus is shown to be greatest during the G₀/G₁ phase of the cell cycle (351). The G₁/S phase arrest induced by MNV may increase translation efficiency rates of MNV proteins and aid in MNV replication.

The recruitment of membranes for norovirus replication is shown to be vital to the synthesis of viral proteins (102). The MNV-1 replication complexes associate with the endoplasmic reticulum and Golgi apparatus (109). During mitosis the endoplasmic reticulum and the Golgi apparatus disassemble and so the G₁/S arrest seen in MNV-1-infected cells may prevent impairment of membrane structures and reduced MNV-1 replication (95, 100).

An induced cell arrest has the potential to protect infected cells from the host immune system. Non-cycling cells are also less likely to be killed by cytotoxic T cells, making for a more persistent infection and longer shedding period (352). Several studies have described links between the cell cycle and apoptosis signalling. A delay in apoptosis is often seen following a cell cycle arrest (353, 354). Furthermore, induction of apoptosis often requires cell cycle progression (355). MNV-1 infection results in a cell cycle arrest for up to 12 hours post-infection (data not shown). At 12 hours post-infection apoptosis is stimulated in RAW264.7 cells, illustrated by a down-regulation of survivin (141). Thus, the MNV-1 induced cell cycle arrest may prevent early death from apoptosis, allowing time for MNV replication.

4.3 The mechanism behind MNV-1 infection and cell cycle effects

*MNV-1 and the IFN response*

Initially it was considered that the changes in the host cell cycle might be a consequence of IFN produced during infection. Type 1 IFN, is produced by various cell types after recognition of antigens. IFN can cause cell cycle arrests in neighbouring cells after it is released in response to a viral infection or other stimuli. It has been observed that some
cells enter into a quiescent $G_0/G_1$ state in response to type 1 IFN (356, 357). There are several pathways that IFN is thought to target, including interactions with p53 and transcription factor c-myc. The p53 protein is transcribed in response to IFN-α and IFN-β and regulates the cell cycle through binding to DNA and inducing expression of p21 (358). The p21 protein directly inhibits the activity of cyclin E/CDK2 and cyclin D/CDK4/6 complexes, inducing a cell cycle block at the G₁ phase. The p21 protein also causes a decrease in phosphorylation of pRb protein, preventing the release of E2F and S phase progression (359). The c-myc protein is a nuclear oncogene transcription factor that induces expression of genes involved in cell cycle entry. Certain cytokines including IFN-α, IFN-β and IL-6 can reduce c-myc expression causing a cell cycle arrest in the $G_0/G_1$ phase (360). Analysis of microarray data from MNV-1 infected RAW264.7 cells found IFN-β to be the mostly highly upregulated cytokine in response to infection (141, 322, 327). Treatment of an asynchronous population of RAW-Blue cells with IFN-β was shown to induce a $G_0/G_1$ phase arrest. In order to study the relationship between IFN and the ability of MNV-1 to induce a G₁ arrest, a cell population with the type 1 IFN response pathway inhibited was used. The IFN response pathway was suppressed with the use of a JAK inhibitor that blocks the action of JAK 1, 2 and 3 proteins. The JAK proteins are an essential part of the signal transduction relay in response to cytokines including IFN. Inhibition of the JAK proteins will lead to a cell non-responsive to IFN, as the JAK proteins will not be able to phosphorylate STAT proteins that carry the signal to the nucleus. MNV-1 infection of this cell line non-responsive to IFN still induced a $G_0/G_1$ arrest, thus implying that the observed cell cycle arrest is independent of the IFN response.

**MNV-1 proteins and the host cell cycle**

As the IFN response was not responsible for effects to the host cell cycle, we hypothesised that the G₁/S arrest may be viral protein driven. MNV is a small virus and only expresses seven non-structural proteins (NS1-2, NS3, NS4, NS5, NS6, NS7 and VF1) and two structural proteins (VP1 and VP2). The VF1 protein of MNV has been documented to down-regulate the innate immune response. Available within our lab was a viable MNV-1 strain with the ORF4 gene knocked out through introduction of a stop codon. The effects on the host cell cycle were compared between the WT MNV-1 strain and the ORF4 knockout virus MNV-4S.
Both showed identical effects on the host cell cycle, indicating that VF1 is not responsible for the observed effects on cell division.

