# Temporal proteomic analysis of BK polyomavirus infection reveals virus-induced G2 arrest and highly effective evasion of innate immune sensing

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46  **Abstract**

47  BK polyomavirus (BKPyV) is a small DNA virus that establishes a life-long persistent infection in

48  the urinary tract of most people. BKPyV is known to cause severe morbidity in renal transplant

49  recipients and can lead to graft rejection. The simple 5.2 kilobase pair dsDNA genome expresses

50  just seven known proteins, thus it relies heavily on host machinery to replicate. How the host

51  proteome changes over the course of infection is key to understanding this host:virus interplay.

52  Here for the first time quantitative temporal viromics has been used to quantify global changes in

53  >9,000 host proteins in two types of primary human epithelial cell throughout 72 hours of BKPyV

54  infection. These data demonstrate the importance of cell cycle progression and pseudo-G2 arrest in

55  effective BKPyV replication, along with a surprising lack of innate immune response throughout

56  the whole virus replication cycle. BKPyV thus evades pathogen recognition to prevent activation

57  of innate immune responses in a sophisticated manner.

58

59  **Importance**

60  BK polyomavirus can cause serious problems in immune-suppressed patients, in particular kidney

61  transplant recipients who can develop polyomavirus-associated kidney disease. In this work, we

62  have used advanced proteomics techniques to determine the changes to protein expression caused

63  by infection of two independent primary cell types of the human urinary tract (kidney and bladder)

64  throughout the replication cycle of this virus. Our findings have uncovered new details of a

65  specific form of cell cycle arrest caused by this virus and importantly we have identified that this

66  virus has a remarkable ability to evade detection by host cell defence systems. In addition, our data

67  provide an important resource for the future study of kidney epithelial cells and their infection by

68  urinary tract pathogens.

## Introduction

BK Polyomavirus (BKPyV) is a small, non-enveloped, double stranded DNA virus that was first identified in 1971 (1). As a ubiquitous pathogen, it establishes a life-long persistent infection in the kidneys of most humans (2). While infection with BKPyV is subclinical in the vast majority of individuals, it is a significant cause of morbidity in the immunosuppressed, in particular kidney and haematopoietic stem cell transplants (HSCT) recipients. Polyomavirus associated nephropathy (PVAN) affects ~8% of kidney transplant patients, however treatment is currently limited to a reduction in immune suppression. Only a small number of anti-BKPyV drugs are available, all exhibiting significant nephrotoxicity, leading to graft decline of function and loss in ~85% of PVAN sufferers (3). In up to 15% of HSCT patients, BKPyV leads to haemorrhagic cystitis (HC) and severely reduced rates of HSCT recovery (4).

As with all polyomaviruses, BKPyV is structurally simple. The dsDNA genome is ~5.2 kilobase pairs long and encodes seven proteins, three of which form the virus capsid (VP1, VP2 and VP3). The four non-structural proteins (large T antigen (LTAg), small T antigen (stAg), truncTAg and agnoprotein) have numerous functions and interact with multiple host factors. For example, LTAg binds members of the Retinoblastoma protein family, inhibiting their regulation of the G1/S phase checkpoint of the cell cycle. As a result, viral infection stimulates cell cycle progression into S phase, facilitating viral DNA genome replication (5, 6). LTAg also binds p53, altering the regulation of both apoptosis and cell cycle progression (7). stAg modulates the phosphorylation of >300 cell cycle proteins and LTAg, through interaction with protein phosphatase 2A (8, 9). The role of agnoprotein is less well understood, although a wide range of activities have been proposed including action as a viroporin, enhancing viral DNA replication through interaction with the processivity factor proliferating cell nuclear antigen (PCNA), and enhancing egress of virions from the nucleus (10, 11).

The limited coding capacity of BKPyV necessitates co-option of multiple host factors in order to replicate and persist. Previous studies investigating how BKPyV infection modulates the host cell environment have primarily been conducted at the level of the transcriptome, which may not be reflected in the proteome. Infection in primary Renal Proximal Tubule Epithelial (RPTE) and Human Umbilical Vein Endothelial (HUV-EC) cells has been studied, either using microarray (12, 13) or RNAseq (14, 15). Such analyses do not provide information about virus-induced changes to cellular proteins. To date there has only been one limited analysis of changes to the host cell proteome in BKPyV infection, where stable isotope labelling with amino acids in cell culture (SILAC) was used to quantify protein changes in nuclei isolated from primary RPTE cells at 3

102  days post infection. In this study ~2000 proteins were quantified, and the effect of infection on
103  proteins outside the nucleus could not be assessed (16).

104  To gain a comprehensive global understanding of changes in host and viral proteins throughout the
105  whole course of BKPyV infection, we conducted a 10-plex quantitative temporal viromic analysis
106  (QTV) of two independent BKPyV-permissive primary human cell types, RPTE and human
107  urothelial (HU) cells. QTV uses tandem mass tags (TMT) and MS3 mass spectrometry to quantify
108  the relative abundance of proteins throughout the whole time course of infection (17). These data
109  have provide the broadest global analysis of proteome changes caused by BKPyV infection, which
110  has provided additional details of a specialised form of cell cycle arrest that is induced by this
111  virus in primary cells. In addition, we have uncovered a complete lack of induction of innate
112  immune responses at the protein level in BKPyV infected cells suggesting that this virus has
113  evolved a sophisticated mechanism for evading pathogen recognition.

114

115  **Results**

116  **Quantitative Temporal Viromic analysis of BKPyV infection**

117  To build a global picture of changes in host and viral proteins throughout the course of BKPyV
118  infection, we infected primary renal (RPTE) and bladder (HU) epithelial cells with BKPyV
119  Dunlop strain. We first used 10-plex TMT and MS3 mass spectrometry to quantify changes in
120  protein expression over three key time points of infection spanning the single-step replication
121  cycle of this virus (0-72 hours) (Experiment 1; Fig. 1A). Cells were infected at a multiplicity of
122  infection (MOI) of 5 infectious units per cell ensuring greater than 90% infection in both RPTE
123  and HU cells (data not shown). In this experiment a total of 8985 cellular and 5/7 viral proteins
124  were quantified in both cell types, providing a global view of changes in protein expression during
125  infection in primary human epithelial cells from the kidney and bladder. Data from all proteomic
126  experiments in this study are shown in Table S1. Here, the worksheet ''Plots'' is interactive,
127  enabling generation of graphs of protein expression of any of the human and viral proteins
128  quantified.

129  In uninfected cells, RPTE and HU cells exhibit differential expression of proteins, as expected
130  from two different cell types. In infected cells, few changes occurred by 24 h of infection, however
131  more substantial differences were seen by 48 and 72 h (Fig. 1B and C). In RPTE cells 191 cellular
132  proteins increased >2-fold, while 149 proteins decreased >2-fold at any time point during BKPyV
133  infection. In HU cells 130 proteins increased >2-fold and 55 decreased >2-fold. Many proteins

showed similar changes in both cell types, although cell type-specific effects were also seen (Fig. 1D; R=0.61). We reasoned that those protein changes which were important for viral replication would be common to different cell types. By combining the two datasets, we found that just 86 cellular proteins, less than 1% of all proteins quantified, were upregulated >2-fold in both RPTE and HU cells (Fig. 1D).

The lack of change in the host cell proteome at the earliest time point of 24 hpi suggested little or no effect of virus binding and penetration. To investigate this further a second TMT-based whole cell proteomics experiment (Experiment 2) was conducted repeating 24 and 48 hpi time points with an additional earlier 12 hpi time point, where RPTE cells were infected with UV-inactivated or unmodified BKPyV at MOI 5 (Fig. 2, Table S1). In Experiment 2 a total of 7698 cellular proteins were quantified, some of which were not detected in Experiment 1, giving a combined total of 9304 cellular proteins quantified across both experiments. Very few changes in protein abundance were observed at 12 or 24 hpi during infection with unmodified BKPyV, while at 48 hpi cellular proteins upregulated were similar to those observed in the first experiment at the same time point (Fig. 2B-D). UV-inactivated virus induced virtually no changes at any time point, suggesting that virus replication is necessary to cause the observed changes in host protein abundance (Fig. 2B).

**Temporal Analysis of BK Polyomavirus Protein Expression**

Expression of the early BKPyV proteins, LTAg and stAg, was observed from 24 hpi, closely followed by late proteins, agnoprotein, VP1 and VP2. Profiles from HU and RPTE cells (both experiments) corresponded well (Fig. 1E and 2E). We were unable to assign peptides to VP3 due to its 100% sequence identity with the C terminus of VP2, and the single unique peptide corresponding to the extreme N-terminus of VP3 was not quantified. Likewise, TruncTAg was not identified due to its similarity to full length LTAg: the only difference in protein sequence are the C-terminal 3 amino acids of TruncTAg, which directly follow a cluster of lysine and arginine residues and so would not be expected to be identified by our mass spectrometry analysis.

**BKPyV does not cause induction of innate immune responses in infected RPTE cells**

One surprising observation from our QTV analyses was an apparent lack of an innate immune response to BKPyV infection. Of the 131 quantified proteins with annotated innate immune functions or the 69 quantified proteins with annotated antiviral functions only five were up- or downregulated >2-fold, and these changes were not consistent between the two independent cell types or experiments (Fig. 3A and Table S2). Despite RPTE cells being capable of mounting a response to type I interferon, the expression of interferon stimulated genes MX1, ISG15, IFIT1,

IFIT2, IFIT3, IRF3, IFI16, and BST2 remained unchanged upon BKPyV infection throughout the time course, as assessed both by proteomics and Western blot (Fig. 3B and C). This was unexpected given that by 72 hpi large amounts of viral DNA and proteins as well as progeny virions were present within cells. This lack of response suggests BKPyV has evolved a highly effective immune-evasion activity, which could be due to either viral DNA and proteins not being recognised by host pathogen recognition receptors (PRRs) in these primary epithelial cells, or suppression of PRR signalling pathways during BKPyV infection.

Activation of RNA and DNA sensors invariably leads to IRF3 phosphorylation and translocation into the nucleus, leading to transcription of type I and III interferons. We analysed whether RPTE cells have functional RNA and DNA sensing pathways, and whether these were activated in response to BKPyV infection. The phosphorylation and localisation of IRF3 was investigated by Western blot and immunofluorescence microscopy following BKPyV infection or treatment with Poly I:C or stimulatory DNA. Poly I:C or stimulatory DNA caused clear nuclear translocation of IRF3 in RPTE cells, with poly I:C having the greatest effect (Fig. 4A). However, BKPyV-infected RPTE cells had no detectable change in IRF3 localisation and appeared no different to mock infected cells apart from characteristic enlarged nuclei in virus infected cells (Fig. 4A). Furthermore, Western blot analysis showed no stimulation of IRF3 phosphorylation in BKPyV-infected RPTE cells, whereas both poly I:C and stimulatory DNA transfection caused robust IRF3 phosphorylation (Fig. 4B). These results suggest that signal transduction pathways that would usually lead to activation of IRF3-specific kinases are not activated in infected cells either due to an inability to sense BKPyV nucleic acids or due to active inhibition by BKPyV.

To investigate whether the lack of viral sensing is due to evasion of nucleic acid detection or active suppression of IRF3 phosphorylation, RPTE cells were mock or BKPyV-infected and subsequently stimulated with Poly I:C or stimulatory DNA at 42 hpi, prior to analysis at 48 hpi. Nuclear translocation and robust phosphorylation of IRF3 was observed in response to both RNA and DNA, irrespective of whether the cells were infected with BKPyV or mock-infected (Fig. 5A and B). This suggests that BKPyV does not actively inhibit nucleic acid sensing pathways, IRF3 phosphorylation or IRF3 nuclear translocation. As BKPyV does not inhibit downstream activation of RNA or DNA sensing pathways this suggests BKPyV evades nucleic acid and other pathogen associated molecular pattern (PAMP) sensing pathways altogether, despite high concentrations of viral DNA, RNA and protein within these primary renal epithelial cells.

