UNIVERSITY OF CALIFORNIA, SAN DIEGO

Study of Anaphase Promoting Complex Inactivation by Human Cytomegalovirus Reveals Cellular Mechanism for Downregulating Anaphase Promoting Complex Subunits

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biology

by

Elizabeth Clark

Committee in charge:

Professor Deborah Spector, Chair
Professor Michael David
Professor Daniel Donoghue
Professor Gentry Patrick
Professor Aleem Siddiqui

2015

This Dissertation of Elizabeth Clark is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

_____

_____

_____

_____

_____
Chair

University of California, San Diego

2015

DEDICATION

This thesis is dedicated to Larry McClellan, my third grade teacher. His enthusiasm for science was contagious, and his confidence in young minds to grasp it was rare and valuable. He taught me to observe and question but also to look for the positive in things. He had the patience to teach life lessons to an 8 year old. I owe him so much.

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENTS

This work was made possible because of the guidance from my advisor and thesis committee and the comradery of my most excellent friends.

Chapter 3, in full, is a reprint of the material as it appears in Studies on the Contribution of Human Cytomegalovirus UL21a and UL97 to Viral Growth and Inactivation of the Anaphase-Promoting Complex/Cyclosome (APC/C) E3 Ubiquitin Ligase Reveal a Unique Cellular Mechanism for Downmodulation of the APC/C Subunits APC1, APC4, and APC5. Journal of Virology, 2015. The dissertation author was the primary investigator and author of this paper.

I thank Don Coen for the UL97 antibody, Dong Yu for the UL21a antibody, and Bradley Moore for salinosporamide A. I especially want to thank Jean-Philippe Belzile for valuable advice and other members of the Spector laboratory for helpful discussions. This work was supported by NIH grants R01CA034729 and R21AI083991 to D.H.S. and training grant T32DK007541.

VITA

2006	Bachelor of Science, Purdue University
2006-2007	Laboratory Technician, Purdue University
2007-2015	Doctor of Philosophy, University of California, San Diego

Publications

Clark E, Spector DH. 2015. Studies on the Contribution of Human Cytomegalovirus UL21a and UL97 to Viral Growth and Inactivation of the Anaphase-Promoting Complex/Cyclosome (APC/C) E3 Ubiquitin Ligase Reveal a Unique Cellular Mechanism for Downmodulation of the APC/C Subunits APC1, APC4, and APC5. J. Virol. **89:**6928-6939.

Sharma A**, Clark E,** McGlothlin JD, Mittal SK. 2015. Efficiency of Airborne Sample Analysis Platform (ASAP) bioaerosol sampler for pathogen detection. Front Microbiol. 2015 May 27;6:512. doi: 10.3389/fmicb.2015.00512

ABSTRACT OF THE DISSERTATION

Study of Anaphase Promoting Complex Inactivation by Human Cytomegalovirus Reveals Cellular Mechanism for Downregulating Anaphase Promoting Complex Subunits

by

Elizabeth Clark

Doctor of Philosophy in Biology

University of California, San Diego, 2015

Professor Deborah Spector, Chair

Human Cytomegalovirus (HCMV) is a ubiquitous pathogen causing illness in immunosuppressed individuals and is the top viral cause of birth defects. HCMV deregulates the cell cycle by several means, including inactivation of the Anaphase Promoting Complex/Cyclosome (APC/C) E3 ubiquitin ligase. Viral proteins UL97and UL21a, respectively, affect the APC/C by phosphorylation of APC/C co-activator Cdh1 and by inducing degradation of subunits APC4 and APC5, which with APC1 form the APC/C platform sub-complex. We have further characterized this mechanism, showing the relative contributions of UL21a and UL97 to APC/C substrate accumulation and to viral growth. Further, we demonstrate that UL21a causes the proteasome-dependent degradation of APC1 in addition to APC4 and

5. We see that in uninfected Human Foreskin Fibroblast (HF) cells in G_0, UL21a expression, but not UL97, leads to accumulation of APC/C substrates. We also demonstrate that there is a previously unreported cellular mechanism for a specific decrease in the levels of all three platform subunits, APC1, APC4, and APC5, upon depletion of any one of these subunits or subunit APC8. A UL21a/UL97 double mutant virus and cells that express UL21a, UL97, or both in the presence of Cre recombinase were made in order to propagate the double mutant while avoiding compensatory mutations. The double mutant is severely growth-restricted at the stage of late protein expression, and depleting subunits 5 or 8 from the APC/C can increase titers of the double mutant. When the double mutant infects complementing cells expressing UL21a and/or UL97, UL97 provides a more complete rescue of viral growth than UL21a, and that the presence of UL21a is more beneficial at low multiplicity of infection (MOI) than at high multiplicity. A method for using the Fucci cell cycle indicator system to screen for inhibitors of the APC/C is presented, along with the method for selecting highly-expressing transduced cells, which proves superior to use of a selectable marker only. Finally, the interaction of the APC/C with UL21a and with Cdh1 appears to be mutually exclusive.

CHAPTER 1

INTRODUCTION

Human Cytomegalovirus

Human Cytomegalovirus (HCMV) is a cause of illness, death and neurological birth defects. It infects a high proportion of the population in countries in which data are available. In the US, 50-90% of adults are infected. Unlike acute viruses, HCMV establishes latency for the lifetime of the host with the possibility of multiple reactivations of infection. Most infections are asymptomatic in immunocompetent individuals, but severe disease is observed in vulnerable populations such as those on immunosuppressive drugs and those with HIV. Neonates are also especially at risk. A CMV infection during pregnancy can result in mild to severe birth defects with greater risk when the infection is primary. These manifest as neural developmental defects ranging from hearing loss to calcification of the developing brain and death at a rate of 1-2 per 1000 newborns. HCMV infection is also the leading congenital cause of deafness.

HCMV is a Betaherpesvirus and member of the virus family Herpesviridae. Viruses in this family are distinguished by their species specificity, relatively large size, and ability to achieve latency. HSVI and II and the Varicella Zoster Virus are other illustrative examples of Herpesviridae. The genome of HCMV is double stranded DNA consisting of unique long and short regions separated by inverted repeats. Due to homology of the repeats, inversion of the long and short regions allows reorientation of the long and short segments of the genome in relation to

each other. The genome is enclosed in a protein capsid that is surrounded by a protein tegument layer that includes viral tegument proteins acquired during the previous round of replication. The virus is enveloped and displays surface glycoproteins on the outer membrane.

The viral life cycle

HCMV has a replication cycle of 72-96 hours for one round of replication. The life cycle of the virus can be loosely divided into several sequential stages: immediate-early, early, and late. Upon infection, the virus enters the host cell and tegument proteins counter cellular antiviral defenses and initiate the expression of viral immediate-early genes. Expression of the immediate-early genes IE1-72 and IE2-86 commences. IE2-86 is a necessary transactivator of early viral gene transcription, and early gene synthesis yields proteins required for further host cell environment modulation and viral DNA synthesis. Late gene expression begins after viral DNA replication, followed by virion maturation and release.

Throughout the replication process, cellular pathways are exploited and deregulated by the virus. Cellular metabolism is reprogrammed, innate immunity is inhibited, and the cell cycle is deregulated. HCMV both alters and is affected by the cell cycle beginning very early in infection.

