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The Human Cytomegalovirus Protein pUL21a Binds to and Degrades Cyclin A via the Proteasome

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by

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Introduction

Human cytomegalovirus (HCMV) is a widespread herpes virus that infects 50-80% of the human population, and establishes a lifelong infection in its host. It is the leading viral cause of birth defects [1] and can cause severe complications in immunocompromised individuals – particularly AIDS patients and transplant recipients. It has also been implicated in facilitating atherosclerosis [2] and in having an oncomodulatory role [3]. There are currently five approved antiviral drugs for HCMV, however prolonged usage can be detrimental and resistant strains have been reported for each of these [4,5], highlighting the urgent need for new therapeutic options.

HCMV is the prototypical β-herpes virus, consisting of a long (~240 kb) double-stranded DNA genome which codes for over 160 genes. Due to its relatively large size and complexity, it is a relatively slow-replicating virus and thus must maintain its host in a favorable state for virus replication over an extended period of time. This is achieved through various means, including modulation of the innate and adaptive immune responses [6,7,8], cellular stress [9,10,11], cell death pathways [11,12], and the cell cycle [13].

HCMV has been shown to arrest cells in late G1, before the onset of host DNA replication [14,15]. This is thought to benefit the virus as the cell contains a large store of nucleotides and is primed for DNA replication. Several viral genes are known to be involved in cell cycle arrest, including UL69 [16,17], IE2-86 [18,19], and UL117 [20]. If any of these are deleted, HCMV infected cells enter S-phase and virus growth is severely attenuated, indicating the importance of cell cycle arrest for virus replication.

Previous work from our lab has shown that the HCMV protein pUL21a is necessary for virus replication [21]. Recently, we have shown that pUL21a binds the anaphase promoting complex (APC), and targets components APC4 and APC5 for degradation, resulting in a non-functional APC [22], and a potential role for UL21a in cell cycle arrest. A proline-arginine (PR) motif at pUL21a residues 122-123 was shown to be necessary for this function - the PR mutant is unable to bind or degrade APC components during infection, resulting in a functional APC. Interestingly, PR mutant virus still grows to wild-type levels despite the loss of APC inhibition.

This suggests that UL21a may have another function, independent of APC inhibition, which is required for wild-type growth.

To probe for other potential UL21a functions, a search was conducted in the Eukaryotic Linear Motif resource [23]. Analysis of the pUL21a sequence identified a cyclin-binding RxL motif in pUL21a at residues 46-48, suggesting a potential means for cell cycle control. Cyclins drive progression through the cell cycle by binding and activating cyclin-dependent kinases (CDKs), which in turn phosphorylate cell cycle stage-specific substrates. G1 is when cyclin A is first expressed, and its steadily rising levels lead CDK2 binding to progress cells through S-phase [24,25]. Cyclin A has been shown to be reduced during HCMV infection [14,26], suggesting active control of the cell cycle by the virus. However, the mechanism by which HCMV reduces cyclin A levels remains to be uncovered.

In this study, we demonstrate that pUL21a binds to cyclin A via an RxL motif, leading to proteasome-dependent degradation of cyclin A. Mutation of the RxL motif demonstrates that this function is independent of APC degradation, and that the ability to reduce cyclin A is necessary for cell cycle arrest and virus growth. These are significant findings in that they identify the viral protein responsible for HCMV reduction of cyclin A, and demonstrate that HCMV is dependent on this function for cell cycle arrest and virus replication. The study explores the relationship between the virus and its host at the molecular level, contributing new knowledge to the HCMV field and viral pathogenesis in general. The novel, unique function of a necessary viral protein with no cellular homologs make it an ideal target for potential therapeutics, and the ability of UL21a to arrest cells in G1 also suggests a potential new tool for studying cancer and the cell cycle.

Results

An RxL motif is necessary for pUL21a to bind cyclin A

A search in the Eukaryotic Linear Motif resource revealed potential cyclin-binding RxL motifs at pUL21a residues 31-33, 42-44, and 55-57. Due to the role of cyclin A in exiting G1, we decided to test for a potential interaction between pUL21a and cyclin A. The high conservation of residues 36-45 between human, chimp, and rhesus CMV pUL21a proteins led us to focus on residues 42-44 (Fig 1a). pUL21a residues 42-44 were mutated to from Arg-Arg-Lys to Ala-Arg-Ala (RxL-AxA), and the N-terminus was tagged with GFP. Cells were transfected to express GFP-tagged wild-type (WT), stop mutant, PR mutant, or RxL mutant pUL21a. pUL21a mutated at the Pro-Arg (PR) motif at residues 122-123 has been shown lose the ability to bind and degrade the APC. Subsequently, extracts from cells transiently expressing UL21a mutants were immunoprecipitated for GFP or Cyclin A (Fig 1b).