**Cell cycle associated proteins are the primary target of BKPyV**

To investigate host cell functions that were modified by BKPyV, the Database for Annotation, Visualisation and Integrate Discovery (DAVID) was used to identify pathways enriched among proteins up- or downregulated during BKPyV infection (18). Amongst upregulated proteins, similar terms were enriched between HU and RPTE cells (both from experiments 1 and 2). 'Cell cycle' and terms related to the cell cycle dominated this analysis (Fig. 6A, Tables S3 and S4). Terms associated with the G2/M phase of the cell cycle were particularly enriched, including: chromosome, microtubule, spindle, sister chromatid cohesion and DNA damage. G2/M phase arrest has previously been observed in a number of different polyomavirus infections (19-22).

Cellular proteins associated with G2/M phase of the cell cycle generally increased in abundance throughout BKPyV infection including: M-phase (CDCA3), spindle formation (CDC20, CDCA2), kinetochore assembly, sister chromatid segregation and cytokinesis (KIF11, CENPK, SKA1, KIF22, ANLN), DNA repair and control of re-replication (HELLS, GMNN) and G2/M-associated cyclins and cyclin-dependent kinases (CDK1, cyclin A2 and cyclin B1) (Fig. 6B). Proteins associated with the G1 phase, such as cyclin D2, were observed to decrease in abundance. As expected, levels of the tumour suppressor p53 were elevated during BKPyV infection; polyomavirus LTAg binds, stabilises and inactivates p53 (5, 23, 24). Interestingly, MDM2, the ubiquitin ligase that normally mediates p53 degradation, was depleted during BKPyV infection (Fig. 6B).

We confirmed these results for a number of cell cycle regulatory proteins by Western blot throughout the time course of BKPyV infection in both RPTE and HU cells (Fig. 6C). Immunofluorescence microscopy of BKPyV or mock-infected RPTE cells further confirmed the increase in cyclin B1 and CDK1 during BKPyV infection, and furthermore that cyclin B1 remained cytoplasmic during BKPyV infection (data not shown). This suggests that infected cells do not proceed into M phase, when cyclin B1 would normally relocalise to the nucleus (25).

**MDM2 and p53 levels are modulated by LTAg and cell cycle arrest**

BKPyV-induced upregulation of p53 and downregulation of MDM2 was also confirmed by immunofluorescence (Fig. 7). The E3 ubiquitin ligase MDM2 is a negative regulator of both p53 and itself, leading to ubiquitinylation and degradation of p53 and MDM2 (26). In addition, p53 is a transcription factor for both itself and MDM2 (27), whose transcriptional activity is governed by the strength of extracellular and intracellular signals, such as cell cycle checkpoints, leading to the establishment of both positive and negative feedback loops (28).

Polyomavirus LTAgs are well established to efficiently bind, stabilise and inhibit p53 (5, 23, 24), leading to increased p53 levels in BKPyV infected cells. Our data now demonstrates that this is

accompanied by a decrease in MDM2 levels. Interplay between BKPyV infection, MDM2 and p53 was investigated using the MDM2 inhibitor Nutlin-3. Nutlin-3 occupies the p53 binding pocket on MDM2 obstructing their interaction and leading to reduced p53 ubiquitinylation. In addition, Nutlin-3 leads to increased transcription of MDM2 due to the release of active p53 (29). Mock or BKPyV infected RPTE cells were treated with Nutlin-3 at 2 hpi, or DMSO as a control, and fixed at 48 hpi. Cells were immunostained for expression of MDM2, p53, and LTAg (infection marker) (Fig. 7A). Low endogenous levels of both MDM2 and p53 were observed in the nuclei of untreated mock infected cells. BKPyV infection lead to a reduction in MDM2, while p53 increased, correlating with the changes observed in the proteomics data. Mock infected cells treated with Nutin-3 showed increased levels of MDM2, accompanied by a slight increase in p53 levels, in accordance with published effects of Nutlin-3 (29). Interestingly, MDM2 levels did not increase in BKPyV infected cells treated with Nutlin-3 and in fact MDM2 levels were observed to reduce, whilst p53 levels were once again increased. This suggests that during infection MDM2 remains able to self-ubiquitinylate, leading to its degradation in the presence of Nutlin-3, however transcription of MDM2 by p53 is apparently inhibited, likely due to p53 sequestration by LTAg.

To investigate whether LTAg expression alone was sufficient to cause the observed MDM2 decrease and p53 increase RPTE cells were transfected with a LTAg expression plasmid. At 2 h cells were treated with Nutlin-3 or DMSO and then in addition some samples were treated at 24 h with a CDK1-specific inhibitor, RO-3306, to simulate BKPyV induced cell cycle arrest. Cells were fixed at 48 h and immunostained for MDM2, p53, and LTAg (Fig. 7B). Expression of LTAg alone was sufficient to reduce MDM2 levels, however p53 levels were increased only slightly suggesting other effects of BKPyV infection in addition to LTAg expression modulate p53 and MDM2 levels. Nutlin-3 treatment did not alter the effects of LTAg on MDM2 or p53 levels. Treatment of LTAg expressing cells with the CDK1 inhibitor RO-3306 lead to a marked increase in p53 expression, while MDM2 levels were once again decreased. Combined Nutlin-3 and RO-3306 treatment further enhanced the increase of p53 in LTAg expressing cells. Taken together, these data suggest LTAg binding to p53 displaces MDM2 leading to p53 stabilisation and MDM2 degradation, but LTAg binding also prevents p53-dependent expression of MDM2 and p53. Furthermore, virus infection or G2/M arrest stimulates p53 expression, possibly via a DNA damage-type response.

### BKPyV induced G2/M phase arrest is prevented by inhibition of CDK1 & CDK2, but not by inhibition of CDK1 alone or CDK4 & CDK6

Given the dysregulation of cell cycle-related proteins during BKPyV replication, we postulated that a virus-induced pseudo-G2 phase may serve a number of roles in BKPyV replication. We

therefore investigated the effect of BKPyV infection on the host cell cycle status in the presence or absence of various CDK inhibitors. Polyomavirus replication is heavily reliant on the host DNA synthesis machinery and it has previously been shown that either BKPyV infection or JCPyV LTAg expression alone can cause cells to arrest in the G2/M phase of the cell cycle (22, 30, 31). However, the impact of CDK inhibitors on BKPyV-induced arrest has not been fully investigated. RPTE cells were infected with BKPyV, treated with CDK inhibitors after 24 hpi to allow sufficient time for virus entry and initiation of early gene expression, then subsequently harvested at 48 hpi and analysed by flow cytometry to compare cell cycle profiles. PD0332991 was used to inhibit CDK4 and 6, which are active in G1 phase, Roscovitine was used to inhibit CDK1 and 2, which are active throughout S, G2, and M phase, and RO-3306 was used to inhibit CDK1, which is active in G2 and M phase. In mock-infected RPTE cells, all three inhibitors produced the expected effects: PD0332991 increased the proportion of cells in G1 from 72% to 84% (*p<0.05*), Roscovitine showed little change in proportion of cells in any cell cycle phase because of its broad effect on S, G2 and M phases, and RO-3306 showed an increased proportion of cells in G2/M (18% to 23%) and reduced proportion in G1 (72% to 65%), although this did not reach statistical significance (Fig. 8A and B). Cell viability remained above 90% for all inhibitor conditions used (Fig. 8C). Infection of RPTE cells with BKPyV in the absence of any inhibitor significantly increased the proportion of cells in G2/M from 18% to 31% and decreased the proportion of cells in G1 (72 % to 56%; *p<0.01)*, consistent with BKPyV-induced G2 arrest observed in previous published data (30). BKPyV-infected cells that were treated with PD0332991 showed a significant decrease in the proportion of cells in G1 compared to uninfected cells treated with PD0332991 (84% to 58%; *p<0.001*). While a slight increase was observed in the proportion of cells in G1 for BKPyV infected and PD0332991 treated cells compared to control BKPyV infected cells (56% to 58%) this did not reach statistical significance. This suggests that inhibition of CDK4 and 6 does not prevent BKPyV driving infected cells through the G1/S checkpoint (due to Rb inactivation by LTAg) or arresting cells in G2/M. In contrast, treatment of infected cells with Roscovitine, which inhibits both CDK1 and 2, appears to severely restrict BKPyV-stimulated S phase entry and G2/M arrest, as the cell cycle status was similar to that of mock-infected cells with no significant change in the proportion of cells in any cell cycle phase. BKPyV infected cells treated with RO-3306 (CDK1 inhibitor) showed a similar cell cycle profile to that of control BKPyV infected cells, with no significant difference in the proportion of cells in any cell cycle phase (Fig. 8A). Comparison of mock infected RO-3306 treated cells with BKPyV infected RO-3306 treated cells showed increased proportion of cells in G2/M and S with a corresponding decrease in G1 (65% to 49%; *p<0.05*). This suggests that BKPyV infection and CDK1 inhibition have a similar and additive effect on the cell cycle, namely induction of G2/M arrest.

### Inhibition of CDK1 and CDK2 or CDK1 alone reduces BKPyV replication

The ability of BKPyV to induce a pseudo-G2 arrest in the presence of CDK1 or CDK4/6 inhibition suggested that virus replication should be unaffected in such conditions, while inhibition of CDK1/2 should perturb viral replication due to inhibition of S phase progression. To investigate if this was the case we next analysed the effect of CDK inhibitors on viral genome synthesis in BKPyV infected RPTE cells. Infected cells were treated with each inhibitor at 24 hpi and harvested at 48 hpi. Viral and host cell DNA was quantified using qPCR to determine viral DNA copy numbers per cell and were normalised to uninhibited controls (arbitrarily set to 1). Inhibition of CDK4/6 had no significant effect on viral genome synthesis, while inhibition of CDK1 and 2 by Roscovitine showed a 7.4-fold reduction in the synthesis of BKPyV genome, likely due to the restriction of cells from entering and progressing through S phase (Fig. 9A). Surprisingly, inhibition of CDK1 alone by RO-3306 also caused a significant, although more modest, 2.3-fold reduction in BKPyV genome synthesis. This suggests that, despite this inhibitor having little effect on BKPyV-driven cell cycle progression, CDK1 activity is important for efficient viral genome synthesis.

The effects of these CDK inhibitors on viral protein synthesis was similarly investigated (Fig. 9B). Inhibition of CDK4 and 6 had no observable effect on viral protein synthesis, while inhibition of CDK1 and 2 by Roscovitine substantially reduced viral protein expression levels. Inhibition of CDK1 alone by RO-3306 showed only small reductions in viral protein levels.

Analysis of infectious virus production in the presence or absence of these CDK inhibitors also demonstrated a similar trend. Inhibition of CDK4 and 6 caused only a slight reduction of infectious titres, whereas inhibition of CDK1 and 2 by Roscovitine resulted in a significant 80-fold reduction of virus production, unsurprisingly given the inhibition of viral DNA and protein synthesis (Fig. 9C). Inhibition of CDK1 alone by RO-3306 caused a significant reduction of infectious virus titre by >3-fold. These data further suggest that CDK1 activity is important for the efficient production of infectious viruses.

### Discussion

By employing the power and sensitivity of TMT-based MS3 mass spectrometry technology, we have been able to uncover BKPyV-induced changes to protein abundance and a global view of protein expression within these human cell types. Importantly, these studies were conducted in primary human cells from epithelial tissue representing the natural sites of replication *in vivo*.

Therefore, this work also provides a comprehensive proteomic resource for future studies on human renourinary epithelial biology.

One of the most surprising findings of this study was just how few of the ~9000 cellular proteins that were quantified changed in abundance in response to BKPyV infection. In fact, just 235 were found to be upregulated and 196 downregulated >2-fold or more across either cell type at any time point, which corresponds to <5% of the total proteome. Previous studies that applied a similar TMT-based approach to infection with human cytomegalovirus (HCMV), another dsDNA virus, revealed that 56% of cellular proteins changed in abundance more than 2-fold during the course of infection (17). This suggests that BKPyV, and presumably other polyomaviruses, are so highly adapted to their host that they only need to induce subtle changes to host gene expression to reprogram cells into virus-producing factories. This also suggests polyomaviruses can very effectively evade detection by host pathogen recognition receptors despite producing high concentrations of foreign (viral) nucleic acid and proteins during productive infection.