The expression of other non-structural proteins were then analysed for their effects on the host cell cycle. The NS7 protein is an RdRp, whose functions are conserved in replication of the viral RNA. The two structural proteins (VP1 and VP2) are expressed later in viral infection and so it is unlikely that they play a role in manipulation of the host cell cycle, as effects seen on the host cell division occur as early as 6 hours post-infection. That left NS1-2, NS3, NS4, NS5 and NS6 as the most likely viral cause of the cell cycle arrest. Available within the Ward lab were *E. coli* cells containing plasmids encoding the individual MNV-1 non-structural proteins. With varying degrees of success, the sequences of viral proteins could be transcribed to RNA, expressed and detected within cells through the use of Neon-based transfections. Viral NS1-2 and NS5 were analysed for their effects on the host cell cycle. Both NS1-2 and NS5 show high expression through RNA transfection and were good candidates for inducing cell cycle effects. NS1-2 was chosen because it is a disordered protein and contains a putative H box/NC sequence motif (95, 100). Disordered proteins are well known for containing multiple functions and the putative H box/NC sequence motif is found in cellular proteins such as H-rev and TIG3 that are both tumour suppression proteins involved in regulation of cellular proliferation (94). The NS5 protein is also a disordered protein and is involved in binding host eIF, to aid in viral protein translation (121, 129). Disorder is also often found in proteins associated with cell division, including p21 and p27 proteins that contribute to the control of G<sub>1</sub>/S progression (361). Initially the hypothesis was that host protein shut off induced by NS5 binding eIF, as seen in plant VPg proteins, could attribute to the cell cycle arrest induced by MNV-1 infection (122).

Expression of NS5 protein in an asynchronous cell culture induced a cell cycle arrest in the G<sub>0</sub>/G<sub>1</sub> phase similar to what occurs during MNV-1 infection. Furthermore, NS5 expression was shown to inhibit G<sub>1</sub>/S progression and inhibit cyclin A accumulation post-G<sub>1</sub> release. Both of the characteristics of cyclin A inhibition and a G<sub>1</sub>/S arrest were also observed during MNV-1 infection and strongly imply that NS5 is responsible for the cell cycle effects of MNV-1. To further narrow down the domain within NS5 responsible for cell cycle effects, three NS5 constructs were expressed and their effects on the cell cycle
investigated. Previous studies had pinpointed functions of NS5 to identified regions of the protein. These were taken into account when designing regions of NS5 to be expressed. Of particular interest was the nucleotidylation site located within the first helix and the eIF binding region located at the C-terminus (120, 123). The NS5 1-62 construct contained the first two helices, while the NS5 63-124 construct contained a middle region of disorder and the eIF binding site. The third construct NS5 11-107 contained the three structural helices but not the first 11 disordered residues and the eIF binding site. Synthetic constructs were obtained from GenScript, who guarantee 100% sequence accuracy. The constructs were meant to complement each other as they each expressed specific regions, so if a particular construct had a cell cycle effect then that could be matched to a region of NS5. The α-NS5 antibody could detect expression of NS5 1-62 and NS5 11-107 but could not detect the NS5 63-124 construct. It is possible that the α-NS5 antibody recognises a motif in NS5 towards the N-terminus and so could not detect the NS5 63-124 protein. Alternatively, the NS5 63-124 construct may not fold correctly due to lack of conserved secondary structures or the protein is not being expressed. To further confirm expression of the construct the proteins were expressed in RAW-Blue cells, separated by SDS-PAGE and analysed by mass spectrometry for detection of protein sequences. Although NS5 1-62 and NS5 11-107 could be detected by Western blot analysis, mass spectrometry failed to detect expression of any NS5 constructs (data not shown). The mass spectrometry approach used was shotgun proteomics, meaning the mass spectrometry spectrum created is made from the most abundant proteins first. This means that low abundance proteins are often missed even if present. The NS5 1-62 and NS5 11-107 constructs could be detected by the α-NS5 antibody and migrate at the expected sizes on a SDS-PAGE gel, implying that these constructs are indeed expressed. The NS5 1-62 construct when expressed in a cell population induced a G₀/G₁ phase arrest, similar to full length NS5, while NS5 63-124 and NS5 11-107 had no effect on the host cell cycle. Although, NS5 63-124 could not be detected by Western blot analysis and NS5 11-107 had a weaker band density then NS5 1-62 because of either lower expression or poorer detection. As NS5 1-62 induced effects on the cell cycle, but NS5 11-107 did not, it appears that residues at the N-terminus are essential to inducing cell cycle effects and furthermore that the cell cycle effects occur independently of the eIF binding region. The first 10 amino acids of NS5 may contain the region solely responsible for the effects of NS5 on the host cell cycle or may be active in conjunction with downstream
regions. The NS5 1-62 protein was further analysed for inhibition of G1/S phase transition. Cells were arrested in the G1 phase, transfected with NS5 1-62 and released from the cell cycle arrest to measure transition into S phase. Initial experiments failed to produce data showing NS5 1-62 inhibited G1/S transition (data not shown). This is likely due to the short half-life of NS5 1-62, as the protein is degraded quickly post-transfection and was undetectable when cell cycle effects of full length NS5 are observed on a G1/S transitioning population. An alternative approach to measuring the effect of NS5 1-62 on G1/S phase transition is needed.