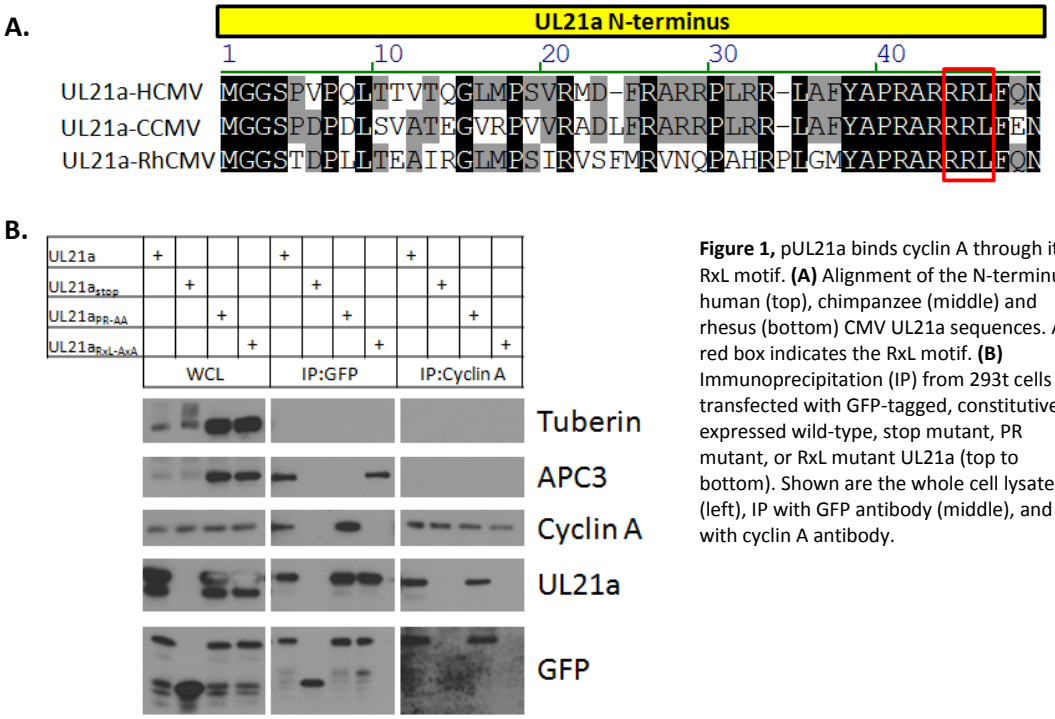

Figure 1, pUL21a binds cyclin A through its RxL motif. **(A)** Alignment of the N-terminus of human (top), chimpanzee (middle) and rhesus (bottom) CMV UL21a sequences. A red box indicates the RxL motif. **(B)** Immunoprecipitation (IP) from 293t cells transfected with GFP-tagged, constitutively expressed wild-type, stop mutant, PR mutant, or RxL mutant UL21a (top to bottom). Shown are the whole cell lysate (left), IP with GFP antibody (middle), and IP with cyclin A antibody.

GFP antibody was able to pull down all UL21a constructs, and WT UL21a pulled down both cyclin A and APC3. The PR mutant was able to pull down cyclin A but not APC3, whereas

the RxL mutant pulled down APC3 but not cyclin A. Accordingly, cyclin A pulled down WT and PR mutant UL21a, but not the RxL mutant. As a specificity control, cyclin A was unable to pull down the abundant cellular protein tuberin. Thus, UL21a independently binds the APC complex and Cyclin A via separable binding sites.

pUL21a is dependent on the RxL motif to reduce cyclin A expression

Because of the interaction between pUL21a and cyclin A and the potentially antagonistic effect of S-phase progression on virus replication, we postulated that pUL21a may inhibit cyclin A activity. Cells were either mock infected or infected with HCMV containing wild-type, deleted, or PR mutant UL21a. Cells were lysed at 24, 48, and 72 hours post infection (hpi) and analyzed by western blot (Fig 2a). Relative to mock, HCMV infected cells displayed reduced cyclin A expression, but this reduction was lost in the UL21a deleted virus. PR mutant virus, however, was able to reduce cyclin A to wild-type levels, despite being unable to reduce APC expression.