For host proteins induced by BKPyV infection, we identified substantial overlap between the two primary cell types, with many of the same or highly related functional clusters identified by DAVID analysis. This includes clusters such as 'DNA damage' and the 'Fanconi Anaemia' pathway, which have been previously described as important during polyomavirus replication to ensure viral genome replication maintains high fidelity (30). Interestingly, the majority of the functional clusters identified as upregulated in BKPyV infection are related to cell cycle activity and regulation, in particular activities associated with G2 and M phases. In fact, BKPyV infection appears to have a similar 'G2/M arrest' effect on cell cycle status as the CDK1-specific inhibitor RO-3306, a drug commonly used to arrest cells in G2. The fact that infected cells do not progress into authentic mitosis is supported by the observation that cyclin B1 remains predominantly cytoplasmic despite higher protein levels in infected cells, and supports previously published data indicating G2/M phase arrest is driven by polyomavirus infection (5-7, 22, 32). Our data now provides a greater understanding of host protein profiles that are associated with polyomavirus-induced G2 arrest, and it would be interesting to compare these observations to the effects of RO-3306 or other specific CDK1 inhibitors on cellular protein expression profiles.

Our data also indicate a specific perturbation of the p53-MDM2 axis by BKPyV infection, where MDM2 is reduced and p53 is increased but kept inactive by LTAg binding. However, these changes require more than just LTAg expression, and are also driven by additional effects of BKPyV infection related to G2/M arrest and potentially DNA damage responses. Our findings suggest the following model: low MDM2 and p53 levels are maintained in uninfected cells due to their poly-ubiquitinylation by MDM2 and subsequent proteosomal degradation (Fig. 10A).

Inhibition of MDM2-p53 interaction by Nutlin-3 releases p53 which then stimulates MDM2 expression (Fig. 10B). Interaction of LTAg with p53 displaces MDM2, thereby causing MDM2 to be destroyed by the proteasome, and so protects p53 from degradation but inhibits p53 transcriptional activity prevent induction of de novo MDM2 expression (Fig. 10C). Therefore, the expression of just LTAg results in decreased MDM2 levels but only a modest increase in p53. During active BKPyV infection, p53 expression is induced by some other effect(s) of virus replication, and these additional copies of p53 are also bound and inactivated by LTAg (Fig. 10D). We predict that stimulation of p53 expression during BKPyV infection is via a DNA damage response pathway, which can be mimicked by inducing a G2 arrest through inhibition of CDK1.

Polyomaviruses have a well-established capacity to drive cells into S-phase by overriding the G1/S checkpoint via the activity of LTAg. It is therefore unsurprising that inhibition of CDK4 and 6 has little-to-no effect on the ability of BKPyV to drive cell cycle progression or to replicate. CDK4 and 6, in complex with cyclin D, are normally responsible for phosphorylation of Rb and release of E2F proteins allowing passage through the G1/S checkpoint (33). This is bypassed through the binding of LTAg to Rb family proteins, releasing E2F proteins enabling S-phase entry that is unconstrained by upstream factors (5, 6).

In contrast Roscovitine, a potent inhibitor of CDK1 and 2, caused a global cell cycle arrest, irrespective of BKPyV infection, and reduced BKPyV replication. Similar effects of Roscovitine on polyomavirus replication have been previously been attributed to inhibition of CDK1 activity alone (34). However, our data suggests the effect of Roscovitine are more likely due to inhibition of CDK2 activity, or the combination of inhibiting both CDK1 and 2. Inhibition of both these cyclin dependent kinases causes a rather global block to cell cycle progression; CDK2 is active in both late G1 and S phase, while CDK1 is active in G2 and M phase (35). CDK2 activity is required immediately after G1 checkpoint clearance and beyond, and so the primary cause of BKPyV inhibition by Roscovitine could be due to a failure to activate S-phase proteins required for viral genome synthesis and consequent protein expression. Interestingly, we also observed reduced expression of LTAg in Roscovitine treated cells. This might be attributed to inhibited progression through S phase, thus leading to reduced viral genome copy numbers from which LTAg is transcribed, although other effects of Roscovitine that inhibit transcription, such as inhibition of CDK7 and 9, may also contribute to this effect (36)

Somewhat more intriguing is the effect of CDK1-specific inhibition on BKPyV infection; RO-3306 caused significant reductions in viral DNA synthesis and infectious virus assembly. This was surprising because CDK1 activity is normally important for the transition through G2 and into M-phase, and so inhibition of CDK1 would not be expected to inhibit progression through S-phase

and thus viral DNA replication. Whether CDK1 activity is required directly or indirectly to enhance DNA synthesis or other S-phase activities required for BKPyV genome replication, or the process of virion assembly, remains to be determined.

Moreover, our data have demonstrated that BKPyV infection of renourinary epithelial cells does not appear to cause the induction of antiviral responses in agreement with published data of RPTE cells (12, 14, 15). Both RPTE and HU cells express the appropriate receptors, signalling pathways and transcription factors associated with sensing and responding to DNA viruses, such as cGAS (MB21D1), IFI16, STING (TMEM173), NFκB, and IRF3, which were readily detected in our mass spectrometry analysis (Table S1). RPTE cells are quite capable of responding to foreign intracellular DNA or RNA, leading to phosphorylation and nuclear translocation of IRF3, and RPTE cells also robustly express antiviral genes in response to type-1 interferon. However, we could not detect activation of these pathways even by three days after BKPyV infection: IRF3 remains unphosphorylated and cytoplasmic and no ISGs were induced. Furthermore, we have also shown that active BKPyV infection within the same cell does not prevent the phosphorylation and nuclear translocation of IRF3 in response to either cytoplasmic RNA or DNA. This suggests that BKPyV is not actively suppressing such antiviral responses, but rather prevents its own detection by pathogen recognition receptors. This evasion of detection may be due to a combination of having a small circular double stranded DNA genome that is associated with histones, thus appearing similar to open chromatin, and tightly regulated entry and egress mechanisms to prevent exposure of viral DNA in the cytoplasm. Whether an inability to sense and respond to BKPyV infection is partly due to the nature of epithelial cells in the renourinary systems and whether this contributes to the natural tropism of BKPyV for these tissue types will be interesting questions for future study.

In summary, we have generated extensive data sets on the protein expression profiles of primary epithelial cells of the kidney and bladder using advanced multiplexed proteomics and provided a detailed understanding of how infection by BKPyV modifies the protein expression profiles in these cells. This research has provided additional details of the specific cell cycle arrest induced by virus infection and revealed the importance of this arrest for BKPyV replication. Furthermore, our findings suggest a surprising ability of BKPyV to evade detection and activation of innate immune responses in cells that are natural sites of lytic virus infection *in vivo*.

**Materials and Methods**

**Cell types, virus and primary antibodies.**

13

HU cells were grown in Urothelial Cell Medium enriched with Urothelial Cell Growth Supplement and penicillin/streptomycin solution (Caltag Medsystems). HU cells were used at passage 4-6 for all experiments. RPTE cells were grown in renal epithelial basal media enriched with REGM bullet kit (Lonza). RPTE cells were used at passage 6-7 for all experiments.

BKPyV (Dunlop strain) inserted into pGEM7Zf(+) vector (kindly provided by M. Imperiale, University of Michigan) was digested with BamHI, purified and re-ligated. Resultant BKPyV-Dunlop genome was transfected into a T150 flask of RPTE cells, one week later the flask was split into three T150 flasks of RPTE cells. After a period of up to four weeks virus was harvested by freeze thawing cells three times. Virus purification by sucrose cushion, followed by caesium chloride gradient and dialysis provided purified BKPyV stocks as described previously (37). Concentration and purity was assessed by FFU assay and coomassie gel stain respectively.

The primary antibodies used in this study were PAb597 against SV40 VP1 (kindly provided by W. Atwood, Brown University), P5G6 against BKPyV VP1 (kindly provided by D. Galloway, Fred Hutchinson Cancer Research Center), ab6160 against Tubulin (Abcam), ab32386 against Cyclin A2 (Abcam), ab32053 against Cyclin B1 (Abcam), MA5-11472 against CDK1 (Thermo Scientific), ab207604 against Cyclin D2, ab1101 against p53 (Abcam), GTX116125 against Geminin (GeneTex), ab16895 against MDM2 (Abcam), 37849 against MX1 (Cell Signalling Technologies), 2758 against ISG15 (Cell Signalling Technologies), PA3-848 against IFIT1 (ThermoFisher), 12604-1-AP against IFIT2 (ProteinTech), SAB1410691 against IFIT3 (Sigma Aldrich), 11904 against IRF3 (Cell Signalling Technologies), ab76493 against IRF3 (phospho S386) (Abcam), ac-8023 against IFI16 (Santa Cruz), 11721 against BST2 (NIH AIDS Reagent Programme), ab53983 against SV40 VP2 + VP3 (Abcam), ab16879 against SV40 LTag (Abcam), and against BKPyV agnoprotein (rabbit polyclonal antibody generated against agnoprotein specific peptide).

## **Cell infections and harvesting virus**

For viral infections RPTE or HU cells were infected with BKPyV at either MOI=5 for TMT and validation experiments, or MOI=3 or 0.5 for all other experiments, diluted in appropriate medium. At 1 hpi media was removed, cells washed twice with PBS and fresh medium was added. For TMT analysis, cells were harvested in TMT lysis buffer (6M Guanidine HCl, 50mM HEPES pH 8.5), vortexed extensively and incubated at room temperature for 10 min. Lysates were then sonicated at 25 W for 30 s, followed by centrifugation at 21,000 g for 10 min, after which supernatant was transferred to a fresh tube. Centrifugation was repeated and supernatants snap-

466　frozen in liquid nitrogen for further processing. For Western blot, cells were harvested by

467　centrifugation at 6,000 g after two PBS washes.

### Transfection

469　RPTE cells were transfected with pcDNA3-LTAg plasmid using TransIT-LT1 Transfection

470　Reagent (Mirus) in Opti-MEM media according to the manufacturers protocol.

### Inhibitors

472　For p53:MDM2 interaction inhibition experiments cells were treated at 2 hpi with Nutlin-3.

473　Nutlin-3 (Sigma) was made up to 20mM in DMSO and used at 5µM. For cell cycle inhibition

474　experiments cells were treated with inhibitors at 24 hpi. PD0332991 (Sigma) was made up to 5mM

475　in $dH_2O$ and used at 1µM, Roscovitine (Sigma) was made up to 20mM in DMSO and used at

476　20µM and RO-3306 (Sigma) was made up to 20mM in DMSO and used at 5µM. Controls were

477　subjected to treatment with an equivalent amount of DMSO at the greatest volume of any inhibitor

478　used. Cells were harvested in 1mL media at 48 hpi and either pelleted by centrifugation at 6,000 g

479　for use in Western blot or qPCR, or frozen for assay by FFU. For analysis by flow cytometry cells

480　were detached from wells by trypsin/EDTA treatment, centrifuged at 6,000 g, washed in PBS and

481　fixed in 70% ice-cold ethanol.

### FFU and immunofluorescence microscopy

483　Fluorescent focus unit (FFU) assays were used to determine the concentration of infectious virus

484　in purified BKPyV stocks or experimental samples. RPTE cells were infected with sample

485　dilutions, fixed at 48 hpi and immunostained for VP1 expression as described in (38). For

486　comparison of inhibitor effects infectious BKPyV levels of uninhibited conditions were arbitrarily

487　set to 1 and inhibited conditions corrected to this control for 7 independent experiments. A one

488　sample t-test was conducted to give *p* values, standard deviation shown with error bars.

489　For immunofluorescence analysis, RPTE cells were fixed in 3% formaldehyde. Fixed cells were

490　then permeabilised and quenched (50mM $NH_4Cl$ and 0.1% Triton X-100 in PBS), blocked in

491　PGAT (0.2% gelatin, 0.01% Triton X-100, 0.02% $NaN_3$ in PBS) and stained with primary

492　antibodies. Secondary antibodies used for immunofluorescence were Alexa Fluor 568 donkey anti-

493　mouse or goat anti-IgG1 mouse and Alexa Fluor 488 donkey anti-rabbit or goat anti-IgG2a mouse.

494　Coverslips were mounted using SlowFade Gold with DAPI (Invitrogen). Samples were imaged

495　using a 63x oil immersion lens on an Olympus IX81 wide-field fluorescent microscope.