Studies on HuNoV GII.4 NS5 showed that deletion of sequences at the N-terminus greatly inhibited nucleotidylation (118). Deletion of the first 3 residues decreased guanylylation by 83%, while deletion of the first 20 residues saw complete inhibition. These results indicate that the disordered N-terminus of norovirus VPg proteins containing a stretch of positively charged amino acids is essential in the nucleotidylation process. This sequence is similar to an NTP-binding motif in the potyvirus (potato A virus) VPg (362). The NTP-binding site may facilitate nucleotidylation at the tyrosine residue, using NTPs as an energy source for guanylylation or the region might facilitate binding to the viral RNA genome. Alternatively, deletion of the N-terminus may disrupt the conformation of the nucleotidylation site, affecting interactions with the viral RdRp (NS7). In regards to the effects of nucleotidylation on the cell cycle, it is difficult to hypothesise feasible mechanisms of inducing cell cycle arrests since the viral RdRp was not present during transfections. Nucleotidylation relies on the catalytic action of the viral RdRp to guanylylate VPg to the viral RNA, and so without expression of the viral RdRp nucleotidylation will not occur. Alternative functions within the N-terminus are more likely the cause of the cell cycle manipulation. This study is the first documentation of a VPg protein inducing changes to the host cell cycle.

4.4 Bioinformatic analysis of MNV-1 NS5

Bioinformatic analysis of MNV-1 NS5 predicted a potential di-Arg ER retrieval and retention motif at the N-terminus. This motif has been documented in HuNoV GI.1 NS4 protein, with an amino acid sequence that mimics a traditional di-acidic ER export signal and may inhibit
cellular secretion (108). Interactions within the ER are too vast to hypothesise to permit any confident prediction of function of the NS5 motif, but it could be involved in interactions with global pathways such as membrane trafficking, protein folding or protein synthesis. ER stress also induces cell cycle arrests in the G₁ and G₂ phases and so effects on G₁/S phase transition may be a global effect to ER stress induced by NS5 expression (363, 364). ER stress induces a G₁ arrest due to activation of protein kinase RNA-like endoplasmic reticulum kinase (PERK), a protein that inhibits cyclin D1 expression (365, 366). Inhibition of cyclin D1 was not observed during MNV-1 infection, suggesting ER stress is not involved in inducing the cell cycle effects.

The di-Arg ER retrieval and retention motif has also been documented in binding to 14-3-3 proteins. If NS5 were binding to 14-3-3 proteins, it would most likely act as a negative regulator. The 14-3-3 proteins are a large family of conserved proteins with very diverse functions and multiple isoforms. The two isoforms of 14-3-3 proteins that can bind to di-Arg ER motifs are epsilon (ε) and zeta (ζ) (335). The 14-3-3ε proteins are involved in signal transduction while the 14-3-3ζ proteins can play a role in cell division (367). The 14-3-3ζ proteins have been shown in murine cells to bind to Wee1 protein, increasing its stability and activity. Wee1 is a negative regulator of CDK1 and phosphorylates the protein, rendering it inactive (368, 369). CDK1 is involved in progression through late cell phases, and is found in complex with cyclin A and cyclin B. Inhibition of CDK1 by 14-3-3-Wee1 leads to a G₂/M phase arrest (369). If the NS5 protein from MNV-1 was binding to 14-3-3ζ and inhibiting its action, this would lead to Wee1 instability, inactivity and loss of CDK1 inhibition. The loss of CDK1 inhibition would allow for cell cycle progression through the late cell phases. MNV-1 induced a cell cycle arrest at the G₁/S restriction point, which is not regulated by Wee1 and so it is unlikely that NS5 is inducing its cell cycle effects through binding to 14-3-3 proteins.

A STAT5 binding motif conserved across MNV-1, HuNoV GI and HuNoV GII was found in the NS5 sequence and could be involved in depletion of cellular STAT5. Depletion of STAT5 would lead to an increase in levels of p16, p21 and p27 leading to a cell cycle arrest at the G₁ phase (337). Furthermore, STAT5 regulates the expression of cdc25A, an oncogene with a minor role in regulating G₁/S progression through activation of cyclin E/A-CDK2
complexes (370). Therefore a decrease in STAT5 may lead to a decrease in cdc25A activity and a G₁ arrest. The LIG_SH2_STAT5 motif found in ELM predictions is promiscuous and commonly found in proteins with arginine residues, however its presence in NS5 may indicate a potential mechanism for an NS5 induced cell cycle arrest.