In order to test the necessity of the RxL motif for cyclin knockdown, the arginine and lysine residues were mutated to alanines in the virus. The mutant virus showed decrease growth relative to wild-type, and its growth was complemented by exogenous expression, ruling out a defect due to secondary mutation (data not shown). Infection was performed as above, and again was analyzed by western blot (Fig 2b). As expected, RxL mutant virus was unable to knockdown cyclin A, similar to the UL21a deletion virus, but was able to knock down APC components. This finding, in combination with the PR mutant data in Fig 2a, suggests that the ability of pUL21a to decrease APC3 and cyclin A is genetically separable, and the functions are independent of one another.

To test whether UL21a by itself is sufficient for cyclin A knockdown, the PH mutant and RxL mutant UL21a were expressed exogenously from a tetracycline-inducible promoter. The PH mutant, described previously [22], behaves essentially like WT UL21a. Cells were serum starved to synchronize at G0, treated with tetracycline to induce expression of the UL21a constructs, and released from G0 with serum induction. Cells were then lysed at 0, 24, and 32 hours post serum

stimulation, and analyzed by western blot (Fig 2c). In overexpression, PH-mutant UL21a was able to knockdown cyclin A whereas RxL mutant was not, and both were capable of knocking down APC components. Thus, UL21a in isolation is sufficient to reduce cyclin A, and depends on the RxL motif for this function.

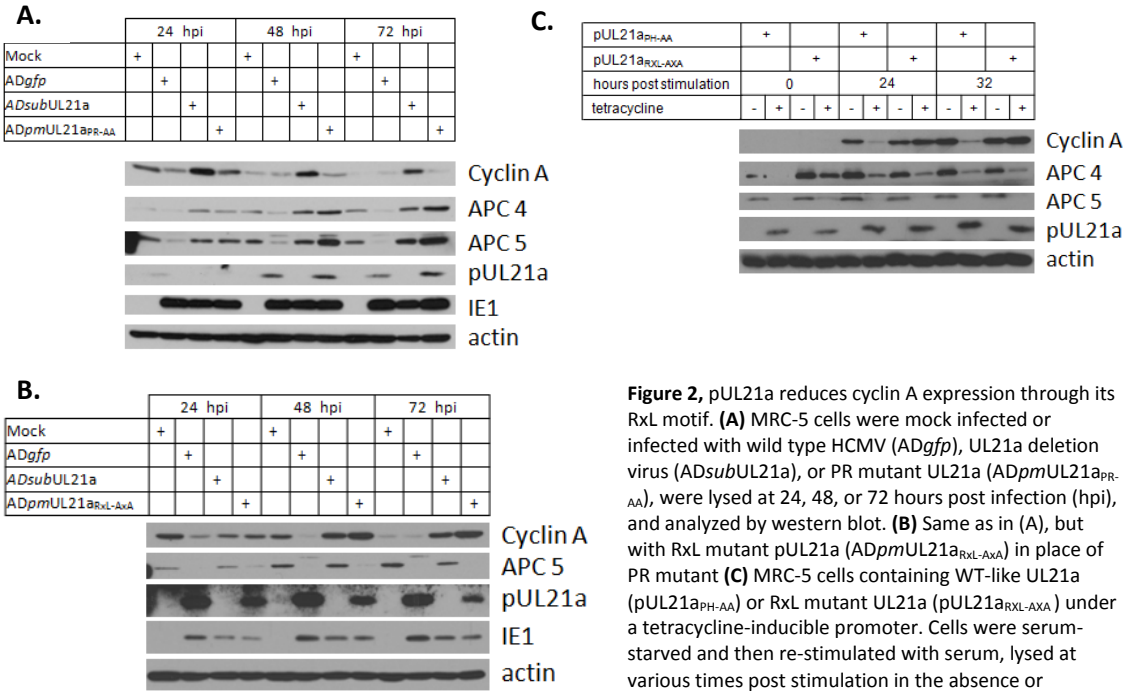

Figure 2, pUL21a reduces cyclin A expression through its RxL motif. **(A)** MRC-5 cells were mock infected or infected with wild type HCMV (AD*gfp*), UL21a deletion virus (AD*sub*UL21a), or PR mutant UL21a (AD*pm*UL21a$_{PR-AA}$), were lysed at 24, 48, or 72 hours post infection (hpi), and analyzed by western blot. **(B)** Same as in (A), but with RxL mutant pUL21a (AD*pm*UL21a$_{RxL-AxA}$) in place of PR mutant **(C)** MRC-5 cells containing WT-like UL21a (pUL21a$_{PH-AA}$) or RxL mutant UL21a (pUL21a$_{RXL-AXA}$) under a tetracycline-inducible promoter. Cells were serum-starved and then re-stimulated with serum, lysed at various times post stimulation in the absence or presence of tetracycline, and analyzed by western blot.