### Western blot

RPTE cells were lysed by suspending in mRIPA (50mM Tris pH 7.5, 150mM NaCl, 1% Sodium Deoxycholate and 1% Triton X-100) supplemented with Complete Protease Inhibitors without EDTA (Roche). Cellular debris were removed by centrifugation at 17,000g. HU cells were lysed by suspending in HU cell Lysis Buffer (20mM HEPES pH 7.6, 250mM Sucrose, 2mM DTT, 2mM EDTA Na$_2$ and 2mM EGTA) supplemented with Complete Protease Inhibitors without EDTA, followed by sonication at 25W for 30 sec. Proteins were separated by SDS-PAGE electrophoresis and transferred to nitrocellulose membranes before blocking in 5% skimmed milk powder in PBS.

Following primary antibody binding, LI-COR IRDye680- (anti-mouse, anti-rabbit or anti-rat) or IRDye800-conjugated (anti-mouse or anti-rabbit) secondary antibodies were used. Membranes were then imaged on a LI-COR Odyssey Infrared Imaging system.

## Real-time PCR (qPCR)

RPTE cell pellets were lysed in 200μL NDA Lysis Buffer (4M Guanidine Thiocyanate, 25mM Tris and 134mM β-mercaptoethanol) and incubated at 56°C for 10 min, after which an equal volume of 100% ethanol was added. DNA was then bound to silica columns by centrifuging at 16,000× g for one min. Columns were washed with Buffer 1 (1M Guanidine Thiocyanate, 25mM Tris pH7 in 10% ethanol), and centrifuged, followed by a final wash in Buffer 2 (25mM Tris pH7 in 70% ethanol). DNA was eluted with nuclease free water by centrifugation at 16,000× g. Primers and probe for BKPyV genome were designed as described in (38). Human TNFα primers and probe were designed and obtained through TIB MOLBIOL (forward primer: AGGAACAGCACAGGCCTTAGTG; reverse primer: AAGACCCCTCCCAGATAGATGG; Taqman probe: CCAGGATGTGGAGAGTGAACCGACATG). 300nM of each primer and 50nM of Taqman probe were used in each qPCR reaction, run on a Rotor-Gene (RG-3000, Corbett Research) and subsequently analysed on Rotor-Gene software. BKPyV genome levels were corrected to the TNFα control for each sample, and uninhibited samples arbitrarily set to 1, 6 independent experiments. A one sample t-test was conducted to give *p* values, standard deviation shown with error bars.

## Flow cytometry

Cellular DNA content was used as an indicator of cell cycle status. Cells were fixed in 70% ethanol for 30 mins, DNA was stained by resuspending each PBS washed cell pellet in 0.2mg RNAse A and 50μg propidium iodide in 1mL PBS and incubated at 37°C for 1 hr. Cells were then centrifuged at 6,000 g, supernatant removed and resuspended in 500μL PBS. Cells were analysed by flow cytometry using BD FACSCantoII with BD FACSDiva Software (BD Biosciences) and further analysed using FlowJo v10.4.2 cell cycle analysis function. A minimum of 10,000 cells

530 were collected for each sample, 3 independent experiments. Two-tailed students t-tests were
531 conducted to assess significance of changes in cell cycle status between samples. Standard
532 deviation error was calculated for each cell cycle status sample.

**Whole cell lysate protein digestion**

534 Cells were washed twice with PBS, and 250 µλ lysis buffer added (6M Guanidine/50 mM HEPES
535 pH 8.5). Cell lifters (Corning) were used to scrape cells in lysis buffer, which was removed to an
536 eppendorf tube, vortexed extensively then sonicated. Cell debris was removed by centrifuging at
537 21,000 g for 10 min twice. Dithiothreitol (DTT) was added to a final concentration of 5 mM and
538 samples were incubated for 20 mins. Cysteines were alkylated with 14 mM iodoacetamide and
539 incubated 20 min at room temperature in the dark. Excess iodoacetamide was quenched with DTT
540 for 15 mins. Samples were diluted with 200 mM HEPES pH 8.5 to 1.5 M Guanidine followed by
541 digestion at room temperature for 3 h with LysC protease at a 1:100 protease-to-protein ratio.
542 Samples were further diluted with 200 mM HEPES pH 8.5 to 0.5 M Guanidine. Trypsin was then
543 added at a 1:100 protease-to-protein ratio followed by overnight incubation at 37°C. The reaction
544 was quenched with 5% formic acid, then centrifuged at 21,000 g for 10 min to remove undigested
545 protein. Peptides were subjected to C18 solid-phase extraction (SPE, Sep-Pak, Waters) and
546 vacuum-centrifuged to near-dryness.

**Peptide labelling with tandem mass tags**

548 In preparation for TMT labelling, desalted peptides were dissolved in 200 mM HEPES pH 8.5.
549 Peptide concentration was measured by microBCA (Pierce), and 25 µg labelled with TMT reagent.
550 TMT reagents (0.8 mg) were dissolved in 43 µl anhydrous acetonitrile and 3 µl added to peptide at
551 a final acetonitrile concentration of 30% (v/v). Samples were labelled as follows. Experiment 1 (9-
552 plex); 126 – mock infection 12 hpi, 127N – mock infection 24 hpi, 127C – mock infection 48 hpi,
553 128N – BKPyV infection 12 hpi, 128C – BKPyV infection 24 hpi, 129N – BKPyV irradiated – 48
554 hpi, 129C – BKPyV irradiated 24 hpi, 130N BKPyV infection 48 hpi. Experiment 2 (10-plex); 126
555 – HU cells mock infection 24 hpi, 127N – HU cells mock infection 72 hpi, 127C – HU cells
556 BKPyV infection 24 hpi, 128N – HU cells BKPyV infection 48 hpi, 128C – HU cells BKPyV
557 infection 72 hpi, 129N – RPTE cells mock infection 24 hpi, 129C – RPTE cells mock infection 72
558 hpi, 130N – RPTE cells BKPyV infection 24 hpi, 130C – RPTE cells BKPyV infection 48 hpi,
559 131N – RPTE cells BKPyV infection 72 hpi. Following incubation at room temperature for 1 h,
560 the reaction was quenched with hydroxylamine to a final concentration of 0.3% (v/v). TMT-
561 labelled samples were combined at a 1:1:1:1:1:1:1:1:1 ratio (experiment 1) and 1:1:1:1:1:1:1:1:1:1
562 ratio (experiment 2). The sample was vacuum-centrifuged to near dryness and subjected to C18

SPE (Sep-Pak, Waters). An unfractionated singleshot was initially analysed to ensure similar peptide loading across each TMT channel, to avoid the need for excessive electronic normalization. Quantities of each TMT labelled sample were adjusted prior to high pH reversed-phase (HpRP) so that normalisation factors were >0.67 and <1.5. Normalisation is discussed in 'Data Analysis', and fractionation is discussed below.

**Offline HpRP fractionation**

TMT-labelled tryptic peptides were subjected to HpRP fractionation using an Ultimate 3000 RSLC UHPLC system (Thermo Fisher Scientific) equipped with a 2.1 mm internal diameter (ID) x 25 cm long, 1.7 μm particle Kinetix Evo C18 column (Phenomenex). Mobile phase consisted of A: 3% acetonitrile (MeCN), B: MeCN and C: 200 mM ammonium formate pH 10. Isocratic conditions were 90% A / 10% C, and C was maintained at 10% throughout the gradient elution. Separations were conducted at 45°C. Samples were loaded at 200 μl/minute for 5 minutes. The flow rate was then increased to 400 μl/minute over 5 minutes, after which the gradient elution proceed as follows: 0-19% B over 10 minutes, 19-34% B over 14.25 minutes, 34-50% B over 8.75 minutes, followed by a 10 minutes wash at 90% B. UV absorbance was monitored at 280 nm and 15 s fractions were collected into 96 well microplates using the integrated fraction collector. Fractions were recombined orthogonally in a checkerboard fashion, combining alternate wells from each column of the plate into a single fraction, and commencing combination of adjacent fractions in alternating rows. Wells prior to the start or after the stop of elution of peptide-rich fractions, as identified from the UV trace, were excluded. This yielded two sets of 12 combined fractions, A and B, which were dried in a vacuum centrifuge and resuspended in 10 μl MS solvent (4% MeCN / 5% formic acid) prior to LC-MS3. 11 set 'A' fractions were used for experiment 1 and 10 set 'A' fractions were used for experiment 2.

**LC-MS3**

Mass spectrometry data was acquired using an Orbitrap Lumos (Thermo Fisher Scientific, San Jose, CA). An Ultimate 3000 RSLC nano UHPLC equipped with a 300 μm ID x 5 mm Acclaim PepMap μ-Precolumn (Thermo Fisher Scientific) and a 75 μm ID x 50 cm 2.1 μm particle Acclaim PepMap RSLC analytical column was used.

Loading solvent was 0.1% formic acid (FA), analytical solvent A: 0.1% FA and B: 80% MeCN + 0.1% FA. All separations were carried out at 55°C. Samples were loaded at 5 μL/minute for 5 minutes in loading solvent before beginning the analytical gradient. The following gradient was used: 3-7% B over 3 minutes, 7-37% B over 173 minutes, followed by a 4 minute wash at 95% B and equilibration at 3% B for 15 minutes. Each analysis used a MultiNotch MS3-based TMT

596    method (39, 40). The following settings were used Th, 120,000 Resolution, $2x10^5$ automatic gain

597    control (AGC) target, 50 ms maximum injection time. MS2: Quadrupole isolation at an isolation

598    width of m/z 0.7, CID fragmentation (normalised collision energy (NCE) 35) with ion trap

599    scanning in turbo mode from m/z 120, $1.5x10^4$ AGC target, 120 ms maximum injection time.

600    MS3: In Synchronous Precursor Selection mode the top 6 MS2 ions were selected for HCD

601    fragmentation (NCE 65) and scanned in the Orbitrap at 60,000 resolution with an AGC target of

602    $1x10^5$ and a maximum accumulation time of 150 ms. Ions were not accumulated for all

603    parallelisable time. The entire MS/MS/MS cycle had a target time of 3 s. Dynamic exclusion was

604    set to +/- 10 ppm for 70 s. MS2 fragmentation was trigged on precursors $5x10^3$ counts and above.

605    **Data analysis**

606    In the following description, we list the first report in the literature for each relevant algorithm.

607    Mass spectra were processed using a Sequest-based software pipeline for quantitative proteomics,

608    "MassPike", through a collaborative arrangement with Professor Steve Gygi's laboratory at

609    Harvard Medical School. MS spectra were converted to mzXML using an extractor built upon

610    Thermo Fisher's RAW File Reader library (version 4.0.26). In this extractor, the standard mzxml

611    format has been augmented with additional custom fields that are specific to ion trap and Orbitrap

612    mass spectrometry and essential for TMT quantitation. These additional fields include ion

613    injection times for each scan, Fourier Transform-derived baseline and noise values calculated for

614    every Orbitrap scan, isolation widths for each scan type, scan event numbers, and elapsed scan

615    times. This software is a component of the MassPike software platform and is licensed by Harvard

616    Medical School.

617    A combined database was constructed from (a) the human Uniprot database (4th February 2014),

618    (b) the BK polyomavirus database (6th October, 2014). The combined database was concatenated

619    with a reverse database composed of all protein sequences in reversed order. Searches were

620    performed using a 20 ppm precursor ion tolerance (41). Product ion tolerance was set to 0.03 Th.

621    TMT tags on lysine residues and peptide N termini (229.162932 Da) and carbamidomethylation of

622    cysteine residues (57.02146 Da) were set as static modifications, while oxidation of methionine

623    residues (15.99492 Da) was set as a variable modification.

624    To control the fraction of erroneous protein identifications, a target-decoy strategy was employed

625    (42, 43). Peptide spectral matches (PSMs) were filtered to an initial peptide-level false discovery

626    rate (FDR) of 1% with subsequent filtering to attain a final protein-level FDR of 1% (44, 45). PSM

627    filtering was performed using a linear discriminant analysis, as described previously (46). This

628    distinguishes correct from incorrect peptide IDs in a manner analogous to the widely used

629 Percolator algorithm (47), though employing a distinct machine learning algorithm. The following
630 parameters were considered: XCorr, ΔCn, missed cleavages, peptide length, charge state, and
631 precursor mass accuracy. Protein assembly was guided by principles of parsimony to produce the
632 smallest set of proteins necessary to account for all observed peptides (46).