The cell cycle

The cell cycle consists of stages G1, S, G2, and Mitosis and leads to duplication of the chromosomes and division of the cell. Procession through the cell cycle is a tightly controlled process, synthesizing inputs from the energy status of the cell and the integrity of the genome at critical decision points to ensure

faithful duplication of the genome or cell cycle arrest/apoptosis in the event of an error. Progression from one phase to the next depends on the activity and cyclin-dependent kinases, (reviewed in (1, 2) which, through phosphorylation of substrate effector proteins, promote the actions needed for the cell cycle. These kinases pair with cyclins, and their activity is controlled by phosphorylation and by the timed sequential degradation of the partner cyclins by means of the ubiquitin proteasome system. In G1 phase, the cell has received a growth-promoting signal and is preparing for division. Cyclin D levels increase during G1 and pair with cdk4 or 6. Cdk4/Cyclin D phosphorylates the transcription repressor Rb, which is bound to the E2F1 transcription factor. Phosphorylation of Rb releases E2F1, which then induces transcription of factors involved in DNA replication.

For DNA replication to take place, the pre-replication complex (pre-RC) must form on DNA origins of replication prior to S phase. The origin recognition complex (ORC) on the origin of replication of the chromosome is recognized by proteins Cdt1 and Cdc6, and their binding recruits the minichromosome maintenance (MCM) proteins. To prevent further copies from being made from the nascent DNA strands during S phase, the protein Geminin inhibits Cdt1 and is then degraded during mitosis to allow origin licensing for the next round of replication (3-5).

Entry into mitosis is tightly regulated by Cyclin B/cdk1, and separation of sister chromatids in mitosis is prevented by the spindle assembly checkpoint until all chromatids are attached. At this time, the Anaphase Promoting Complex/Cyclpsome (APC/C) E3 ubiquitin ligase targets the protein Securin for

degradation, allowing separation of the sister chromatids. At the end of mitosis, cyclins are degraded, and kept at low levels to prevent unscheduled entry into the next cycle, also by the APC/C.

The ubiquitin-proteasome system

Progression through the cell cycle is controlled in part by the ubiquitin-proteasome system through the appropriately timed degradation of Cyclins to silence the activity of cdks. E1, E2, and E3 enzymes conjugate the small ubiquitin moiety to a lysine residue on substrate proteins, targeting the substrate to the proteasome for degradation. The E1 ubiquitin-activating enzyme forms a covalent linkage with ubiquitin in order to pass it to an E2 ubiquitin carrier, and E2 proteins can interact with their specific E3 ubiquitin ligase. The E3 ligase recruits both the E2 carrying the ubiquitin and the protein substrate, bringing them into proximity and catalyzing the transfer of the ubiquitin to the substrate. After conjugation of the initial ubiquitin, a chain of ubiquitins linked at one of several lysine residues form on the substrate. The specific lysine through which the linkage forms determines the fate of the substrate, and different ubiquitin ligases catalyze different types of linkages. Ubiquitination generally targets the substrate to the proteasome for degradation.

The ubiquitin proteasome system and the cell cycle

Two large multisubunit ubiquitin ligases are responsible for regulation of progression through the cell cycle: the SCF complex (reviewed in (6)) and the APC/C. The SCF Skp2 complex first degrades cdk inhibitors p21 and p27, which allows progression from G1 to S phase, and then cyclin E. The APC/C causes

degradation of Cyclin A, Geminin, and other substrates during G1 and degradation of securin during mitosis to allow separation of the sister chromatids.

The APC/C

The APC/C was discovered in 1995 by the Hershko group as the agent responsible for the proteasome-dependent cyclical degradation of the cyclins (7). The APC/C is a large multi-subunit cullin-ring type ubiquitin ligase that is conserved from yeast to mammals. Twenty years after its characterization, discoveries are still being made about its complex structure and function. The large, complex structure of the APC/C makes it unique among ubiquitin ligases. It is the largest known ubiquitin ligase, and its structure has been studied in detail. Recent publications from the Barford group show cryo-electron microscopy images of the mammalian APC/C, bound or not bound to co-activator Cdh1 (8) and inhibitor Emi1. The APC/C is comprised of APC1, the largest scaffold protein, APC2 and 11, (the catalytic core,) APC3, 6, 7, and 8, (the homodimeric tetratricopeptide repeat (TPR) subunits,) APC10, (necessary for coactivator binding (9)), and APC12, 13, and 15, which are small core proteins. The coactivator proteins Cdh1 or Cdc20 bind to APC3, 8, and 1 and confer activity and substrate specificity to the APC/C. While the TPR subunit area of the APC/C is rigidly static, the catalytic core and adjacent region is mobile and shifts upon binding of Cdh1 (8). The APC/C interacts with two E2-Ubiquitin enzymes for the initial substrate ubiquitination (UbcH5 and UbcH10) and with a processivity E2 (Ube2S) to conjugate additional ubiquitin moieties. These E2s contact the APC/C through APC11, bringing the

ubiquitin in proximity of the substrate. Uniquely, the APC/C forms K11 linked ubiquitin chains (10, 11).

Substrate recognition by the APC/C is multifactorial. The coactivators Cdh1 or Cdc20 as well as APC10 participate in substrate binding. Substrates of the APC/C may possess one or both of two major degrons. APC/C-Cdc20 recognizes substrates containing the D-Box (RXXLXXXXN) (12-14), and APC/C-Cdh1 recognizes those with either a D box or a KEN box (KEN.) (15, 16). Approximately 69% of the proteins in the human genome have one or both of these degrons, and they are not considered sufficient for targeting by the APC/C (17) . A recent publication shows evidence that substrate recognition and ubiquitination that leads to degradation by the proteasome requires multiple rounds of interaction between the substrate and the APC/C and that initial ubiquitination greatly increases the affinity for the substrate, allowing it to outcompete other proteins which possess APC/C degrons(s) but lack a lysine to accept ubiquitination (17) .

Activity of the APC/C oscillates throughout the cell cycle. APC/C-Cdh1 activity in G1 prevents unscheduled entry into the cell cycle by degrading Cyclins (18). During S phase, the APC/C is inhibited to allow accumulation of substrates necessary to promote cell cycle progression. Accumulation of geminin causes inhibition of Ctd1, preventing ORC formation on the nascent strands of DNA during S phase that would lead to multiple rounds of DNA replication and genomic instability. The APC/C is inactivated at the G1/S border by phosphorylation and subsequent dissociation of coactivator Cdh1 (19). The expression of Emi1 (20) also inhibits the APC/C by preventing substrate binding and ubiquitin chain

elongation (21). The APC/C (bound to Cdc20) regains activity at the transition to anaphase when all chromosomes are aligned at the metaphase plate and the spindle assembly checkpoint is relieved.

The initiation of mitosis is controlled by Cyclin B/cdk1, and in metaphase, the protein Securin inhibits the protease that will cleave the bonds holding the sister chromatids together at the metaphase plate. During mitosis, the APC/C, which had been inhibited by the spindle assembly checkpoint, becomes active and bound to the mitotic co-activator, Cdc20. Cyclin B and Securin are degraded, leading to exit from mitosis and the separation of the sister chromatids (22-25).

Recently, it was found that the APC/C is also active in terminally differentiated cells such as neurons (26, 27) and plays a completely different role. APC/C activity was shown to be required for proper differentiation (28). Axonal growth and morphogenesis also appear to be controlled by APC/C (29-32). APC-Cdh1 activity is necessary for the survival of neurons, as it targets metabolic substrates Pfkfb3 and gsl1 in order to prevent high levels of glycolysis and reactive oxygen species, which cause toxicity in these cells. More recently APC-Cdh1 activity has been shown to be involved in synaptic plasticity (33).