Out of the first ten amino acids at the N-terminus of MNV-1 NS5 protein, five are lysines and one is arginine, both of which have positively charged side chains. Positively charged amino acids are often involved in nucleic acid interactions, suggesting the positive N-terminus of NS5 may interact with host mRNA or act as a transcription factor or transcription repressor on host DNA (371, 372). Bioinformatic analysis of the MNV-1 NS5 sequence predicted potential poly-nucleotide interaction sites and a nuclear localisation signal (NLS) sequence within the N-terminus. The NLS sequences are recognised by the importer protein importin-alpha and then cargo-loaded importin complexes are translocated through the nuclear pore into the nucleus. The NS5 protein could then engage in inducing or inhibiting transcription of genes through its predicted poly-nucleotide binding region within its N-terminus, inducing downstream effects causing a G₁/S arrest. These interactions between nucleic acids and proteins occur as nucleic acid is generally negatively charged and so electrostatic interactions or hydrogen bonds appear between the nucleic acid phosphodiester backbone and positively charged lysines, arginine and occasionally histidine side chains (373). This potential NLS sequence was also observed at the N-terminus of human GI but not human GII noroviruses. The importation sequence predicted in MNV-1 NS5 is a monopartite NLS, containing a short basic cluster of lysines and arginines. Multiple virus proteins include a nuclear importation sequence in order to hijack the host nuclear importation system to transport viral proteins to the nucleus (374-377). It is possible that MNV NS5 protein might be transported to the nucleus and bind DNA through its predicted binding sites at its N-terminus. However, localisation studies found NS5 to be localised in the perinuclear region, suggesting that if NS5 is transported into the nucleus, it must also be exported out (109). Bioinformatic analysis also did not find evidence of a nuclear export signal, suggesting the predicted poly-nucleotide binding regions may facilitate binding to host mRNA, interfering with expression of genes involved in cell division. It is possible that the MNV-1 NS5 protein binds to and inhibits cyclin A
mRNA expression or other mRNA that would lead to inhibition of G₁/S phase progression and a decrease in cyclin A expression.

Sequence alignments of caliciviral NS5 proteins showed very conserved regions at the N-terminus of the protein, including conservation of positively charged amino acids, predicted to be involved in nucleotide binding. Furthermore, the amino acids at the N-terminus of MNV-1 NS5 (1-10) were shown to be essential in inducing cell cycle effects. It is therefore possible that the effects of MNV-1 NS5 on the cell cycle are conserved among other caliciviruses. Further analysis of the NS5 protein of other caliciviruses will be required to address this possibility.

4.5 Conclusions

This research is the first documentation of a calicivirus manipulating the host cell cycle, in order to favour its own replication. This study showed that MNV-1 infection of RAW-Blue cells leads to change in host cyclin levels and consequently a cell cycle arrest, leading to an increase in the G₀/G₁ population. Furthermore this arrest occurred at the G₁/S restriction point, as infected cells showed reduced progression into S phase. This effect on the host cell division created an environment beneficial to viral reproduction, as MNV-1 replication was increased in a G₁ progressing population. The manipulation of the host cell cycle may be a strategy used by the virus to increase viral propagation in macrophage populations during infection. These effects on the host cell division were linked to the activity of viral NS5 protein, as expression on this single protein induced a cell cycle arrest, identical to that observed during MNV-1 infection. Furthermore, the arrest could be induced by the first 62 amino acids of NS5 with the N-terminus (1-10 amino acids) playing an essential role. Sequence similarities at the N-terminus between norovirus NS5 sequences suggest the cell cycle manipulation may be a conserved function.
5 Future Directions

Research using MNV as a model for HuNoV characterised the relationship between viral replication and the host cell cycle. The purpose for the use of MNV as a model virus is that interactions with the host may be conserved with HuNoV. There are close similarities in the sequences of NS5 between noroviruses, especially at the N-terminus, which is linked to the effects on the host cell cycle. It is possible that other caliciviruses are capable of inducing cell cycle effects through the actions of NS5. Experiments mimicking those undertaken in this thesis could be carried out on other caliciviruses, such as other MNV strains or FCV that can grow readily and produce clear cytopathic effects in cultures of established cell lines, such as Crandell's feline kidney cells. Other caliciviruses are not as easily replicated in cell culture, however transfection of caliciviral NS5 transcripts from different strains into cells could be used for analysis of cell cycle effects. This could be done through transfection of caliciviral RNA or expression of viral genes from a DNA plasmid in a cell population. HuNoV has recently been documented to replicate in B-cells in the presence in coliform bacteria (53). Experiments examining HuNoV infected B-cells could be analysed for effects on the host cell cycle. Alternatively the HuNoV GI and GII NS5 could be expressed individually within cells and the effects on the host cell cycle measured.