633 Proteins were quantified by summing TMT reporter ion counts across all matching peptide-
634 spectral matches using "MassPike", as described previously (39, 40). Briefly, a 0.003 Th window
635 around the theoretical m/z of each reporter ion (126, 127n, 127c, 128n, 128c, 129n, 129c, 130n,
636 130c, 131n, 131c) was scanned for ions, and the maximum intensity nearest to the theoretical m/z
637 was used. The primary determinant of quantitation quality is the number of TMT reporter ions
638 detected in each MS3 spectrum, which is directly proportional to the signal-to-noise (S:N) ratio
639 observed for each ion (48). Conservatively, every individual peptide used for quantitation was
640 required to contribute sufficient TMT reporter ions (minimum of ~1250 per spectrum) so that each
641 on its own could be expected to provide a representative picture of relative protein abundance (39).
642 An isolation specificity filter was additionally employed to minimise peptide co-isolation (49).
643 Peptide-spectral matches with poor quality MS3 spectra (more than 9 TMT channels missing
644 and/or a combined S:N ratio of less than 250 across all TMT reporter ions) or no MS3 spectra at
645 all were excluded from quantitation. Peptides meeting the stated criteria for reliable quantitation
646 were then summed by parent protein, in effect weighting the contributions of individual peptides
647 to the total protein signal based on their individual TMT reporter ion yields. Protein quantitation
648 values were exported for further analysis in Excel.

649 For protein quantitation, reverse and contaminant proteins were removed, then each reporter ion
650 channel was summed across all quantified proteins and normalised assuming equal protein loading
651 across all channels. For further analysis and display in figures, fractional TMT signals were used
652 (i.e. reporting the fraction of maximal signal observed for each protein in each TMT channel,
653 rather than the absolute normalized signal intensity). This effectively corrected for differences in
654 the numbers of peptides observed per protein. For all TMT experiments, normalised S:N values
655 are presented in Table S1 ('Data' worksheet).

656 Hierarchical centroid clustering based on uncentered Pearson correlation, and k-means clustering
657 were performed using Cluster 3.0 (Stanford University) and visualised using Java Treeview
658 (http://jtreeview.sourceforge.net) unless otherwise noted.

659

660 **Author Contributions**

661    L.G.C., R.A. and C.T.R.D. acquired and analysed the experimental data. L.G.C., C.T.R.D., P.J.L.,

662    M.P.W. and C.M.C. conceived and designed the experiments, interpreted the data and contributed

663    to writing the manuscript. M.P.W. and C.M.C. supervised the project.

664

665    **Competing Interests**

666    None of the authors have competing interests.

667

673

674    **References**

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804

805 ## <u>Figure Legends</u>

806 **Figure 1. Quantitative temporal analysis of BK virus lytic infection.**

(A) Schematic of experimental workflow. RPTE and HU cells were infected at MOI 5 or mock infected. Whole cell lysates (WCL) were harvested at 24, 48, and 72 h (infected samples) or 24 and 72 h (mock infected samples).
(B) Hierarchical cluster analysis of all quantified proteins.
(C) Scatter plots of all proteins quantified at 24, 48 and 72 hpi in RPTE and HU cells. Fold change is shown in comparison to the average corresponding mock for the same cell type. Benjamini-Hochberg-corrected significance B was used to estimate p-values (50).
(D) Scatter plot showing the correlation between protein abundance changes in BKPyV infected RPTE and HU cells, and overlap of proteins up and downregulated by > 2-fold between RPTE and HU cells.
(E) Temporal profiles of the 5 viral proteins identified, normalised to a maximum of one.

**Figure 2. Repeat quantitative temporal profiling in RPTE cells (Experiment 2).**
(A) Schematic of experimental workflow for experiment 2.
(B) Scatter plots of all proteins quantified. Fold change is shown in comparison to the average corresponding mock. Benjamini-Hochberg-corrected significance B was used to estimate p-values (Cox and Mann, 2008).
(C) Overlap of proteins quantified between Experiment 1 and Experiment 2.
(D) Scatter plot showing the correlation between experiments 1 and 2 in RPTE cells, for proteins quantified by ≥2-peptides.
(E) Temporal profiles of the 5 viral proteins quantified, normalised to a max of one.

**Figure 3. Proteins involved in the innate antiviral immune response remain unchanged during BKPyV infection.**
(A) Up- or down-regulation of a minority of proteins with innate antiviral function (Uniprot keywords: 'Innate immunity' and 'Antiviral').
(B) Example protein profiles from (A).
(C) Validation of temporal profiles shown in (B) by Western blot. RPTE cells were mock infected or infected with BKPyV at MOI 3 or stimulated with IFNα2A ($10^4$ U/mL) and analysed by Western blot for the proteins shown.

**Figure 4. RPTE cells phosphorylate and translocate IRF3 in response to cytoplasmic RNA and DNA but fail to do so upon BKPyV infection.**
(A) Immunofluorescence microscopy analysis of IRF3 localisation changes upon stimulation. RPTE cells infected with BKPyV (MOI 0.5) or mock infected were fixed at 48 hpi. RPTE cells stimulated with Poly I:C (2 µg/mL) or stimulatory DNA (2 µg/mL) were fixed at 6 h after stimulation. DAPI was used as a nuclear marker and anti-VP1 as a marker of infection.
(B) Analysis of IRF3 phosphorylation by Western blot. RPTE cells infected with BKPyV (MOI 3) or mock infected and harvested at 48 hpi. RPTE cells stimulated with Poly I:C (2 µg/mL) or stimulatory DNA (2 µg/mL) were harvested at 6 h after stimulation.

**Figure 5. BKPyV and mock-infected RPTE cells do not differ in their responses to cytoplasmic RNA and DNA.**
(A) Immunofluorescence microscopy analysis of IRF3 localisation changes upon stimulation in BKPyV infected or uninfected cells. RPTE cells infected with BKPyV (MOI 0.5) or mock infected were stimulated with Poly I:C (2 µg/mL) or oligomeric DNA (2 µg/mL) at 42 hpi and fixed at 48 hpi. DAPI was used as a nuclear marker and anti-LTAg as a marker of infection.
(B) Analysis of IRF3 phosphorylation upon stimulation in BKPyV infected or uninfected cells by Western blot. RPTE cells infected with BKPyV (MOI 3) or mock infected were