pUL21a is necessary for cell-cycle arrest by HCMV

Cyclin A plays a crucial role in progressing cells through S phase, leading us to hypothesize that pUL21a may be important for inducing cell cycle arrest in infected cells. To test this, cells were mock infected or infected with wild-type, UL21a-deletion, or PR mutant HCMV and analyzed by flow cytometry for total DNA content (Fig 3). Uninfected cells displayed a characteristic bimodal distribution, with the left peak indicating cells in G1 and the right peak cells in G2. At 24 hours post infection, all samples showed a cell-cycle profile similar to uninfected cells. However, by 48 hours wild-type virus showed a more robust G1 peak whereas UL21a

deletion virus showed a dramatic shift towards S phase and G2, and PR mutant showed a profile similar to wild-type virus. This indicates that UL21a is necessary for arresting infected cells in G1, and that this function is independent of its ability to degrade the APC.

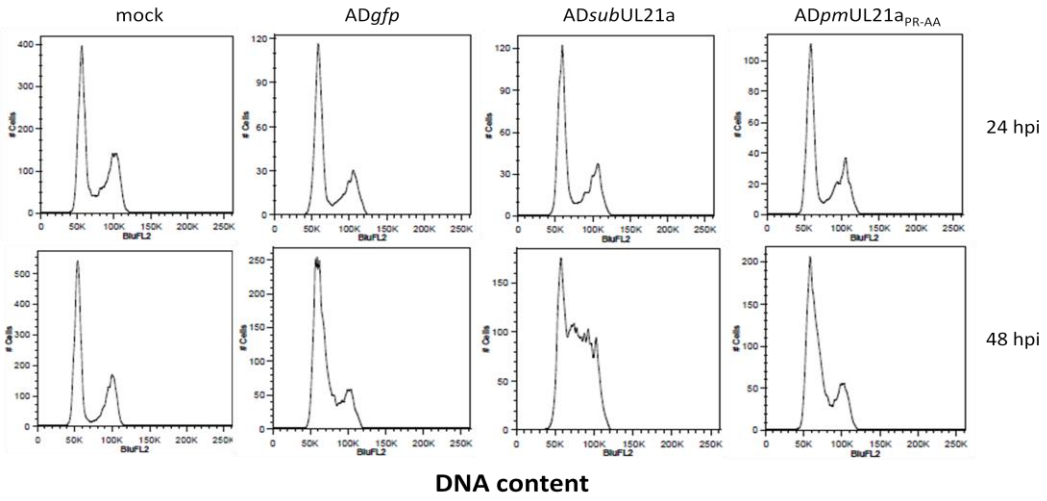

Figure 3, UL21a is necessary for HCMV-induced cell cycle arrest at G1. MRC-5 cells were mock infected or infected with wild-type, Ul21a deletion, or PR mutant HCMV. At 24 (top) and 48 (bottom) hours post infection, cells were fixed and DNA was stained with propidium iodide for analysis by flow cytometry. Additionally, infected cells were identified by pUL44 staining, and with the exception of mock all samples were gated for UL44-positive cells only.

Cyclin A reduction by pUL21a is required for virus replication

We postulated that pUL21a-mediated degradation of cyclin A was necessary for efficient virus replication, and that this function accounted for UL21a's role in virus replication. In order to test this theory, cyclin A was knocked down artificially via siRNA. As a control, cells were treated with an siRNA to luciferase. Cells were then infected with wild-type or UL21a deletion virus and assayed for viral growth (Fig 4a) and viral late gene expression (Fig 4b). In a high-MOI infection, deletion mutant virus showed an approximately 10-fold growth defect relative to wild-type virus on control cells. However, treatment with cyclin A siRNA was able to rescue UL21a deletion virus to within wild type titers. Similarly, the deletion mutant showed a defect in expression of the viral gene IE2, which was fully restored by treatment with cyclin A siRNA. Thus, the ability of UL21a to

knock down cyclin A is necessary for virus replication, and largely accounts for UL21a mutant virus growth defect.