Studies to identify the mechanism behind the effects of the NS5 protein on the cell cycle would add valuable knowledge and may identify important host proteins involved in interactions with viral proteins. The two most characterised functions of NS5 are nucleotidylation to the viral genome and aiding in translation of viral proteins. These two functions can be knocked out through the use of point mutations. Residues Y26 and F123 have been documented to be essential in nucleotidylation and binding of host eIF respectively. Substitutions to alanines generally have minimal effects on protein structure, but can completely abolish the nucleotidylation and translational activity of NS5 (121, 123). Generating two NS5 constructs with F123 to A123 and Y26 to A26 mutations and analysing their effects on the host cell cycle could easily address if the known functions of NS5 are involved in cell cycle dysregulation.
The NS5 protein may induce cell cycle manipulation through interactions with cellular host proteins. To identify interactions between host proteins and viral NS5, an immunoprecipitation of NS5 and NS5 1-62 using the α-NS5 antibody and detection of interactions via mass spectrometry would identify potential binding partners. Mass spectrometry of full length NS5 will identify host eIF already documented to be involved in viral translation, but analysis of the NS5 1-62 construct will avoid the confounding effects of eIF co-precipitation as it does not contain the eIF binding domain (64, 121, 123).

Bioinformatic analysis of MNV-1 NS5 protein gave insights into possible mechanisms of cell cycle manipulation. Analysis of the NS5 1-62 protein showed potential poly-nucleotide binding sites at the N-terminus. It is possible that NS5 is binding to host mRNA and effecting protein expression of genes involve in cell regulation. RNA-NS5 interactions could be studied through various methods such an RNA pull-down assay using the α-NS5 antibody and sequencing of the mRNA transcripts. If NS5 targets were identified these could be confirmed by Northern blot analysis or the proteins detected by Western blotting.

Alternatively, the NS5 protein might be involved in interactions with DNA. Despite the presence of a predicted N-terminal NLS, localisation studies of MNV NS5 detected its presence in the perinuclear region, where viral replication occurs (102, 128). These experiments assessing MNV proteins expressed from a tetracycline-regulated plasmid in HEK293 cells (128). It would be interesting to see if the same localisation is observed following transfection of RNA transcripts into either RAW-Blue or RAW264.7 cells. Localisation studies on MNV-1-infected cells would also be interesting. Such studies could include the use of inhibition of nuclear export with leptomycin B to examine the possibility that NS5 shuttles between the cytoplasm and nucleus. If NS5 was detected in the nucleus then it is likely that it is interacting with host DNA. Protein-DNA interactions could then be detected through several means such as via chromatin immunoprecipitation.

Additional confirmation of the effects of NS5 1-62 on the cell cycle would confirm the N-terminus as the key functional region of NS5. Previous experiments addressing the effect of NS5 1-62 expression on G1/S phase progression failed potentially due to the short half-life of NS5 1-62, compared to full length NS5. A way of guaranteeing expression of the
truncated protein over a longer time course is through a second transfection at a later time, thus keeping expression high and avoiding the decrease in protein levels seen at end time points (378). Alternatively, transfection of NS5 1-62 in a plasmid may increase protein expression but at the cost of RAW-Blue cell activation. Transfections with plasmids could also be tested in alternative cell lines that do not activate upon transfection. A decrease in cyclin A protein expression was linked to MNV-1 infection and NS5 full length expression. To further confirm the effects of NS5 1-62 on the cell cycle, experiments comparing cyclin A expression in an NS5 1-62 transfected population to a mock-transfected population would supplement the studies on cell division and confirm the N-terminus as the functional region. This could be done by analysis of cyclin A levels after transfection of NS5 1-62 via Western blot in an asynchronous cell population or cells progressing from G₀/G₁ to S phase.

The effects of MNV-1 on the cell cycle could be analysed in additional cell lines. Although the cells that noroviruses infect in vitro are not well documented, MNV has been shown to infect dendritic cells as well as monocytes/macrophages (76, 77). Analysis of the effects of MNV-1 infection or MNV NS5 expression on the host cell cycle could be tested in primary dendritic cells. Alternatively a gut epithelial cell line could be transfected with full length NS5 or full length MNV-1 and cell cycle effects analysed.

This research is the first documentation of a VPg protein interacting with the host cell cycle. VPg proteins are found in some strains of viruses from the Picornaviridae, Astroviridae, Secoviridae, Potyviridae, Futeoviridae and Sobemovirus families (115, 119). Although the VPg protein comes in a range of sizes (2-22 kDa), it is possible that the ability to manipulate the cell cycle is conserved among certain strains.
6 References


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201. **Lees EM, Harlow E.** 1993. Sequences within the conserved cyclin box of human cyclin A are sufficient for binding to and activation of cdc2 kinase. Mol Cell Biol **13:**1194-1201.