858    stimulated with Poly I:C (2 µg/mL) or oligomeric DNA (2 µg/mL) at 42 hpi and fixed at 48
859    hpi.
860
861 **Figure 6. Upregulated proteins are enriched in cell cycle functions.**
862    (A) DAVID enrichment analysis of proteins upregulated or downregulated >2-fold against a
863        background of all 8985 human proteins quantified in Experiment 1. No significantly
864        enriched downregulated clusters were observed for HU cells.
865    (B) Example protein profiles for selected cell cycle-related proteins for both RPTE and HU
866        cells. Proteins families are separated by coloured boxes.
867    (C) Validation of selected temporal profiles shown in (B) by Western blot (RPTE cells, MOI
868        3). Tubulin was used as a loading control and VP1 as a control for infection.
869
870 **Figure 7. MDM2 and p53 are modulated by BKPyV via LTAg-dependent and -independent**
871 **activities.**
872    (A) Expression of MDM2, p53 and LTAg in RPTE cells infected with BKPyV (MOI 1) or
873        mock infected, then treated with 5 µM Nutlin-3 or DMSO as a control at 2 hpi, fixed at 48
874        hpi. DAPI was used as a nuclear marker.
875    (B) Expression of MDM2, p53 and LTAg in RPTE cells transfected with BKPyV LTAg, then
876        treated with 5 µM Nutlin-3 or DMSO as a control at 2 h and subjected to cell cycle
877        inhibition (RO-3306 5 µM) at 24 h, fixed at 48 h post transfection. DAPI was used as a
878        nuclear marker. Five example cells shown for each condition
879
880 **Figure 8. Cell cycle inhibitors have variable effects on BKPyV induced G2/M phase cell cycle**
881 **arrest.**
882    (A) The cell cycle status of RPTE cells was determined in a number of different experimental
883        conditions. RPTE cells were infected with BKPyV (MOI 3) or mock infected, then
884        subjected to CDK4/6 inhibition (PD0332991 1 µM), CDK1/2 inhibition (Roscovitine 20
885        µM) or CDK1 inhibition (RO-3306 5 µM) at 24 hpi, and subsequently collected for
886        analysis at 48 hpi. Collected cells were stained with propidium iodide (PI) and analysed by
887        flow cytometry (n=3). Error bars represent standard deviation. *p < 0.05; **p < 0.01; ***p
888        < 0.001; ns = not significant; two sample t-test for changes in proportion of cells in G1.
889    (B) Histograms of PI stain for each experimental condition of a single experiment shown.
890    (C) Cell viability tests. RPTE cells were treated with 1 µM PD0332991, 20 µM Roscovitine or
891        5 µM RO-3306 for 24 h then subjected to a Trypan Blue exclusion assay according to
892        manufacturers protocol (n=3).
893
894 **Figure 9. CDK1 and 2 inhibitors impede BKPyV replication in RPTE cells.**
895        RPTE cells infected with BKPyV (MOI=3) were subjected to CDK4/6 inhibition (1 µM
896        PD0332991), CDK1/2 inhibition (20 µM Roscovitine) or CDK1 inhibition (5 µM RO-
897        3306) from 24 hpi and harvested for analysis at 48 hpi.
898    (A) qPCR to determine viral DNA copy numbers per cell. DNA was extracted from each
899        condition, BKPyV genome copy number was determined, normalised to host gene (TNFα)
900        copy number and compared to the uninhibited control, which was arbitrarily set to 1 (n=6).
901    (B) Expression of viral proteins VP1, VP2, VP3, Agno, and LTAg was determined by Western
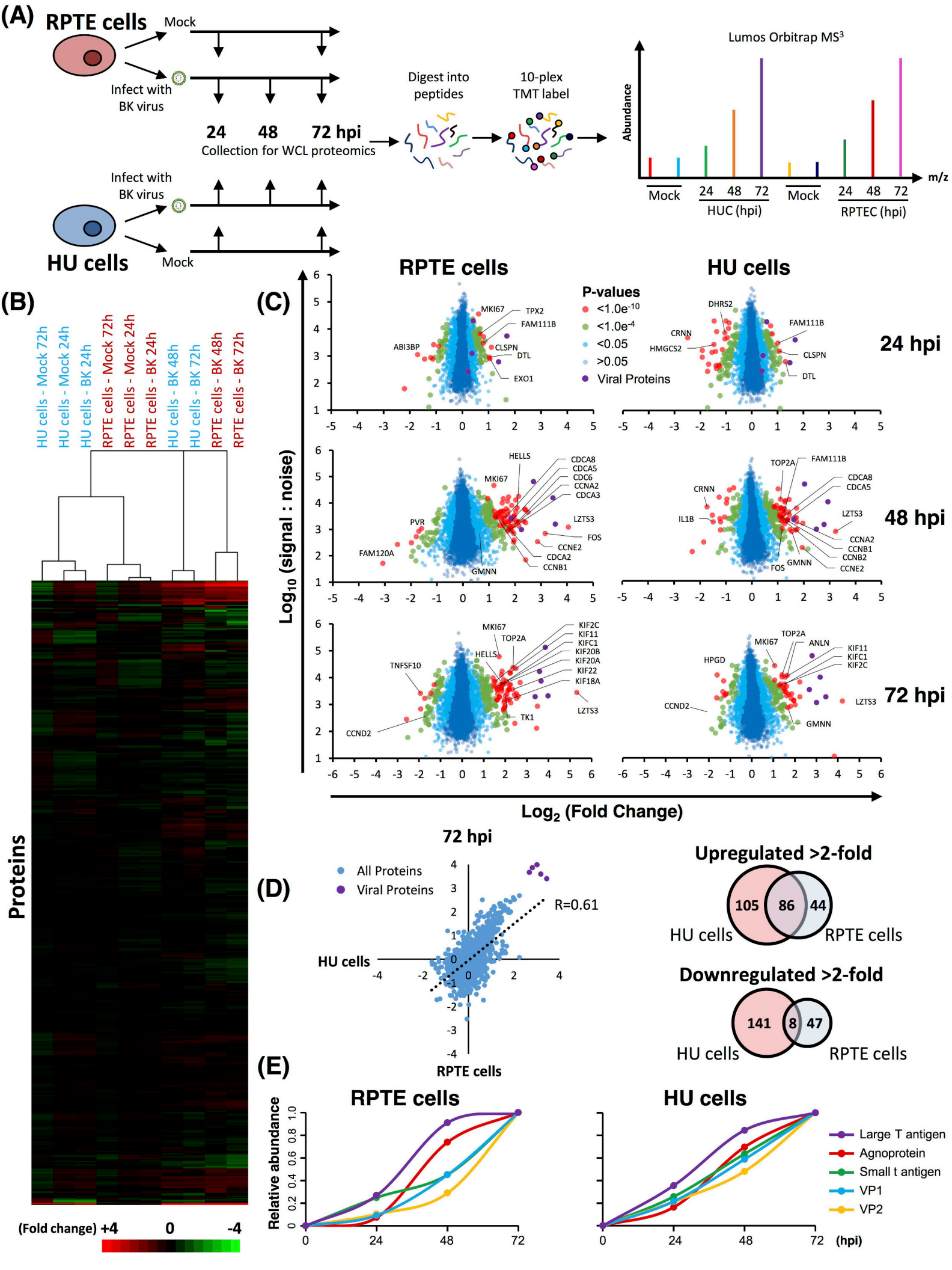902        blot. Tubulin was used as a loading control.
903    (C) Infectious BKPyV produced in each experimental condition was determined by fluorescent
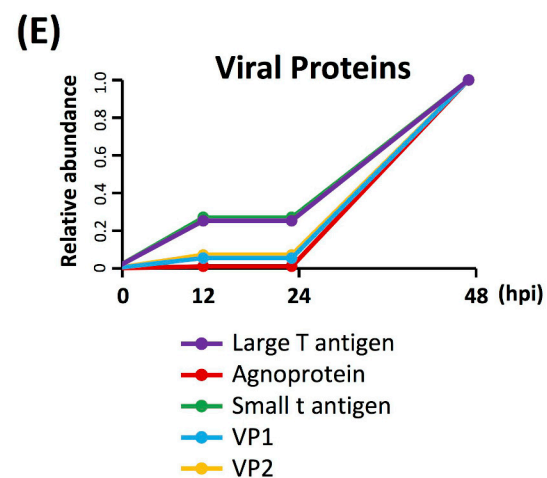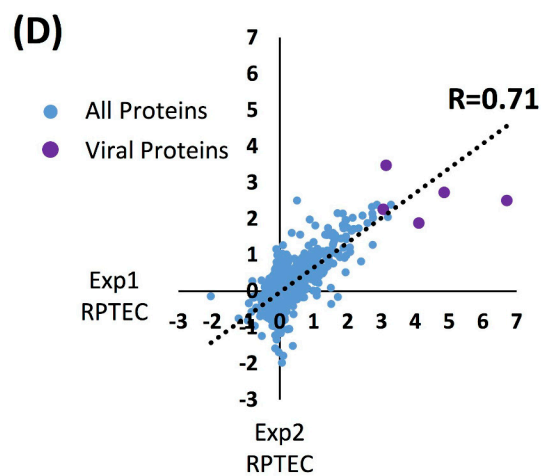904        focus unit (FFU) assay and normalised to uninhibited control (arbitrarily set to 1) (n=7).
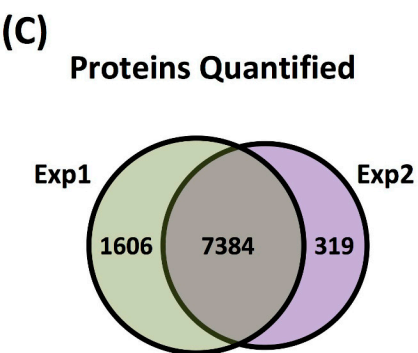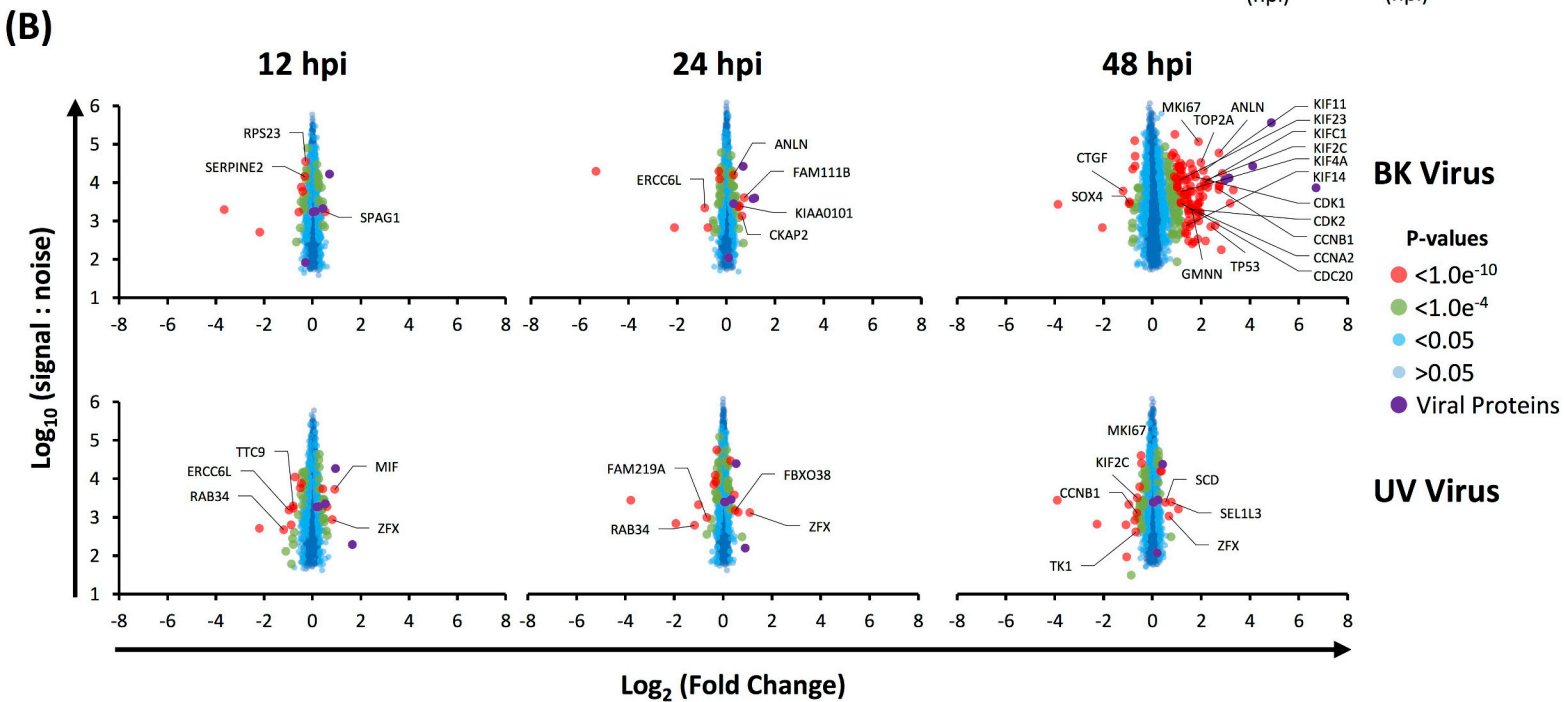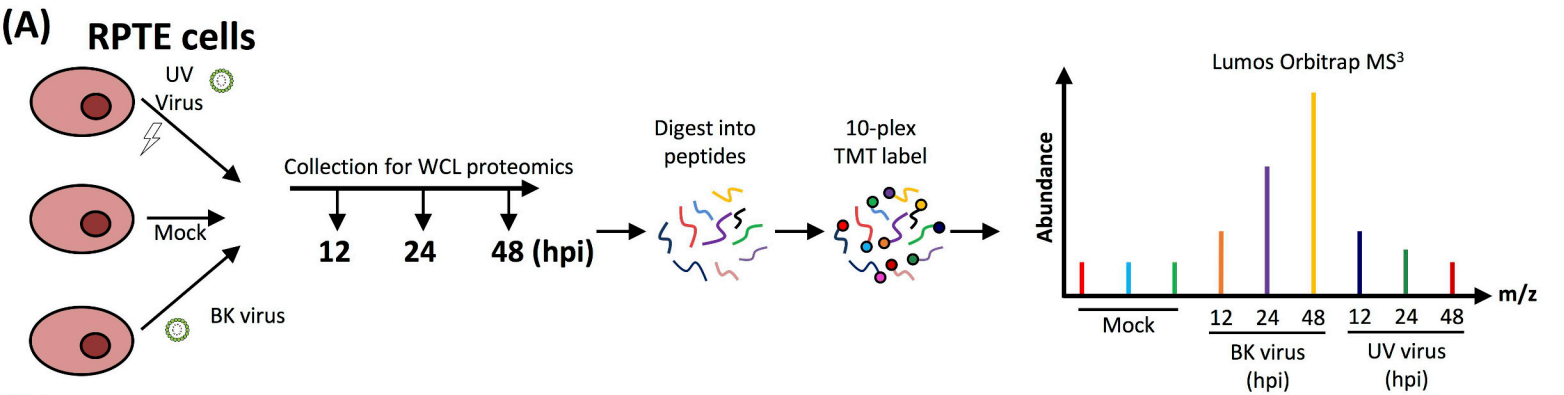905        Error bars represent standard deviation. *p < 0.05; ***p < 0.001; ****p < 0.0001; ns = not
906        significant; one sample t-test experimental conditions versus control.
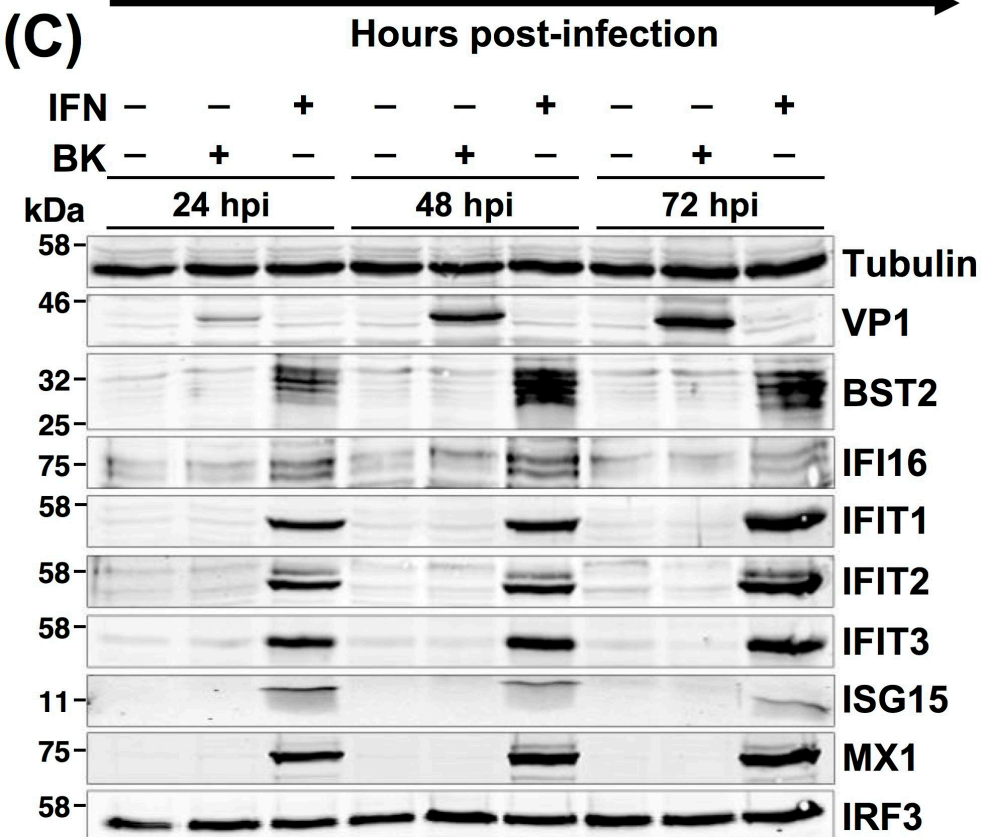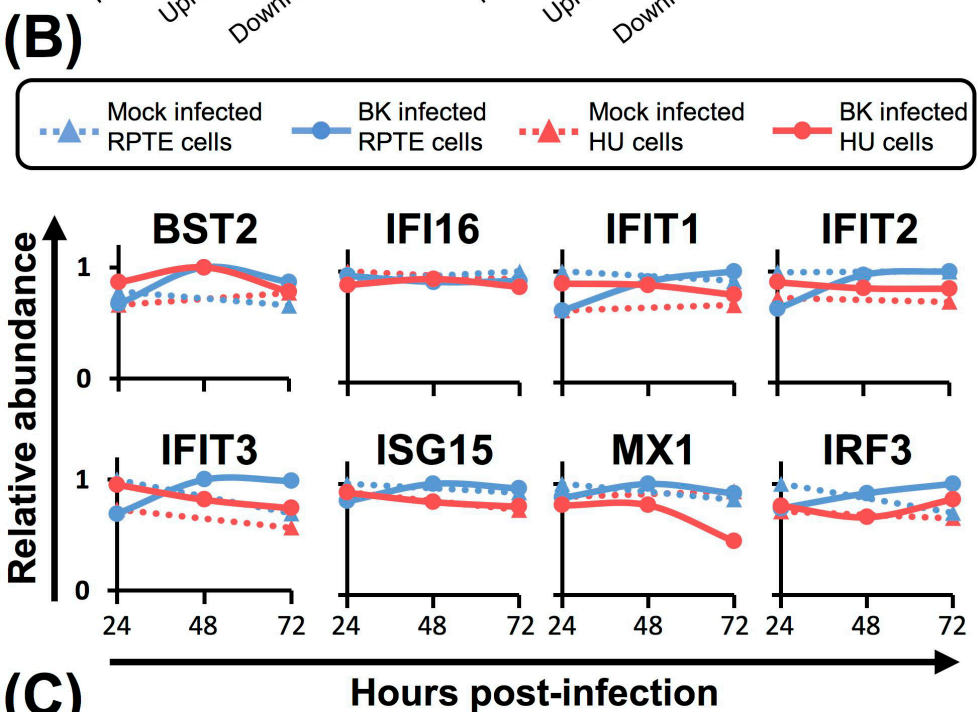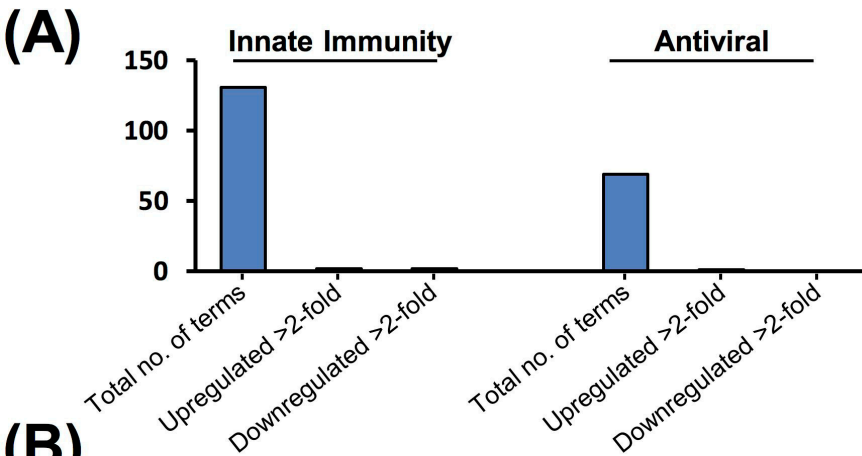907
908 **Figure 10. Proposed mechanism of interplay between MDM2 and p53 levels in the presence**
909 **of LTAg**