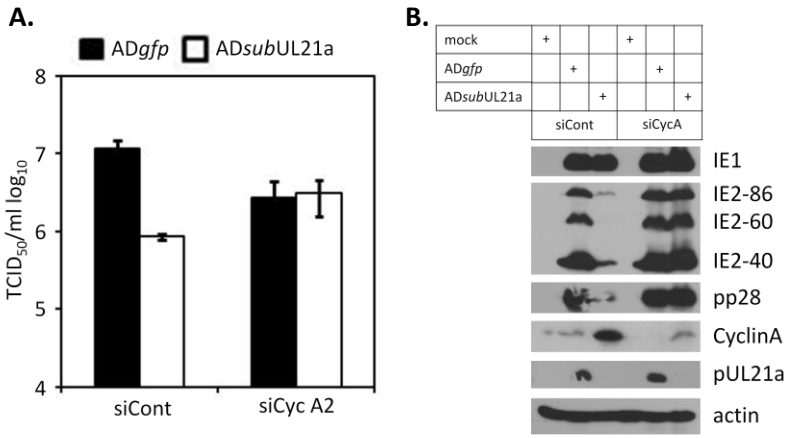

Figure 4, Cyclin A knockdown rescues UL21a mutant virus. MRC-5 cells were treated with siRNA against cyclin A (siCyc A) or luciferase (siCont). Treated cells were infected, and at 96 hours post infection supernatant was collected for titering virus progeny **(A)** or cells were lysed for western blot analysis **(B)**. Virus titering was performed in duplicate by tissue culture infectious dose 50 ($TCID_{50}$)

UL21a degrades cyclin A via the proteasome

To begin investigating the mechanism by which UL21a leads to cyclin A degradation, we decided to test whether pUL21a affects cyclin A transcript levels. Cells were infected and collected at 6 hpi, or treated with or without the proteasome inhibitor MG132 at 6 hpi and collected at 18 hpi. Total RNA was extracted and reverse transcribed for real-time PCR analysis (Fig 5a), or cells were lysed for western blot analysis (Fig 5b). At 6 hpi, cells infected with wild-type vs UL21a-deletion virus showed comparable levels of cyclin transcript, with deletion virus having perhaps slightly higher levels. At 18 hpi the cyclin A transcript levels between WT and deletion levels remained close, and in the presence of MG132 all cyclin A levels decreased drastically. In contrast, MG132 fully restored cyclin A protein in wild-type virus to the level of pUL21a-deletion virus. Interestingly, cyclin A protein levels remained elevated during treatment with MG132, despite the drastic reduction of transcript levels seen in Fig 5a. This suggested that

cyclin A is degraded by UL21a at the protein level, and more specifically is targeted to proteasome.

To further probe whether pUL21a-mediated degradation of cyclin A is proteasome dependent, cells were treated with MG132 for a shorter period of 6 hours and were additionally treated with epoxomicin, a highly specific proteasome inhibitor [27] (Fig 5c). Treatment with MG132 or epoxomicin again resulted in full restoration of cyclin A in wild-type virus, further supporting the proteasome-dependence of cyclin A degradation by pUL21a.