HCMV and the cell cycle

HCMV has an intimate relationship with the cell cycle (reviewed in (34). Studies from several groups show that HCMV deregulates normal cell cycle progression and cell cycle protein levels. Furthermore, the state of the cell cycle at the start of infection and the activity of cyclin dependent kinases are critical for normal progression of infection. During the 1990's, several papers from different

groups, including our lab, showed that HCMV-infected cells experience a block in cell cycle progression. The first suggested a G2/M arrest, (35) whereas subsequent studies also showed a G1/S block (36-38). These studies used various lab-adapted strains of HCMV infecting primary fibroblasts of lung or foreskin origin and relied on serum starvation to synchronize cells in G0/G1 phase of the cell cycle. It was next shown that HCMV gene expression was affected by the cell cycle state of the cells at the time of infection. HCMV preferentially infected cells in G0/G1 state, and if infection began in S or G2, IE gene expression would not take place until after a round of mitosis (39). Several HCMV proteins contribute. The immediate early protein IE2 is capable of causing G1 arrest when overexpressed (40), as is the RNA export protein UL69 (41). Activity of the major cell cycle regulating E3 ubiquitin ligase, the APC/C, is inhibited and several of its substrates accumulate. Despite this inhibition, levels of the APC/C substrate cyclin A are reduced.

HCMV and cyclin A

HCMV appears to take great effort to reduce levels of cyclin A in cells, encoding several proteins that sense and lead to its degradation. It was demonstrated that unlike other cyclins that are substrates of the APC/C, cyclin A protein remains low (35, 37). Cyclin A transcript was also strongly downregulated at early times in infection, in contrast to cyclin B, which did not change, and cyclin E, which was strongly upregulated. HCMV uses a mechanism to sense levels of cyclin A in the cell as a decision branch point between productive infection and quiescence (42). The tegument protein pp150 interacts with cyclin A upon viral

entry into the host cell. Pp150 complexed with cyclin A becomes phosphorylated and prevents IE expression. When the cyclin A binding site RXL in pp150 is mutated, the cell cycle state dependent permissivity to HCMV infection is abolished. It was also demonstrated recently that failure of viral protein UL21a to degrade cyclin A is sufficient to release the HCMV-induced S phase block (43) and send host cells into a state of mitotic catastrophe and reduced infection efficiency (44).

HCMV inhibits the APC/C

Consistent with the larger pattern of cell cycle deregulation, HCMV inhibits activity of the APC/C early in infection Our lab demonstrated that APC/C substrates cyclin B (35) and geminin (45) accumulated during infection, but the first report of reduced APC/C ubiquitin ligase activity came from the Hagemeier lab with the publication showing that APC/C that was immunoprecipitated from lysates of infected cells had lost the ability to ubiquitinate Cyclin B1 in vitro between 4 and 8 hours postinfection (hpi) (46). Release of Cdh1 from the complex occurred within the same timeframe postinfection. Our lab showed that Cdh1 is phosphorylated in infected cells (47), and that the APC/C dissociated during infection, with TPR subunits relocalizing to the cytoplasm and APC1 remaining nuclear. Later, it was demonstrated that UL97 was phosphorylating Cdh1, but a UL97 mutant virus was still capable of inhibiting the APC/C (48). It was shown that not only is the APC/C from infected cells lacking the coactivator, its subunits 4 and 5 levels dropped dramatically 6-12 hours post-infection. Using a UL97 null mutant it was shown that UL97 was not required for this event. The down-modulation appeared to be

proteasome dependent, as addition of proteasome inhibitor would halt the decrease. When the reversible proteasome inhibitor MG132 was added to infected cells, APC/C subunit depletion would pause, resuming when the MG132 was washed out. The additional viral factor causing this degradation was unknown but eventually found to be the viral protein UL21a (49).

HCMV protein UL21a

UL21a is a protein of 123 amino acids with no sequence homology to any known mammalian protein. It was first mentioned in a transposon mutation screen (50) as an open reading frame that, when mutated, leads to reduced viral growth efficiency. The initial characterization of this protein by Anthony Fehr in the lab of Dong Yu showed that it is expressed with early kinetics and localizes to the cytoplasm when tagged with GFP (51). A mutant virus lacking the protein shows defects in viral DNA replication and late gene production (52). The function of the protein was revealed to include inhibition of the anaphase promoting complex (49). Data showed that tagged UL21a co-precipitated APC/C subunits, overexpression of UL21a reduced levels of APC/C subunits 4 and 5, and that a UL21a null mutant inhibited the APC/C with greatly reduced efficiency. Further, the Yu group isolated the residues responsible for interacting with the APC/C as amino acids PR at position 109-110.

The first indication of the APC/C-inhibiting function of UL21a came from the identification of APC/C subunits among the binding partners in a pulldown of GFP-UL21a in which binding partners were identified by mass spectrometry. Prior to the release of this information, the reciprocal experiment was being performed in

our lab to identify the viral factor responsible for APC/C inactivation. UL21a was not found to interact with the APC/C in these screens, as UL21a was shown to be very unstable and proteasome inhibitor was not used. When proteasome inhibitor was added, UL21a co-immunoprecipitated with the APC/C in our hands (unpublished).

It has been recently shown that the APC/C is not UL21a's only target. As stated above, HCMV possesses multiple proteins to sense and respond to the inhibitory cyclin A early in infection, and it was demonstrated that UL21a causes the proteasome dependent degradation of cyclin A (43). The responsible domain, amino acids 42-44 (RRL), is separable from the site that targets the APC/C. It was further shown that the UL21a-mediated reduction of cyclin A levels is necessary to prevent a state of genomic instability in HCMV-infected cells (44).

HCMV protein UL97

The other protein implicated in HCMV-mediated inactivation of the APC/C is UL97. UL97 is a multifunctional viral kinase. It has been reported to mimic Cdk1 activity in phosphorylating cellular Lamins to aid viral nuclear egress (53, 54). Early in infection, it phosphorylates the E2F transcriptional repressor Rb (55) on CDK consensus sites (56), allowing transcription from E2F target genes. It has also been shown to phosphorylate HCMV early gene UL44 (57). This protein has clinical relevance as the kinase that phosphorylates the HCMV antiviral drug Gancyclovir (58, 59), and mutations in UL97 that lead to inability to phosphorylate ganciclovir are associated with drug resistance (Reviewed in (60)). Deletion of UL97 result in a severely growth defective virus and can result in

compensatory mutations in protein UL27 (61). Highlighting its importance in inactivating Rb, UL97 deletion growth can be partially restored by complementation with the human papillomavirus 16 inactivator of RB, E7 (62). It was show that in addition to its other targets, UL97 phosphorylates Cdh1 (48), causing its dissociation from the APC/C.