25
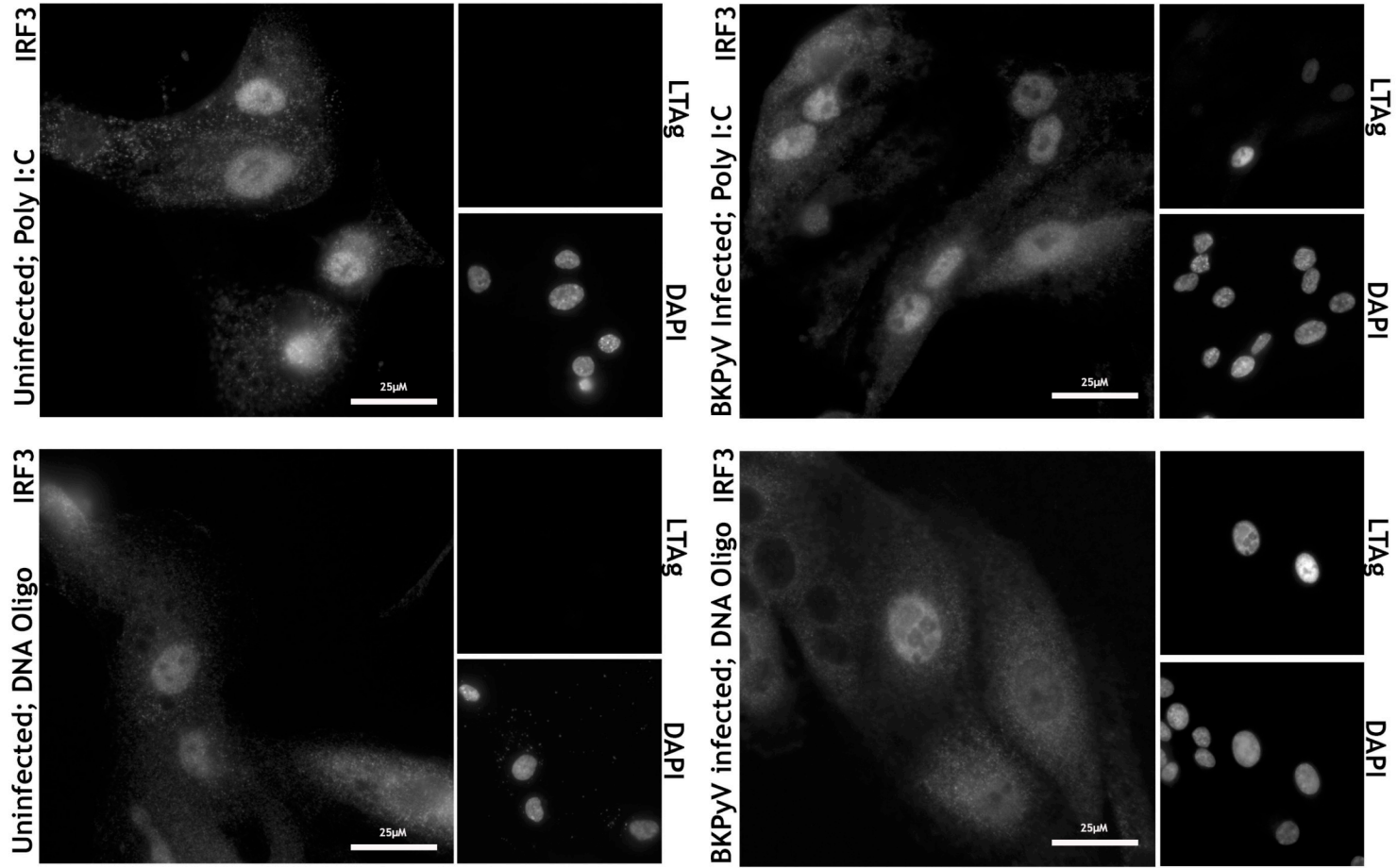
910      (A) Untreated cells.
911      (B) Uninfected, untransfected cells inhibited with Nutlin-3.
912      (C) Cells expressing LTAg in the absence of infection.
913      (D) Cells BKPyV infected, or cells expressing LTAg combined with cell cycle arrest/DNA
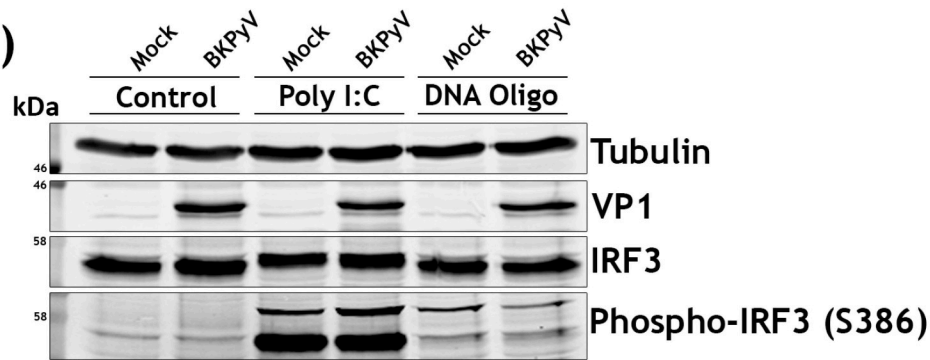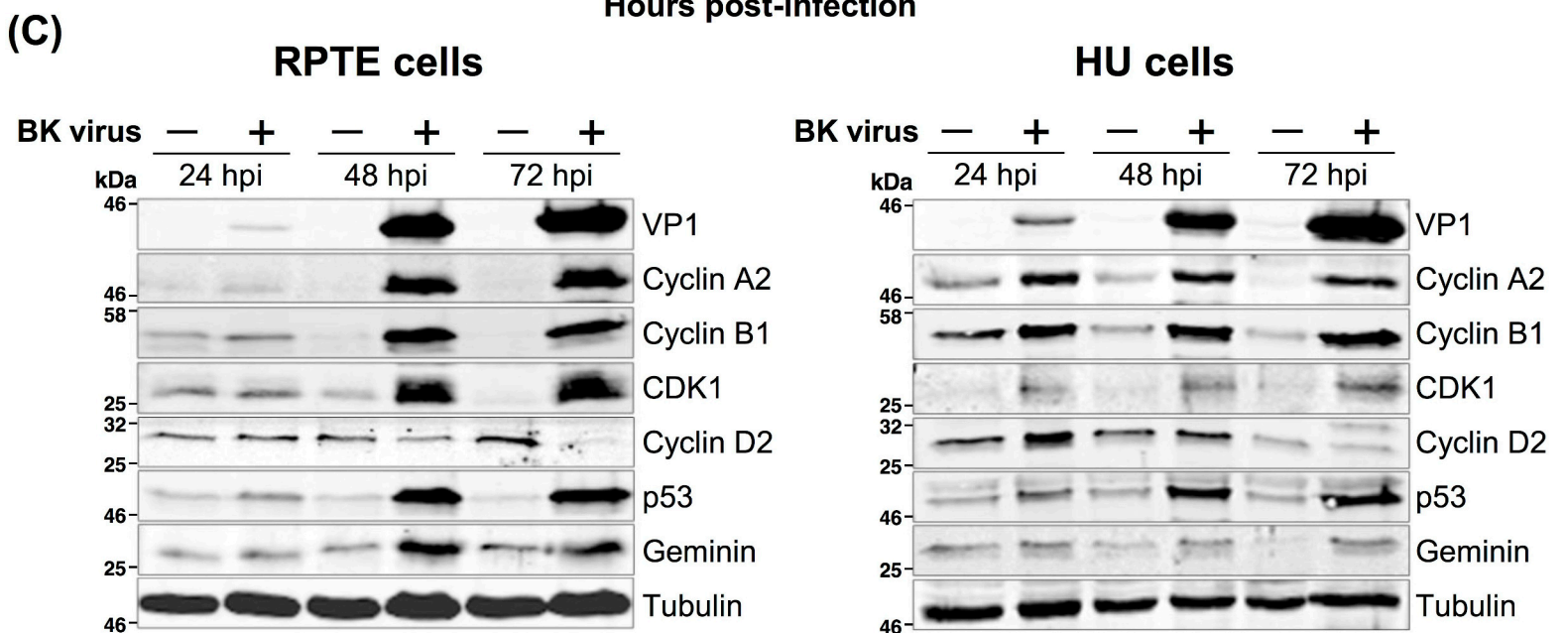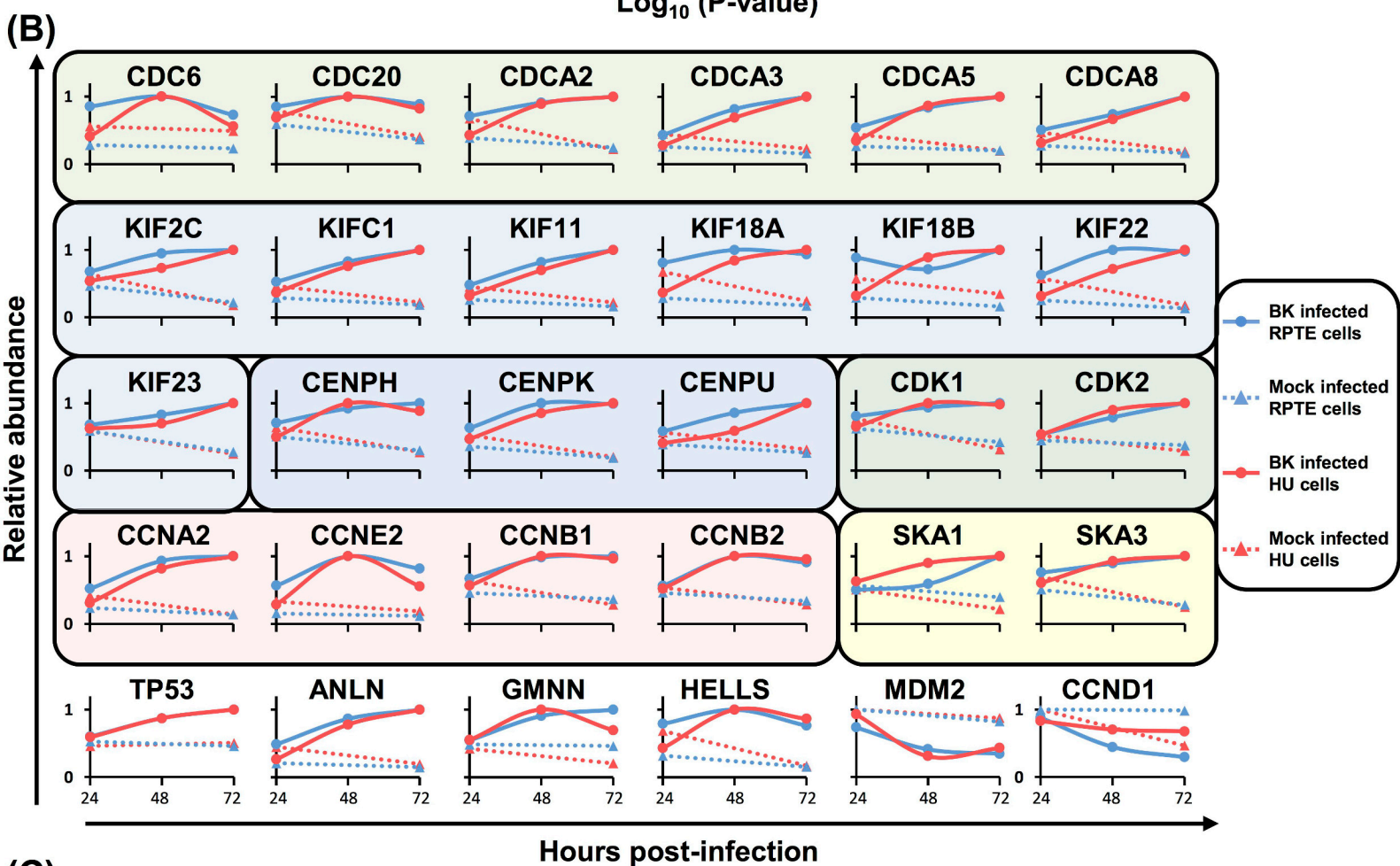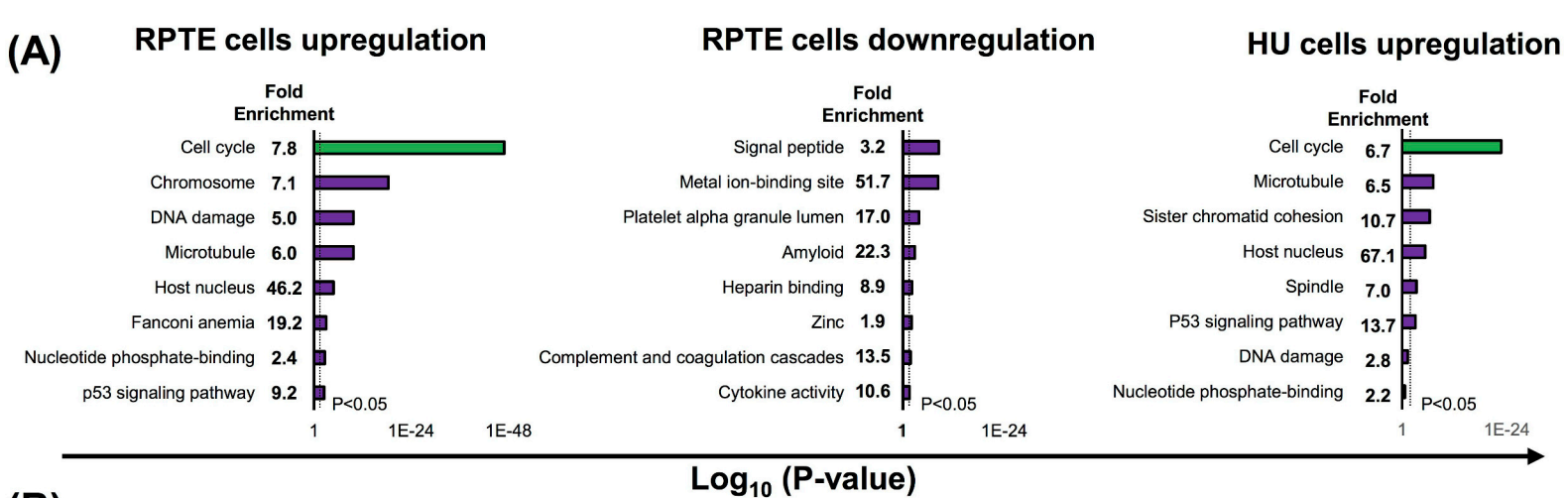914            damage.