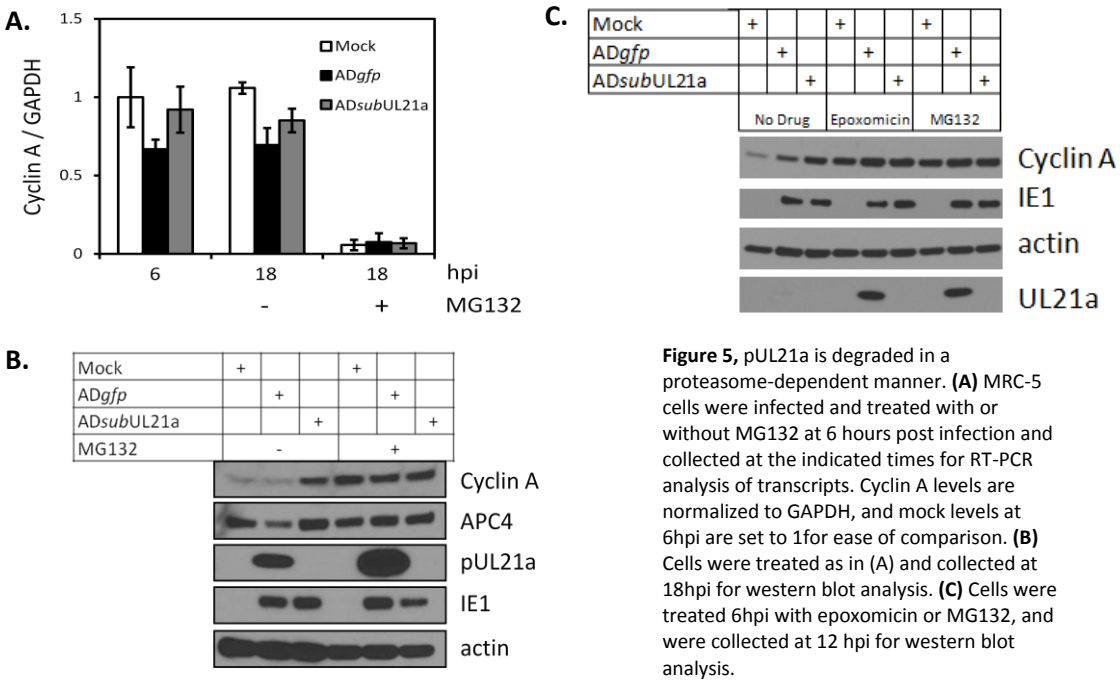

Figure 5, pUL21a is degraded in a proteasome-dependent manner. **(A)** MRC-5 cells were infected and treated with or without MG132 at 6 hours post infection and collected at the indicated times for RT-PCR analysis of transcripts. Cyclin A levels are normalized to GAPDH, and mock levels at 6hpi are set to 1for ease of comparison. **(B)** Cells were treated as in (A) and collected at 18hpi for western blot analysis. **(C)** Cells were treated 6hpi with epoxomicin or MG132, and were collected at 12 hpi for western blot analysis.

Discussion

In this study, we have demonstrated that pUl21a binds to cyclin A via an RxL motif, leading to cyclin A degradation. We have also demonstrated that this function is independent of its ability to bind and induce degradation of the anaphase promoting complex. UL21a was necessary to induce HCMV cell cycle arrest, and cyclin A degradation was necessary for efficient viral replication. Lastly, we showed that cyclin A degradation by pUL21a is primarily proteasome dependent.

Previous studies from our lab have shown that pUL21a induces APC degradation via a PR motif, and that APC degradation is also proteasome dependent. Furthermore we have also shown that treatment with proteasome inhibitors dramatically increases pUL21a levels. Thus, in our current model, cyclin A and the APC are bound by UL21a via independent sites, and all three proteins are degraded by the proteasome (Fig 6).

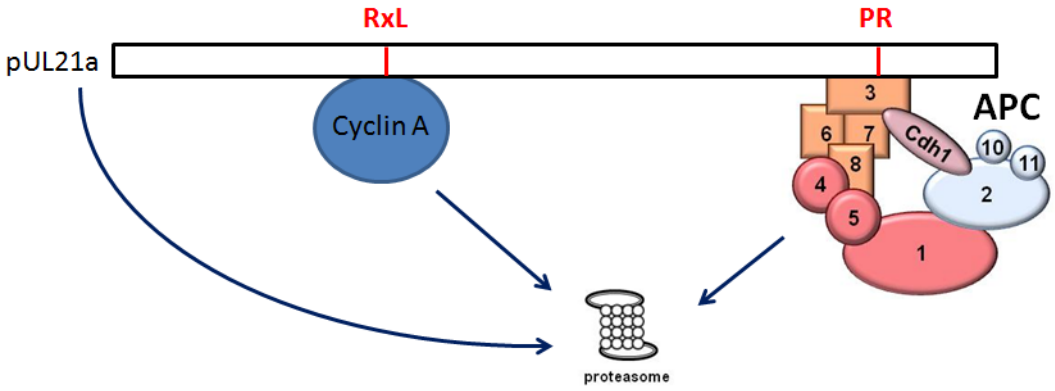

Figure 6, Current model of UL21a function and mechanism. pUL21a independently binds cyclin A and the APC complex via its RxL and PR motifs, respectively. Cyclin A and the APC are similarly independently degraded in a proteasome-dependent manner, as is UL21a.

To further understand the mechanism by which UL21a induces degradation of cyclin A and the APC, it is important to address the signal by which these enzymes are transported to the proteasome. By far the most common proteasome-targeting tag is the poly-ubiquitin chain. In this system, ubiquitin is activated by an E1 enzyme and is transferred to an E2, which binds an E3

specificity factor to target ubiquitin to the molecule to be degraded [28]. Interestingly, a previous study from our lab has found that proteasome degradation of pUL21a persists in temperature sensitive knockout of an E1 enzyme in mouse ts20 cells (tony 2010), which is also necessary for p53 degradation. This suggests that the degradation of UL21a may occur in an ubiquitin-independent manner, as well as possibly the degradation of its substrates. However, this remains to be established and pUL21a may induce cyclin A and APC degradation in a manner different from its own.