Other viruses and the APC/C

HCMV is not unique in targeting the APC/C, and several other viruses have been shown to alter its activity by various mechanisms (reviewed in (63, 64)). The literature includes several reports showing viral proteins altering the activity of the APC/C, but many of them were intended only to characterize the interaction of the viral protein with the APC/C or the effect of overexpressing that protein on the cell cycle or cell survival. Studies on Chicken Anemia Virus (CAV) protein Apoptin in various transformed cells show that it binds to APC1, the scaffold subunit, and recruits it to PML bodies (65, 66). Expression of Apoptin is sufficient to induce accumulation of APC/C substrates cyclin B1 and Plk. The focus of the work was on the effect of Apoptin on cell survival and cell cycle, so experiments were not done to determine the effect of disrupting this interaction on the CAV viral life cycle. Several members of the poxvirus family have been found to express a poxvirus anaphase promoting complex regulator (PACR) that mimics APC11 but lacks ubiquitin ligase activity (67, 68). The protein PACR contains sequence homology to APC11 and binds to APC2, as shown by cotransfection of tagged constructs into transformed 293 cells. While PACR associates with the APC/C, it does not promote ubiquitin ligase activity, thereby acting as a competitive mimic. Deletion

of this protein from a wildtype Orf poxvirus results in reduced replication efficiency. However no experiment was performed to show rescue of the PACR null mutant with some exogenous APC/C inactivation method. To date, the only virus found to induce activity of the APC/C is Human T cell Lymphotropic virus (HTLV-1). It activates the APC through the versatile viral transcription transactivator protein Tax, inducing G1 arrest (69-71).

Goal of the Ph.D. Thesis Research

The APC/C is a major regulator of the cell cycle that is targeted by HCMV and several other viruses by various mechanisms. The overall goal of this work is to further understand how and why HCMV inactivates the APC/C. We first developed a method for screening viral genes for ability to inactivate the APC/C when overexpressed in HF cells. This method addressed the challenges of overexpressing genes in a hard-to-transfect cell type and the potential for false positives caused by natural APC/C inactivation that happens during the cell cycle.

HCMV is the only virus known to have two proteins that inactivate the APC/C. A major aim was to elucidate the relative requirements for these proteins and their relative ability to induce accumulation of APC/C substrates during infection. To achieve this aim, we characterized the behavior of a mutant virus lacking both proteins involved with APC/C inactivation and the activities of each protein when expressed alone or together in confluence-synchronized cells. We showed that inactivation of the APC/C during this mutant virus infection increased viral output in a single cycle of infection, showing the previously underappreciated importance of APC/C inactivation. We also understand in more detail the physical interaction between HCMV protein UL21a and the APC/C, having demonstrated that the APC/C cannot bind to both UL21a and to its co-activator, Cdh1, at the same time.

During the course of this work, we uncovered a novel cellular mechanism by which specific APC/C subunits are down-modulated. The final aim of this study was to explore this new concept in cell biology. We aimed to characterize which

subunits were involved in this mechanism. We reveal that any disruption to the bridge subcomplex (APC1, 4, 5, and 8) causes the swift down-modulation of APC1, 4, and 5. Perturbing the TPR subunits APC3 or 6 did not result in this reduction of levels of other subunits, and it appears that these TPR subunits are also immune to the effects of disrupting the bridge subcomplex.

CHAPTER 2

UL21a causes fluorescence accumulation in FUCCI-derived system for screening for APC/C-inactivating agents in primary fibroblasts.

Introduction

The aim of this study was identification of the viral factor or factors that caused degradation of APC4 and 5, and the work was initiated prior to identification of UL21a. We sought to optimize a method for screening overexpressed HCMV open reading frames for ability to inhibit the APC/C. A construct encoding a red fluorescent mKO2-tagged-Geminin fragment had been described and used in concert with a GFP Cdt1 to visualize progression through the cell cycle (72). These constructs are now commercially available as the Fucci system. Geminin is a target of the APC/C-Cdh1, and both entry into S phase and HCMV infection induce accumulation of Geminin, making it useful as an indicator of APC/C inactivation. This chapter describes our adaptation of the geminin half of the Fucci system for this purpose in primary fibroblasts and shows that UL21a induces the expected mKO2 fluorescence accumulation.

Materials and Methods

Constructs

pLV-EF1alpha-TK-Hygro-mKO2-Geminin was created by introducing the first 110 amino acids of human Geminin downstream of mKO2. This fragment was ligated into the vector pLV-EF1alpha-TK-Hygro. pLV-UL21a-IRES-EGFP was created by ligating the UL21a open reading frame amplified from HB5 BAC into unique SalI and BamHI sites of pLV-IRES-EGFP. All cloning was confirmed by sequencing. Lentiviral particles were generated as described in Chapter 3. Lentiviral IRES-EGFP and UL21a-IRES-EGFP titers (infectious units/mL) were determined by infection of 293FT cells with serial dilutions of lentiviral stock and counting of EGFP positive cells 48 hpi by flow cytometry.

Cell culture and lentiviral transductions to establish mKO2-Geminin cell line was performed as in Chapter 3.

Overexpression of UL21a

HF cells were infected 24 hours after reseeding with 10 infectious units/cell of lentiviruses encoding UL21a-IRES-EGFP or just IRES-EGFP. At 48 hpi, cells were harvested for analysis of EGFP and mKO2 fluorescence by flow cytometry.

Flow Cytometry

Cells were harvested by trypsinization and fixed for 15 minutes in 1% Formaldehyde in DPBS. Cells were analyzed by on BD FACS Canto II (UCSD VA AIDS Research Center). Side and forward scatter were used to ignore debris and doublets. Cells were scored for GFP and mKO2 positivity.

Live cell sorting

One day after passaging, cells were treated with 2.5uM MG132 for 6 hours prior to sorting. Cells were trypsinized, washed with PBS and resuspended in HF media with 2% FBS at a concentration of 10 million cells/mL. mKO2-positive cells were collected on a BD FACSAria II. Sorted cells were cultured in complete medium without selection.

Immunofluorescence

Cells are plated on slips in 24-well plates. At indicated times after treatment, slips are fixed for 10 minutes at room temperature with 3.7% Formaldehyde. Antibody staining for immunofluorescence and image acquisition is described in Chapter 3.

Results

Selection of Strongly-responding cells

Initial attempts at using mKO2-geminin cells for screening of HCMV cDNAs were complicated by both high background and heterogeneity of mKO2-Geminin levels in the hygromycin resistant cell population. Figure 2-1A shows enrichment of cells expressing high levels of mKO2-geminin after sorting cells that had been treated with proteasome inhibitor to allow mKO2-geminin accumulation. Confluent cells that had been transduced with mKO2-geminin previously were treated with 2.5uM MG132, a reversible proteasome inhibitor, for 6 hours. Cells were then trypsinized, and sorted by FACS. The mKO2-positive population was collected and further cultured without hygromycin. The collected population showed more homogeneous mKO2 expression, as nearly all cells responded to further proteasome inhibition by the irreversible proteasome inhibitor SalA while background in the untreated cells remained low at confluence (Figure 2-1B).