7 Appendix I: Recipes

All solutions are stored at room temperature, unless otherwise stated.

**Crystal violet plaque assay**

*Crystal violet stain solution*
0.2 g crystal violet
100 ml milliQ water
Filter sterilised.

*3.7% formaldehyde solution*
5 ml 37% formaldehyde
45 ml milliQ water

**SDS-PAGE electrophoresis**

*2x SDS-PAGE sample buffer*
5 ml 10% SDS
2 ml glycerol
1.2 ml 1M Tris-HCl, pH 6.8
100 µl 1% Bromophenol Blue
Made up to 9 ml with milliQ.
Before use, 10% β-mercaptoethanol was added.

*Resolving buffer*
1.5 M Tris, pH 8.8
0.4% SDS

*Stacking buffer*
0.5 M Tris, pH 6.8
0.4% SDS
**Resolving gel (10% acrylamide gel)**
1.875 ml 40% acrylamide
1.875 ml resolving buffer
3.75 ml milliQ water
37.5 µl 10% ammonium persulfate
7.5 µl TEMED (Sigma-Aldrich)
Mixed the first three ingredients before adding the polymerising agents.

**Resolving gel (12.5% acrylamide gel)**
2.34 ml 40% acrylamide
1.875 ml resolving buffer
3.28 ml milliQ water
37.5 µl 10% ammonium persulfate
7.5 µl TEMED (Sigma-Aldrich)
Mixed the first three ingredients before adding the polymerising agents.

**Stacking gel (4% acrylamide gel)**
0.25 ml 40% acrylamide
1.25 ml stacking buffer
1 ml milliQ water
17.5 µl 10% ammonium persulfate
3.5 µl TEMED (Sigma-Aldrich)
Mixed the first three ingredients before adding the polymerising agents.

**10x electrophoresis running buffer**
144 g Glycine
30 g Tris
10 g SDS
Made up to 1000 ml with distilled H₂O.
**RIPA buffer**
25 mM Tris-HCl (pH 7.6)
150 mM NaCl
1% NP-40
1% sodium deoxycholate
0.1% SDS
Stored at 4 °C.

**Colloidal Coomassie blue stain solution**
0.08% Coomassie Blue G-250
1.6% orthophosphoric acid
20% Methanol
5% Ammonium sulfate
Dissolved Coomassie and ammonium sulfate in methanol before adding milliQ water then acid.

**Destain solution**
10% methanol
10% acetic acid

**Western Blot**

10 × dPBS pH 6.2 *(for Western blots)*
8% NaCl
0.2% KCl
1.44% Na$_2$HPO$_4$
0.24% KH$_2$PO$_4$
Adjust to pH 6.2 and autoclave.

**Anode buffer I**
0.3 M Tris
10% methanol
Adjusted to pH 10.4 and stored 4 °C.
Anode buffer II
25 mM Tris
10% methanol
Stored at 4 °C.

Cathode buffer
25 mM Tris
40 mM glycine
10% methanol
Adjusted to pH 9.4.
Stored at 4 °C.

Blocking solution
0.1% casein
100 ml 0.2 x dPBS

BSA solution
1% BSA in 1X dPBS

Wash buffer
1X dPBS
0.1% Tween-20

Flow Cytometry

FACS Buffer
1X dPBS
0.01% Sodium Azide
0.1% BSA

RNase A stock
10 mg/ml RNase A
10 mM Sodium Acetate
Boil for 15 minutes and pH to 7.4 with 1 M Tris-HCL (pH 8.0).
Competent cell preparation

Luria-Bertani (LB) agar plates
10 g/l BactoTM Tryptone (BD BioSciences, Sparks, MD, USA)
5 g/l BactoTM Yeast Extract (BD)
5 g/l NaCl
15 g/l Agar Bacteriological (Scharlau Chemie, Barcelona, Spain)
Autoclave, cool to 50 °C, add required antibiotic(s), and pour into petri dishes. Store at 4°C.
Omit agar for LB broth.

Transformation buffer 1
30 mM Potassium acetate
10 mM Calcium chloride
50 mM Manganese chloride
100 mM Rubidium chloride
15% Glycerol
Adjust to pH 5.6 with acetic acid, filter sterilise.

Transformation buffer 2
10 mM MOPS
75 mM Calcium chloride
10 mM Rubidium chloride
5% Glycerol
Adjust to pH 6.5 with potassium hydroxide and filter sterilise.

DNA agarose gel electrophoresis

50 x TAE (tris-acetate) buffer
242 g Tris
57 ml Glacial acetic acid
100 ml 0.5 M EDTA, pH 8
Made up to 1000 ml with distilled H₂O.
0.8% Agarose gel
800 µl 50 x TAE stock solution
40 ml distilled H₂O
0.32 g DNA grade agarose LE
Heated until dissolved. Poured into gel cradle containing comb and left to set.