During the cell cycle, cyclin A is primarily degraded by the APC, which ubiquitinates a region of cyclin A known as the D-box [29]. Thus, one would predict that depletion of the APC would result in an increase of cyclin A. It is technically possible that UL21a degrades cyclin A by bringing it into proximity with the APC. However, this seems unlikely as this study finds that the pUL21a PR mutant, which is unable to bind APC, is still capable of inducing cyclin A degradation. Furthermore, it seems more likely that UL21a would have a single mechanism for degrading both substrates rather than two different mechanisms. Nonetheless, the potential dependence on the APC for degradation of cyclin A by pUL21a can be tested for by deleting the cyclin A D-box to make an APC-resistant cyclin A [29]. It has also recently been shown that cyclin A is acetylated to facilitate ubiquitination [30], thus representing another potential regulatory mechanism.

It is interesting to postulate that pUL21a may degrade Cyclin A by directly transporting it to the proteasome. GFP-tagging of UL21a in isolation and during infection shows diffuse expression throughout the cytoplasm [21], making co-localization difficult. Nonetheless, it may be of interest to investigate a possible association between pUL21a and the proteasome. UL21a also intriguingly contains a diglycine motif at its N-terminus, which may suggest proteasome targeting. The diglycine motif is found on the C-terminus of ubiquitin and many ubiquitin-like proteins including ISG-15, SUMO, and FAT-10, and has been found play different important functions in these proteins [31,32,33].

Although several examples exist of viral proteins that alter cyclin A levels [34,35], none of them are known to do so through a direct interaction. Human papillomavirus E7 protein and

Epstein-Barr virus EBNA3C protein both bind cyclin A, with EBNA3C inhibiting its kinase activity [36,37]. This study elucidates the first viral protein capable of directly binding cyclin A to induce its degradation. The importance of this function is highlighted by the necessity of pUL21a reduction of cyclin A for efficient viral replication. Although future experiments are needed, this study strongly suggests that the inability to arrest in G1 is responsible for the UL21a mutant growth defect. This novel interaction brings new light to HCMV biology, and pUL21a represents a potential target for developing new therapeutics, particularly as there are no known human homologs. Because of its ability to arrest cell cycle, pUL21a also represents a potentially powerful tool for studying the cell cycle or as a cancer therapeutic – unlike chemical cell cycle inhibitors pUL21a has presumably been evolving alongside of cyclin A for millions of years. Overall, we believe this study contributes to HCMV biology and viral pathogenesis and provides new tools for the study of the cell cycle and cell biology in general.

Materials and Methods

Plasmids and Reagents

Human Foreskin Fibroblasts (HFFs), primary embryonic lung fibroblasts (MRC-5s), and 293t cells were propagated in Dulbecco's modified Eagle medium (DMEM) containing 10% Fetal Bovine Serum and penicillin-streptomycin. Transfection of plasmids into 293t cells was achieved using 1mg/ml polyethyleneimine (PEI) in Opti-MEM media or 150 mM NaCl.

Point mutants were constructed by incorporating mutation into complementary primers, amplifying fragments of the UL21a gene, and combining fragments by PCR with overlapping templates using outer primers containing restriction sites. The RxL mutant construct was cloned by restriction digest and ligation into a pLPCX-derived retroviral vector (pYD-C235) containing 5' end GFP to make GFP-tagged RxL mutant (pYD-C760), or into a pLKO-based lentiviral vector under a CMV-TetO_2 promoter (pYD-C639 – generous gift from Roger Everett, University of Glasgow) to make pYD-C762. All new constructs were verified by sequencing, and all other vectors have been previously described [22].

Lentivirus was produced by PEI transfection as described above of pYD-C639-derived constructs along with appropriate packaging plasmids into 293t cells. Lentivirus was harvested at 48 and 72 hours and syringed through a .45 µM filter to remove cellular debris, and was frozen, thawed, and applied to MRC-5 cells stably expressing GFP-TetR [22].Selection with 2 µg/µL puromycin resulted in stable cell lines containing UL21a constructs under a tetracycline-inducible promoter.

The primary antibodies used included anti-β actin (AC-15, Abcam) anti-GFP (3E6 and A6455, Invitrogen); anti-APC3 (610454, BD); anti-APC4 (A301-176A, Bethyl laboratories); anti-APC5 (A301-026A, Bethyl laboratories); anti-UL21a [21]; anti-IE2 (mAB8140, Chemicon); anti-UL44 (virusys, 10D8); anti-Cyclin A (B-8 and H-432, Santa Cruz); anti-tuberin (EP1107Y, epitomics);and anti-IE1 (generous gift from Thomas Shenk, Princeton University).