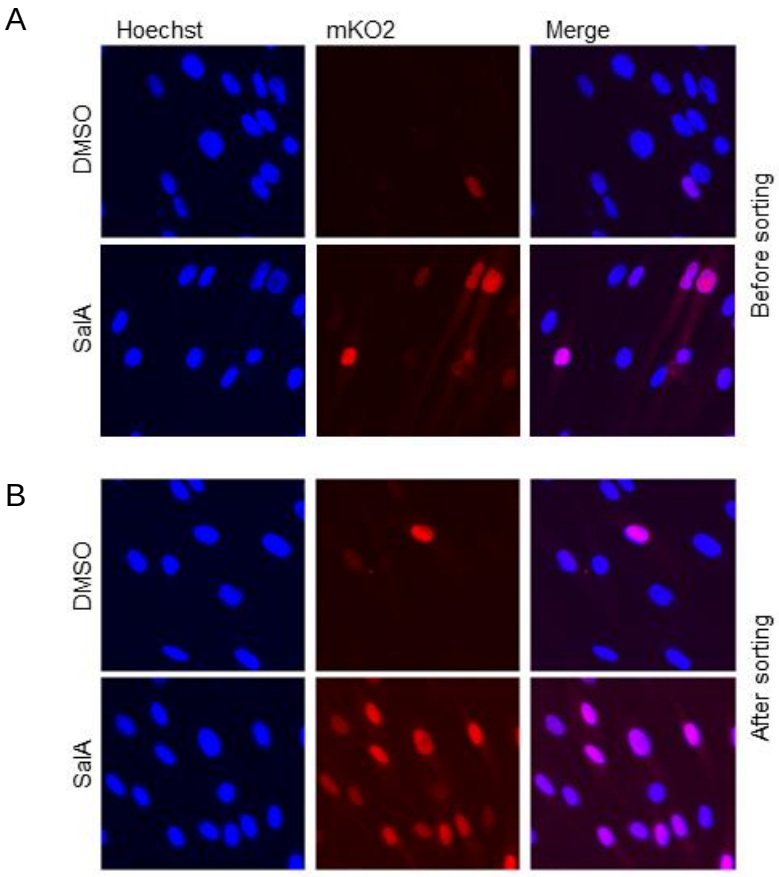

Figure 2-1. Comparison of responsiveness of mKO2-Geminin cells to proteasome inhibition before and after selection of high-responding cells.
mKO2-Geminin cells at 3 days post-confluence before (A) and after (B) selection of high-responding cells. Cells were treated with 100nM Salinosporamide A or DMSO for 12 hours. Slips were harvested and counterstained with Hoechst to visualize nuclei.

Indicator cells fluoresce when infected with HCMV.

Response of the purified population to HCMV infection was characterized. mKO2-Geminin HFs were allowed to grow to confluence and were maintained for three days to establish G1 synchronization. Cells were infected at an MOI of 0.1 without re-seeding and maintained in complete medium. 48 hours after infection, slips were harvested and processed for fluorescent microscopy. Figure 2-2 shows that almost all cells expressing viral proteins accumulate high levels of mKO2-geminin. Very few uninfected cells showed the mKO2 signal.

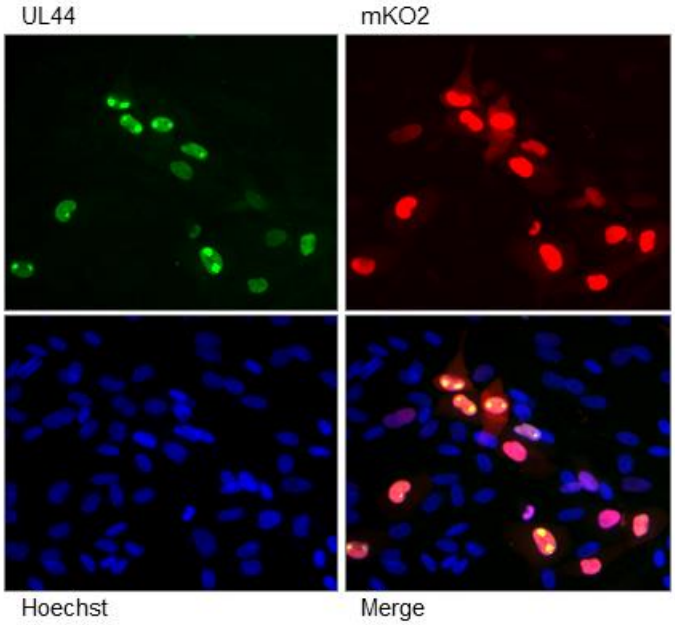

Figure 2-2. HCMV infection induces accumulation of mKO2-Geminin fluorescence. mKO2-Geminin cells were infected 3 days post-confluence at MOI of 0.1. Slips were harvested and stained with UL44 (48hpi). Nuclei were counterstained with Hoechst.

Indicator cells fluoresce when UL21a is overexpressed.

During the course of this thesis the HCMV protein UL21a was discovered to be responsible for the APC/C subunit degradation phenotype. We wished to test the gene in our indicator cells to show proof-of-concept of our adaptation of this technology. Transfection efficiencies at confluence in HF cells proved to be low (15-20%) when quantified by co-transfection with EGFP (unpublished) so we chose to express UL21a upstream of IRES-EGFP via lentiviral transduction. One day after reseeding, cells were transduced with 10 infectious units/cell of LV-EF1alpha-UL21a-IRES-EGFP or LV-EF1alpha-IRES-EGFP. 48 hours later, cells were harvested and processed for flow cytometry. Positive and negative controls included indicator cells that were treated with Salinosporamide A (an irreversible proteasome inhibitor), and HF cells lacking the mKO2 construct. Cell analysis was as follows: Forward scatter (FSC) vs side scatter (SSC) plots were used to gate out doublets and debris (Figure 2-3, bottom row). This chosen population of cells was analyzed for EGFP expression (Figure 2-3, middle row, histogram of GFP signal) and for a comparison of GFP to mKO2 signal (Figure 2-3, top row, mKO2 vs GFP). Figure 2-3A, B, and D (middle row) show positive GFP signal indicative of EGFP expression in the transduced cells compared to C and E, which received no transduction. Panel B shows that there is very low mKO2 background in these cells, and panel C demonstrates that mKO2-geminin accumulation can be induced by proteasome inhibition. The comparison of panels A and B shows that UL21a expression specifically increases mKO2 fluorescence. This signal in panel A appears to increase proportionally to the GFP signal, indicating greater

accumulation of mKO2-geminin in cells expressing higher levels of the UL21a-EGFP construct in cells but not the vector expressing only IRES-EGFP.