10 x Loading dye
25 mg Bromophenol Blue (BDH, VWR International Ltd)
2.5 g Ficoll-400 (Pharmacia, LKB)
Dissolved in 10 ml distilled H₂O.

Ethanol precipitation

TE buffer
100 mM Tris (pH 8.0)
1 mM EDTA (pH 8.0)
Made up to 50 ml with milliQ water and filter sterilise through a 22 nm syringe.

Neon Transfection

Resuspension buffer
1x dPBS
250 mM sucrose
1 mM MgCl₂
8 Appendix II: Vector maps and NS5 constructs

Fast-Bac vector containing the MNV genome

Figure 8.1. pFB MNV-RVmut.

pUC8 vector used for RNA construct generation for NS1-2 and NS5

Figure 8.2. pUC8 vector used for RNA construct generation.
NS1-2 and NS5 sequences were previously cloned into pUC8 vectors and used as the template for the generation of RNA constructs. The NS1-2 and NS5 sequences were added using primers that added an EcoRI and AvaI site respectively at the 3’ end of the viral sequences. AmpR, ampicillin resistance gene (β-lactamase). lacZ, lac operon sequences. Plac, lac operon promoter. pBR322 ori, pBR322 origin. MCS, multiple cloning site.
**pUC57**-simple vector used for RNA construct generation of NS5 1-62, NS5 63-124 and NS5 11-107.

**Figure 8.3. Plasmid map of pUC57.**

The NS5 construct sequences were cloned into the EcoRV site within the lacZ gene. The plasmid contains an ampicillin resistant gene (bla(Ap')) and an origin of replication (rep (pMB1)) and nucleotide numbering is indicated around the plasmid. Figure adapted from www.genscript.com.
9 Appendix III: Microarray Analysis

Analysis of Bok et al, 2009 (141) microarray data for changes to cell cycle related genes