Cyclin A transient knockdown was performed using Thermo Scientific siGENOME siRNA against cyclin A (M-003205-02) or, as a control, luciferase (D-001210-04-05). Primary MRC-5

cells were transfected using siLENTFECT (BIO-RAD 170-3360) as per manufacturer's instructions and infected 48 hours post-transfection.

Recombinant HCMV virus

Point-mutated HCMV was created by two-step homologous recombination into the pAD*gfp* bacterial artificial chromosome (BAC) [38]. Briefly, the UL21a open reading frame was replaced by a GalK-Kan cassette, selected for with kanamycin, and subsequently replaced with mutated UL21a amplified from pYD-C762 (described above) and negatively selected for loss of the cassette. Recombinant BAC was confirmed by PCR, restriction digest, and sequencing. All other viruses used are described previously[22]. Viral stocks were harvested from cell culture supernatant and concentrated by ultracentrifugation through 20% D-sorbitol. Virus titers were determined by duplicate tissue culture infectious dose 50 ($TCID_{50}$) assay on HFFs. Relative viral genome numbers were determined by extracting virion DNA and performing real-time qPCR (quantitative PCR)[21]. Briefly, virions were treated with DNAse I, lysed with proteinase K and SDS, and DNA was extracted with phenol/chloroform/isoamyl alcohol and precipitated with ethanol. Real-time qPCR was performed on the extracted DNA with a taqman probe and primers specific to the viral gene UL54.

HCMV infection

Confluent MRC-5 cells were split 24 hours previous to infection, and all infections were conducted at multiplicity of infection (MOI) equal to 3 infectious units/cell for wild-type virus, with an equal genome number for mutant viruses [39]. Cells were inoculated for 1 hour with virus and then replenished with fresh media. Virus assayed for growth was additionally washed with PBS before the addition of fresh media after inoculation. For cell cycle profiling, cells were treated with phosphonoacetic acid (PAA, 100 µg/ml) immediately after infection. For proteasome inhibition, cells were treated with epoxomicin (10 µM) or MG132 (10 µM). Virus growth was measured by

removing culture media, centrifuging at 4000 RPM to remove cellular debris, and assaying by $TCID_{50}$.

Protein Analysis

For immunoprecipitation, cells were lysed with NP-40 lysis buffer (0.5% NP-40, 50 mM Tris-Cl pH 8.0, 125 mM NaCl, supplemented with protease and phosphatase inhibitors), gently rotated for 1 hr at room temperature, and centrifuged to remove cellular debris and produce whole cell lysate. Antibodies were conjugated to protein A dynabeads (Invitrogen 100.02D) with BS^3 (Thermo Scientific 21580) as per manufacturer's instructions, and rotated gently with whole cell lysate for 1 hr at 4°C. Antibodies used in IP included 1 µg mouse anti-GFP and 2 µg mouse anti-Cyclin A (described above). Beads were washed once with lysis buffer and twice with PBS, and protein was eluted in reducing sample buffer by incubating at 55°C for 20 min. Whole cell lysate was similarly mixed with sample buffer and incubated at 90°C for 5 min.

For immunoblotting, cells were washed with PBS, lysed with reducing sample buffer containing protease and phosphotase inhibitors, and collected after scraping with a cell scraper. Proteins were resolved on a poly-acrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with 5% milk or bovine serum albumin (BSA) followed by incubation with primary antibody and subsequently horseradish peroxidase-conjugated secondary antibody. Signal was observed by SuperSignal West Pico chemiluminescent substrate (Thermo Scientific).

Reverse Transcription Coupled-Quantitative PCR Analysis (RT-qPCR)

Total cellular RNA was extracted with TRIzol (Invitrogen), treated with TURBO DNA-free (Ambion) to remove DNA contaminants, and reverse transcribed with random hexamer primers. Transcript levels were then assessed by qPCR using SYBR green SYBR Advantage qPCR Premix (Clontech) with primers for the cellular genes Cyclin A or GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Six arbitrary samples were mixed together and serially diluted to

make a standard curve. The primers used are GCATGTCACCGTTCCTCCTT (forward) and CAGGGCATCTTCACGCTCTAT (reverse) for cyclin A, and CTGTTGCTGTAGCCAAATTCGT (forward) and ACCCACTCCTCCACCTTTGAC (reverse) for GAPDH.

Analysis of Cellular DNA content

To determine cellular DNA content, cells were trypsinized, washed with PBS, and fixed in 70% ethanol. Fixed cells were then stained with propidium iodide for DNA and anti-pUL44 antibody to identify infected cells, and were subjected to flow cytometry. Analysis was performed with FlowJo software.

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