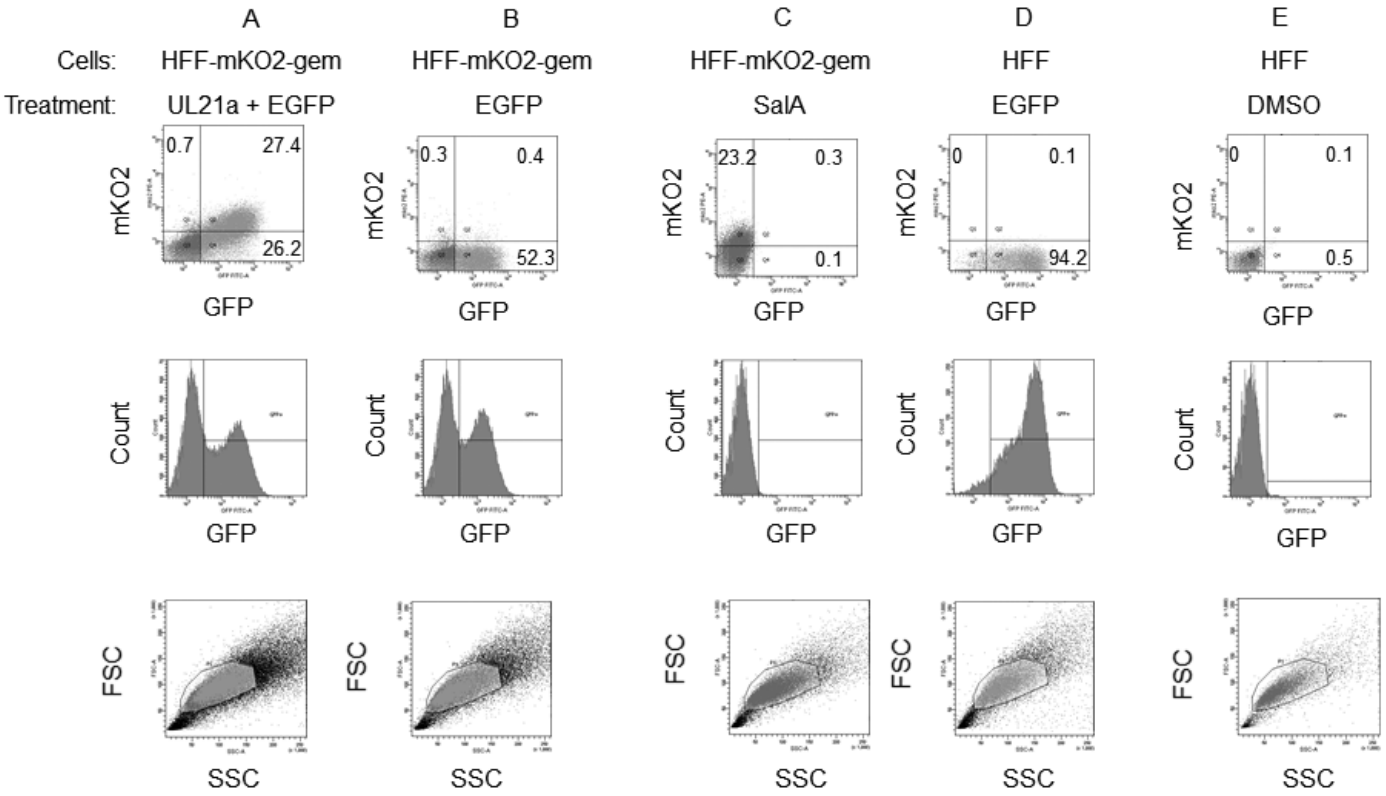

Figure 2-3. UL21a overexpression induced mKO2-Geminin fluorescence accumulation.

HF and HF-mKO2-Geminin cells were reseeded from confluence. 24 hours later, cells were treated as indicated: The indicated cells were transduced with 10 infectious units/cell of lentivirus encoding (A) UL21a-IRES-EGFP (UL21a + EGFP) or (B, D) IRES-EGFP (EGFP), washed and incubated 48 hours. 12 hours before harvest, cells in (C) and (E), which did not receive a transduction, were treated with (C) 100nM Salinosporamide A (SalA) or (E) DMSO. All cells were harvested and prepared for flow cytometry. Live single cells were chosen via Forward Scatter vs Side Scatter (Lower row). Histograms of GFP signal (middle row) show transduction efficiency, and top row shows plot of EGFP expression and mKO2 accumulation.

Discussion

We have presented an adaptation of the Fucci technology to the goal of identification of genes that inactivate the APC/C. This required using a single construct of the two construct system, purifying the strongly-fluorescent population of mKO2-geminin cells after proteasome inhibition and overexpressing the candidate gene via high-titer lentiviral transduction. We showed proof-of-concept for the application of the technology, unique from the original use of monitoring the cell cycle, as a tool for assaying APC/C inactivation by testing the confirmed APC/C inactivator HCMV UL21a. During the generation of these data, the construct has become commercially available and has been used to evaluate various Herpesvirus proteins for ability to cause a G1/S arrest (73). Interestingly, this study by Paladino et al in which many HCMV genes were screened for promotion of G1/S arrest did not identify UL21a. This concurs with the study instead that UL21a will induce G2/M arrest when overexpressed in cells (49).

While convenient, use of mKO2 geminin as an APC/C activity indicator has several limitations. First, it is subject to false positives, as anything causing arrest in S, G2, or mitosis prior to anaphase will result in a naturally inactive APC/C and accumulation of the substrate. This is a necessary trade off to avoid false negatives caused by focusing on proteins that cause arrest in a specific stage of the cell cycle. Second, disruption of the ubiquitin-proteasome system downstream of the ubiquitination event will result in accumulation of fluorescence, even in the presence of an active APC/C. Therefore, use of the construct to screen for ubiquitin ligase activity of the APC/C must be followed by a more direct assay.

Third, use of this construct in cells that are not synchronized in G1 will result in a higher level of background. During optimization, non-synchronized HFs were used to screen select HCMV genes (UL27, UL44, UL56, UL69, UL82, UL84, UL97, UL97 + UL69, UL105, and UL112-113), but the low transfection efficiency of the cells combined with the requirement that they be transfected at low confluence resulted in difficult-to-interpret data (Unpublished). Overcoming either the low transfection efficiency problem or the cell cycle problem is required, and our best data was gathered under conditions of G0/G1 and overexpression via high-titer lentiviral transduction.

The overexpression of UL21a specifically induced the predicted effect in the mKO2-geminin cells, demonstrating a proof of concept that these cells act as indicators of APC/C inactivation, and use of this system is not limited to genes. When used upstream of a more direct assay for APC/C activity, this method is a fast and high throughput method for screening compounds or other pathogens for ability to inactivate the APC/C. Both of these uses would likely be an easier process not requiring the additional transduction step needed for overexpression of a gene.

CHAPTER 3

Studies on the Contribution of Human Cytomegalovirus UL21a and UL97 to Viral Growth and Inactivation of the Anaphase Promoting Complex/Cyclosome (APC/C) E3 Ubiquitin Ligase Reveal a Unique Cellular Mechanism for Down-modulation of the APC/C Subunits APC1, APC4, and APC5.

Abstract

Human Cytomegalovirus (HCMV) deregulates the cell cycle by several means, including inactivation of the Anaphase Promoting Complex/Cyclosome (APC/C) E3 ubiquitin ligase. Viral proteins UL97 and UL21a, respectively, affect the APC/C by phosphorylation of APC/C co-activator Cdh1 and by inducing degradation of subunits APC4 and APC5, which with APC1 form the APC/C platform subcomplex. The aim of this study was to further characterize the mechanism of APC/C inactivation and define the relative contributions of UL21a and UL97 to APC/C substrate accumulation and to viral growth. We show that in uninfected cells UL21a, but not UL97, can disrupt APC/C function, leading to accumulation of substrates. We find that UL21a is necessary and sufficient to induce degradation of APC1, in addition to the previously reported APC4 and APC5. We also demonstrate that there is a previously unreported cellular mechanism for a specific decrease in the levels of all three platform subunits, APC1, APC4, and APC5, upon depletion of any one of these subunits or subunit APC8. Finally, we show that at low MOI either UL97 or UL21a can partially complement a growth-defective mutant virus lacking both UL21a and UL97, with

significantly greater benefit afforded by expression of both proteins. This double mutant also can be partially rescued by inactivation of the APC/C using siRNA against specific subunits. These results further our understanding of HCMV's interaction with the cell cycle machinery and reveal a new cellular pattern of APC/C subunit down-modulation.

Importance

HCMV lytic infection subverts the host cell cycle machinery in multiple ways. A major effect is inactivation of the APC/C, which plays a central role in control of cell cycle progression. This study provides further insight into the mechanism of inactivation. We discovered that the APC1 subunit, which along with APC4 and APC5 form the platform subcomplex of the APC/C, is an additional target of the degradation induced by HCMV protein UL21a. This study also shows for the first time that there is a unique cellular process in uninfected cells whereby depletion of APC1, APC4, APC5, or APC8 recapitulates the pattern of HCMV-mediated APC/C subunit degradation.

Introduction

Human Cytomegalovirus (HCMV) infects the majority of the human population, causing significant morbidity and mortality in immunocompromised individuals such as transplant patients and those with HIV. HCMV is also the leading viral cause of birth defects. These manifest as neural developmental defects ranging from hearing loss to calcification of the developing brain and death at a rate of 1-2 per 1000 newborns.