915

**(A)** RPTE cells

UV Virus

Mock

BK virus

Collection for WCL proteomics

12    24    48 (hpi)

Digest into peptides

10-plex TMT label

Lumos Orbitrap MS³

Abundance

m/z

Mock    BK virus (hpi)    UV virus (hpi)
12  24  48    12  24  48

**(B)**

**12 hpi**

RPS23
SERPINE2
SPAG1

**24 hpi**

ANLN
ERCC6L
FAM111B
KIAA0101
CKAP2

**48 hpi**

MKI67    ANLN    KIF11
CTGF    TOP2A    KIF23
SOX4    KIFC1
KIF2C
KIF4A
KIF14
CDK1
CDK2
CCNB1
CCNA2
GMNN    TP53    CDC20

**BK Virus**

**P-values**
● <1.0e⁻¹⁰
● <1.0e⁻⁴
● <0.05
● >0.05
● Viral Proteins

TTC9    MIF
ERCC6L
RAB34    ZFX

FAM219A    FBXO38
RAB34    ZFX

MKI67
KIF2C    SCD
CCNB1    SEL1L3
ZFX
TK1

**UV Virus**

Log₁₀ (signal : noise)

Log₂ (Fold Change)

**(C)** Proteins Quantified

Exp1    Exp2
1606    7384    319

**(D)**

● All Proteins
● Viral Proteins

R=0.71

Exp1 RPTEC

Exp2 RPTEC

**(E)** Viral Proteins

Relative abundance

0    12    24    48 (hpi)

— Large T antigen
— Agnoprotein
— Small t antigen
— VP1
— VP2

**(A)** Innate Immunity / Antiviral — Total no. of terms, Upregulated >2-fold, Downregulated >2-fold

**(B)**

Legend:
- Mock infected RPTE cells
- BK infected RPTE cells
- Mock infected HU cells
- BK infected HU cells

BST2, IFI16, IFIT1, IFIT2, IFIT3, ISG15, MX1, IRF3

Relative abundance

Hours post-infection

**(C)**

| IFN | – | – | + | – | – | + | – | – | + |
| BK | – | + | – | – | + | – | – | + | – |

kDa — 24 hpi / 48 hpi / 72 hpi

Tubulin, VP1, BST2, IFI16, IFIT1, IFIT2, IFIT3, ISG15, MX1, IRF3

**(A)**

**(B)**

**(A)**

Uninfected; Poly I:C — IRF3, LTAg, DAPI — 25μM

BKPyV Infected; Poly I:C — IRF3, LTAg, DAPI — 25μM

Uninfected; DNA Oligo — IRF3, LTAg, DAPI — 25μM

BKPyV infected; DNA Oligo — IRF3, LTAg, DAPI — 25μM

**(B)**

| kDa | Control | | Poly I:C | | DNA Oligo | | |
|---|---|---|---|---|---|---|---|
| | Mock | BKPyV | Mock | BKPyV | Mock | BKPyV | |
| 46 | | | | | | | Tubulin |
| 46 | | | | | | | VP1 |
| 58 | | | | | | | IRF3 |
| 58 | | | | | | | Phospho-IRF3 (S386) |

**(A)**

RPTE cells upregulation

| Category | Fold Enrichment |
|---|---|
| Cell cycle | 7.8 |
| Chromosome | 7.1 |
| DNA damage | 5.0 |
| Microtubule | 6.0 |
| Host nucleus | 46.2 |
| Fanconi anemia | 19.2 |
| Nucleotide phosphate-binding | 2.4 |
| p53 signaling pathway | 9.2 |

P<0.05

RPTE cells downregulation

| Category | Fold Enrichment |
|---|---|
| Signal peptide | 3.2 |
| Metal ion-binding site | 51.7 |
| Platelet alpha granule lumen | 17.0 |
| Amyloid | 22.3 |
| Heparin binding | 8.9 |
| Zinc | 1.9 |
| Complement and coagulation cascades | 13.5 |
| Cytokine activity | 10.6 |

P<0.05

HU cells upregulation

| Category | Fold Enrichment |
|---|---|
| Cell cycle | 6.7 |
| Microtubule | 6.5 |
| Sister chromatid cohesion | 10.7 |
| Host nucleus | 67.1 |
| Spindle | 7.0 |
| P53 signaling pathway | 13.7 |
| DNA damage | 2.8 |
| Nucleotide phosphate-binding | 2.2 |

P<0.05

$Log_{10}$ (P-value)

**(B)**

Relative abundance

CDC6, CDC20, CDCA2, CDCA3, CDCA5, CDCA8
KIF2C, KIFC1, KIF11, KIF18A, KIF18B, KIF22
KIF23, CENPH, CENPK, CENPU, CDK1, CDK2
CCNA2, CCNE2, CCNB1, CCNB2, SKA1, SKA3
TP53, ANLN, GMNN, HELLS, MDM2, CCND1

Legend:
- BK infected RPTE cells
- Mock infected RPTE cells
- BK infected HU cells
- Mock infected HU cells

Hours post-infection

**(C)**

RPTE cells

BK virus − + / − + / − +
24 hpi / 48 hpi / 72 hpi

VP1, Cyclin A2, Cyclin B1, CDK1, Cyclin D2, p53, Geminin, Tubulin

HU cells

BK virus − + / − + / − +
24 hpi / 48 hpi / 72 hpi

VP1, Cyclin A2, Cyclin B1, CDK1, Cyclin D2, p53, Geminin, Tubulin

**(A)**

Mock | MDM2 | p53 | LTAg | DAPI | RBG
BKPyV | MDM2 | p53 | LTAg | DAPI | RBG
Mock +Nutlin | MDM2 | p53 | LTAg | DAPI | RBG
BKPyV +Nutlin | MDM2 | p53 | LTAg | DAPI | RBG

**(B)**

LTAg Plasmid | MDM2 | p53 | LTAg | DAPI | RBG
LTAg Plasmid +Nutlin | MDM2 | p53 | LTAg | DAPI | RBG
LTAg Plasmid +Ro3306 | MDM2 | p53 | LTAg | DAPI | RBG
LTAg Plasmid +Nutlin +Ro3306 | MDM2 | p53 | LTAg | DAPI | RBG

(A)

(B)

(C)