<table>
<thead>
<tr>
<th>ASYMMETRIC_3PRIME_PNT_ID</th>
<th>GENE_NAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>1429418_at</td>
<td>CDC14 cell division cycle 14 homolog B (S. cerevisiae)</td>
</tr>
<tr>
<td>1433430_at</td>
<td>CDC23 (cell division cycle 23, yeast, homolog)</td>
</tr>
<tr>
<td>1418334_at</td>
<td>DRF4 homolog (S. cerevisiae)</td>
</tr>
<tr>
<td>1455792_at, 1420434_at</td>
<td>E2F transcription factor 2</td>
</tr>
<tr>
<td>1420634_at</td>
<td>MAD1 homolog 2 (Drosophila)</td>
</tr>
<tr>
<td>1460348_at</td>
<td>MAD2 mitotic arrest deficient 3 (yeast)</td>
</tr>
<tr>
<td>1424162_at</td>
<td>MAD21 homolog (S. pombe)</td>
</tr>
<tr>
<td>1419660_at, 1449232_at, 1437033_at, 1460297_at</td>
<td>S-phase kinase-associated protein 2 (p5s)</td>
</tr>
<tr>
<td>1449172_at</td>
<td>Tkt protein kinase</td>
</tr>
<tr>
<td>1416773_at</td>
<td>WEE 1 homolog 1 (S. pombe)</td>
</tr>
<tr>
<td>1417326_at, 1410459_at</td>
<td>anaphase promoting complex subunit 1</td>
</tr>
<tr>
<td>1424046_at</td>
<td>anaphase promoting complex subunit 2</td>
</tr>
<tr>
<td>1447685_at, 1416961_at</td>
<td>budding uninhibited by benomyl subunits 1 homolog (S. cerevisiae)</td>
</tr>
<tr>
<td>1444673_at</td>
<td>budding uninhibited by benomyl subunits 3 homolog (S. cerevisiae)</td>
</tr>
<tr>
<td>1444214_at</td>
<td>cell division cycle 2 homolog A (S. pombe)</td>
</tr>
<tr>
<td>1416664_at</td>
<td>cell division cycle 25 homolog (S. cerevisiae)</td>
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<tr>
<td>1417127_at</td>
<td>cell division cycle 26 homolog A (S. pombe)</td>
</tr>
<tr>
<td>1416573_at</td>
<td>cell division cycle 42 homolog (S. cerevisiae)</td>
</tr>
<tr>
<td>1417019_at</td>
<td>cell division cycle 6 homolog (S. cerevisiae); predicted gene 9430; similar to cell division cycle 6 homolog</td>
</tr>
<tr>
<td>1426002_at</td>
<td>cell division cycle 7 (S. cerevisiae)</td>
</tr>
<tr>
<td>1449708_at, 1450677_at</td>
<td>checkpoint kinase 1 homolog (S. pombe)</td>
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<td>1417910_at, 1417911_at</td>
<td>cyclin A5</td>
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<tr>
<td>1450920_at</td>
<td>cyclin B2</td>
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<tr>
<td>1416492_at</td>
<td>cyclin E1</td>
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<tr>
<td>1450293_at, 1450294_at</td>
<td>cyclin H</td>
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<td>extra spindle pole-like 1 (S. cerevisiae)</td>
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<td>1416481_at</td>
<td>formin cell division cycle 20 related 1 (Drosophila)</td>
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<tr>
<td>1440900_at</td>
<td>histone deacetylase 2</td>
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<td>1440777_at</td>
<td>minichromosome maintenance deficient 2 mitin (S. cerevisiae)</td>
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<td>1437076_at, 1415214_at</td>
<td>minichromosome maintenance deficient 3 homolog (S. cerevisiae)</td>
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<tr>
<td>1436808_at, 1415945_at</td>
<td>minichromosome maintenance deficient 5; cell division cycle 46 (S. cerevisiae)</td>
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<tr>
<td>1416261_at, 1429892_at</td>
<td>minichromosome maintenance deficient 6 (MMS5 homolog, S. pombe) (S. cerevisiae)</td>
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<tr>
<td>1415031_at, 1415030_at, 1439269_at, 1438330_at</td>
<td>minichromosome maintenance deficient 7 (S. cerevisiae)</td>
</tr>
<tr>
<td>1417037_at</td>
<td>origin recognition complex subunit 8-like (S. cerevisiae)</td>
</tr>
<tr>
<td>1422241_at</td>
<td>predicted gene 7395; transcription factor Do 1; similar to Transcription Factor Dsp1 (E. coli; DNA-polymerase 1)</td>
</tr>
<tr>
<td>1448295_at, 1416067_at, 1429942_at</td>
<td>predicted gene 8418; predicted gene 9293; cyclin B1; similar to cyclin B1; predicted gene 9372</td>
</tr>
<tr>
<td>1437608_at, 1437609_at</td>
<td>predicted gene, EF554165; predicted gene 2423; hypothetical protein LOC91211; tyrosine-dependent kinase 3-microsomal activation protein, beta polypeptide</td>
</tr>
<tr>
<td>1424158_at</td>
<td>retractable-like 1 (p107)</td>
</tr>
<tr>
<td>1425961_at</td>
<td>retractable-like 2</td>
</tr>
<tr>
<td>1422439_at, 1422440_at</td>
<td>similar to Cell division protein kinase 4 (Cyclin-dependent kinase 4) (BAC3); cyclin-dependent kinase 4</td>
</tr>
<tr>
<td>1422495_at</td>
<td>similar to Staq1p antigen 1</td>
</tr>
<tr>
<td>1422440_at</td>
<td>similar to spindle assembly checkpoint protein MAD2 mitotic arrest deficient-like 1 (yeast)</td>
</tr>
</tbody>
</table>

Figure 9.1. Changes to genes involved in cell cycle regulation at 12 h.p.i.

DAVID analysis of microarray data from Bok et al, 2009. Data was analysed for changes to cell cycle related genes that were either up or down regulated >2-fold in MNV-1-infected RAW264.7 cells compared to mock-infected populations.
Figure 9.2. Graphic of DAVID analysis using KEGG pathway mapping of genes involved in cell cycle related processes.

Genes that were up or down regulated by MNV-1 infection in RAW264.7 cells in microarray data from Bok et al was graphed with DAVID analysis. Genes that showed >2-fold changes are shown by red stars next to the gene code.
10 Appendix IV: Publications and Presentations

Publications


Presentations

Oral presentation: Murine Norovirus Replication Inhibits Proliferation of Cells at the G₁/S Restriction Point.

Otago School of Medical Sciences Postgraduate Symposium, Dunedin, NZ. 2015.
Oral presentation: Murine Norovirus Manipulation of the Host Cell Cycle.

Winner of oral speaker prize

Microbiology Department Research Retreat, Dunedin, NZ. 2015.
Oral and poster presentations: Murine Norovirus and the Cell Cycle.

Microbiology Department Research Retreat, Dunedin, NZ. 2014.
Oral and poster presentations: Murine Norovirus and the Cell Cycle

7th Australasian Virology Society Meeting, Queenstown, NZ. 2013.
Poster presentation: Murine Norovirus and the Host Cell Cycle.

Winner of best poster prize

Microbiology Department Research Retreat, Dunedin, NZ. 2013.
Oral and poster presentations: Murine Norovirus and the Cell Cycle.

Microbiology Department Research Retreat, Dunedin, NZ. 2012.
Oral and poster presentations: Murine Norovirus and the Cell Cycle.