HCMV lytic infection both modulates and is influenced by the host cell cycle. The virus preferentially infects cells in G_0 or G_1. Infection in other phases of the cell cycle results in a delay of immediate-early gene expression until completion of mitosis in the case of a G_2 infection. Infection during S phase remains unproductive in a certain percentage of cells. Early in infection, the virus causes a stimulation of resting cells into the cell cycle and subsequent arrest at the G_1/S border (36-38). The infection inhibits host DNA replication, affects cyclin levels (35), prevents host DNA replication licensing (45, 74, 75), and inhibits the Anaphase Promoting Complex/Cyclosome (APC/C) (46-48).

The APC/C is a large multi-subunit E3 ubiquitin ligase that targets a growing list of proteins for degradation by the proteasome. The APC/C orchestrates progression through the cell cycle by targeting the cyclins and other cell cycle associated proteins for degradation to allow cells to proceed though cell cycle checkpoints. Its own activity is cyclical, showing activity in G_1 and at anaphase and inhibition from S-phase until the chromosomes are properly aligned in metaphase and the spindle assembly checkpoint is released. The APC/C also plays an

important role in non-cycling cells and is required to maintain low levels of cyclins to prevent unscheduled entry into the cell cycle. In post-mitotic neurons, the APC/C is required for proper axon growth and morphogenesis (30, 76), for neural cell survival (77) and for maintenance of low levels of PFKFB3, a regulator of the rate of glycolysis whose accumulation can lead to exitotoxicity in neurons (78).

A 3D reconstruction at a resolution of 7.4 Å has recently been determined for a ternary complex of recombinant human APC/C with the coactivator Cdh1 and a high affinity substrate, Hsl1 (8). It consists of three major subcomplexes: the tetratricopeptide repeat (TPR) subcomplex (subunits 3, 6, 7, and 8) that interacts with APC/C co-activators Cdh1 or Cdc20 to mediate substrate specificity, the catalytic E3 subcomplex (subunits 2 and 11), and the base or platform subcomplex (subunits APC1, APC4, and APC5) that attaches the TPR subunits to the catalytic core. Other subunits are: APC15, which bridges APC5 and APC8 and is required for APC/C-bound mitotic checkpoint complex-dependent Cdc20 autoubiquitylation and degradation (79), the TPR accessory subunits APC12, APC13, and APC16, and APC10, which assists the regulatory subunits in substrate recognition. The interaction of TPR subunit APC8 with the platform subcomplex of APC1, APC4, and APC5 was found to require all of these subunits to maintain the structure of the subcomplex (80).

The APC is targeted by several viruses, (see ref. (63) for review), but all appear to do so by different mechanisms. We and others have found that upon HCMV infection, the APC4 and APC5 subunits are degraded, the TPR subunits dissociate from the catalytic core and Cdh1, Cdh1 is hyperphosphorylated, and

APC/C substrates accumulate (45-47). We also demonstrated that the viral kinase UL97 is capable of phosphorylating the APC/C co-activator Cdh1 on several sites, but in the context of the infection, UL97 is not required for APC/C inactivation and disassembly (48). It has since been reported that the HCMV protein responsible for degradation of APC4 and APC5 is UL21a, a small unstable protein expressed with early kinetics (49, 51, 52). Other viruses inactivate the APC/C by a variety of mechanisms, including competitive mimicry of APC11 by Orf virus PACR (67, 68) and binding of APC/C co-activators by HPV E2 protein (81).

In this study, we aim to further elucidate the mechanism by which the APC/C is inactivated and the relative contributions of UL21a and UL97 to APC/C inactivation and to viral growth, as both proteins have multiple functions during the infection. We show that in the absence of other viral proteins UL21a, but not UL97, can disrupt APC/C function and cause accumulation of substrates. Our findings demonstrate that UL21a expression alone leads to degradation of APC1 as well as APC4 and APC5. Our investigation into this viral process has also led us to a novel finding regarding APC/C subunit interdependency that points to a possible mechanism of UL21a-mediated APC/C subunit degradation. We show that destruction of APC1, APC4, and APC5 can be mediated by a cellular process in the absence of infection when either APC1, APC4, APC5, or APC8 is depleted by siRNA. We finally demonstrate that exogenous inhibition of APC/C activity via siRNA against APC5 or APC8 is able to partially rescue a mutant virus lacking both UL21a and UL97.

Materials and Methods

Cells culture and infection with HCMV

Human foreskin fibroblasts (HFs) were cultured in HF growth media (minimal essential medium (Gibco) supplemented with 10% FBS, 1.5 μg/ml amphotericin B, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin). Cells were incubated at 37°C with 7% CO2. To prepare for infections, cells were allowed to come to confluence and maintained for three days prior to infection to allow exit from the cell cycle. Virus was diluted in HF growth media at the indicated multiplicity of infection (MOI) and added to cells. After 6 h, virus was removed, cells were washed with PBS, and fresh HF growth media was added. For siRNA experiments, infection took place 96 h after siRNA transfection. For proteasome inhibition experiment, cells were treated with Cre and 100nM salinosporamide A (SalA; gift from Bradley Moore, Scripps Institute of Oceanography, University of California, San Diego) or vehicle and harvested 18 h later.

Lentiviral constructs

The UL21a and UL97 open reading frames were amplified from the pHB5 BAC and inserted downstream of the second loxp sequence in pLV-EF1α-loxp-Neo-loxp and pLV-EF1α-loxp-Puro-loxp respectively (82). To accomplish this, UL21a was amplified using forward and reverse primers 5'-GATCGAGTCGACATGGGAGGTAGCCCTGTTCC-3' and 5'-GATCGGATCCTTAAAACTGGTCCCAATGTTCTTC-3', placing a SalI site at the 5' end. The amplicon was digested with SalI and then phosphorylated and ligated

into the pLV-EF1α-loxp-Neo-loxp vector, which had been digested with unique SalI and SmaI sites. UL97 was amplified using forward and reverse primers 5'-GGGATCCACCGGCCCACTATGTCCTCCGCACTTCGGT-3' and 5-CTTTACTTGTACCCCGCCTTTCCCCTCAGCAACCG-3' and inserted into the SmaI-linearized pLV-EF1α-loxp-Puro-loxp backbone using the InFusion ligation-independent cloning kit (CloneTech).

Complementing cell lines

Complementing cell lines were created by transducing HFs passage 12-15 with lentivirus expressing the following bicistronic constructs. HF-21a received pLV-EF1α-loxp-Neo-loxp-UL21a. HF-97 received pLV-EF1α-loxp-Puro-loxp-UL97. HF-21a/97 received first pLV-EF1α-loxp-Neo-loxp-UL21a followed by pLV-EF1α-loxp-Puro-loxp-UL97 several days later. Transductions were separated to avoid lentiviral recombination. Lentiviral particles were made by transfecting the lentiviral constructs into 293FT cells with ViraPower lentiviral packaging mix (Life Technologies) and transductions were performed as previously described (82). HF-97 cells were selected with 1 μg/mL Puromycin, HF-21a cells were selected with 400ug/mL G418, and HF-21a/97s were selected with both.

Construction of BAC recombinants

pHB5-IE-Cre-1.2-flp has been described (82) and was used as the starting BAC for Δ21a and Δ21a/97. To construct deletions, the UL21a open reading frame was replaced by AmpR, and the UL97 ORF was replaced by TetR. AmpR and promoter sequence were amplified from pcDNA3 using the following primer sequences: 5'-AGCCATGCAGCGTGCGGC

GCCTCTCTCATGGATCCACTGTCACCGTCGCGGTGCGCGGAACCCCTATTT G-3’ and 5’-ATACAGACTTTTATATGATCCTTGTACAGATGTAAATAAAATGTTTTTATGGG GTCTGACGCTCAGTGG-3’. TetR was amplified from the pACYC184 vector using the following primer sequences:

5’-GTCGGTGTGGTAGCTAGTGCAGCCTTAGGAACAGGGAAGACTGTCGCCAC GCCCCATACGATA TAAGTTG-3’ and 5’-GTACCTTCTCTGTTGCCTTTCCCCTCAGCAACCGTC ACGTTCCGCGTCCCGGACAGGAGCACGATCATG-3’. Recombination of the insert into the BAC was carried out in SW105 via heat-inducible recombinase. Clones were selected and confirmed by restriction endonuclease digestion and FIGE (Fig 3-5B) and by PCR. To grow deletion viruses, complementing cell lines were replated at low density. One day later, they were harvested and electroporated with the BAC along pcDNA3-pp71 as described previously (82). Viral supernatants were harvested periodically and titered on the HF-21a/97 complementing cell lines.

Preparation of Cell-Permeable Cre

Cell-permeable Cre recombinase was prepared as described in (83). Briefly, His-Tat-NLS-Cre protein was purified on NT-Nickel Superflow resin and dialyzed against HEPES-buffered saline and HEPES-buffered glycerol, both tissue culture-grade. Amount of Cre required to induce recombination in nearly all cells

was determined by treating HF-97's with dilutions of Cre, followed by anti-UL97 immunofluorescence.

Depletion of APC/C subunits

Cells were seeded at low density in antibiotic-free growth medium. The next day, depletion of a specific APC/C subunit was performed with 75 pmol siGENOME Smartpool siRNA (Dharmacon/Thermo Fisher) per well in 6-well dishes using Lipofectamine RNAiMax (Life Technologies). Transfection mix was removed 4-6 h after transfection and HF growth media was added. Oligos targeted the following sequences.

APC1: ggacaagugugcacaauug, gguuacaauccacgagaaa, gagugguucgggugggaaa, ggcauuggcaguuuauaaa.

APC3: caugcaagcugaaagaaua, caacacaaguaccuaauca, ggagauggauccuauuuac, ggaaauagccgagagguaa.

APC4: ggacauagaugaugaaugg, caacacagcuggcgagguu, gcucaaaucuucgucaugu, gaggaugaaucaaaucuuc.

APC5: gaaggcgaguugaaggaua, ugucagagcucaucgauau, uaggaaagcgguuguauua, gaacaggccuuaagucuuc.

APC6: guagauggcuugcaagaga, Gcuacaagcuuacuucugu, uggaagagcccaucaauaa, cuauggaccugcauggaua.

APC8: gcaguugccuaucacaaua, ugaaacaguugauagcuua, guagaaacgugcuguguaa, gaaauuaaauccucgguau.

Non-Targeting Pool #1: uagcgacuaaacacaucaa, uaaggcuaugaagagauac, aguauuggccuguauuag, augaacgugaauugcucaa.

Western Blot

Cells were harvested by trypsinization, washed with PBS and pelleted. Pellets were lysed in Laemmli buffer with protease inhibitor cocktail (Sigma) and Halt Phosphatase inhibitor cocktail (Thermo Scientific). Lysates were sonicated to shear DNA and equal cell equivalents were run on Tris-Glycine SDS gels and transferred to nitrocellulose. Membranes were probed with the following antibodies: Rb anti-Geminin (Clone FL-209, Santa Cruz Biotechnology), Ms anti-Cdc6 (Neomarkers), Ms anti-Cdh1 (Calbiochem), Ms anti-Tubulin (Sigma), Rb anti-APC1 (A301-653A; Bethyl), Rb anti-APC3 (Thermo Scientific), Rb anti-APC4 (A301-176A; Bethyl), Rb anti-APC5 (A301-026A; Bethyl), Rb anti-APC6 (A301-165A; Bethyl), Rb anti-APC8 (A301-181A Bethyl) and Rb anti-Cyclin A (Clone H-432, Santa Cruz Biotechnology). Antibodies to HCMV proteins included Ms anti-IE2 (Chemicon), Ms anti-IE1/2 (CH160; Virusys), Ms anti-pp28 (Virusys), Ms anti UL44 (Virusys), Rb anti-GST-UL97 (a kind gift from Don Coen), Rb anti-UL21a (A kind gift from Dong Yu), Ms anti-UL57 (Virusys), and Ms anti-pp65 (Virusys).

Determination of Viral Titers

Infected cell supernatants were centrifuged to remove cell debris, mixed with 1% DMSO and stored at -80ºC. Plaque assay was performed using the HF-21a/97 complementing cells to efficiently titer the UL21a/UL97 double mutant.

Immunofluorescence

Cells grown on coverslips were fixed for 10 min at room temperature with 3.7% formaldehyde in PBS. Cells were permeablized for 5 min with 0.2% Triton-X

in PBS, washed twice, and blocked with 10% Normal Goat Serum in PBS. Primary antibodies against UL21a or UL97 were diluted in 5% Normal Goat Serum and incubated with slips for 25 min at room temperature. After washing, slips were incubated in Alexafluor (Life Technologies) secondary antibodies and nuclei were stained with Hoechst in 5% Normal Goat Serum and mounted on slides with Prolong Gold Antifade (Life Technologies). Images were acquired on an epifluorescence microscope.

Real-time RT-PCR

RNA was isolated from pellets using the DNA/RNA/Protein extraction kit (Norgen) according to manufacturer's instructions. Reverse transcription was performed on 1 µg of RNA per sample using Thermoscript reverse transcriptase (Life Technologies). APC1 (5'-tcatggctggctcaggaaacctaa-3' and 5'-acagagaagagcggcaatggaaga-3' and Tubulin (sequence described previously) (84) were amplified using SYBR Green (Life Technologies). APC5 (sequence described previously (48) was amplified with Taqman polymerase (Life Technologies). Real-time PCR was performed on an ABI Prism 7000 (Life Technologies) and relative quantifications were determined by comparison to standard curve for each cDNA. APC1 and APC5 transcript levels were normalized to Tubulin level.

Results

Creation of cell-permeable Cre-inducible fibroblast cell lines expressing UL21a, UL97 alone or together.

In order to study the individual and combined contributions of UL97 and UL21a on APC/C function both in the absence of other viral proteins and in the context of infection with a mutant lacking both genes, we used a Cre-recombination-based system to conditionally express UL21a and UL97 in HF cells (Fig 3-1A). HFs were transduced with bicistronic lentiviruses encoding a selectable marker flanked by loxp sites upstream of UL21a or UL97. Translation of this constitutively produced transcript yields the selectable marker gene product. In the presence of Cre recombinase, the selectable marker is recombined out, and translation of the viral protein commences. This allows expression of each viral protein, alone or in combination, upon treatment with cell-permeable Cre (His-Tat-NLS-Cre) (83) or infection with the Cre-expressing WT or mutant HCMV. These cells are referred to as HF-21a, HF-97, and HF-21a/97. Figure 3-1B shows that treatment of HF-21a and HF-97 cells with cell-permeable Cre results in expression of the corresponding viral proteins in a high percentage of the cells.

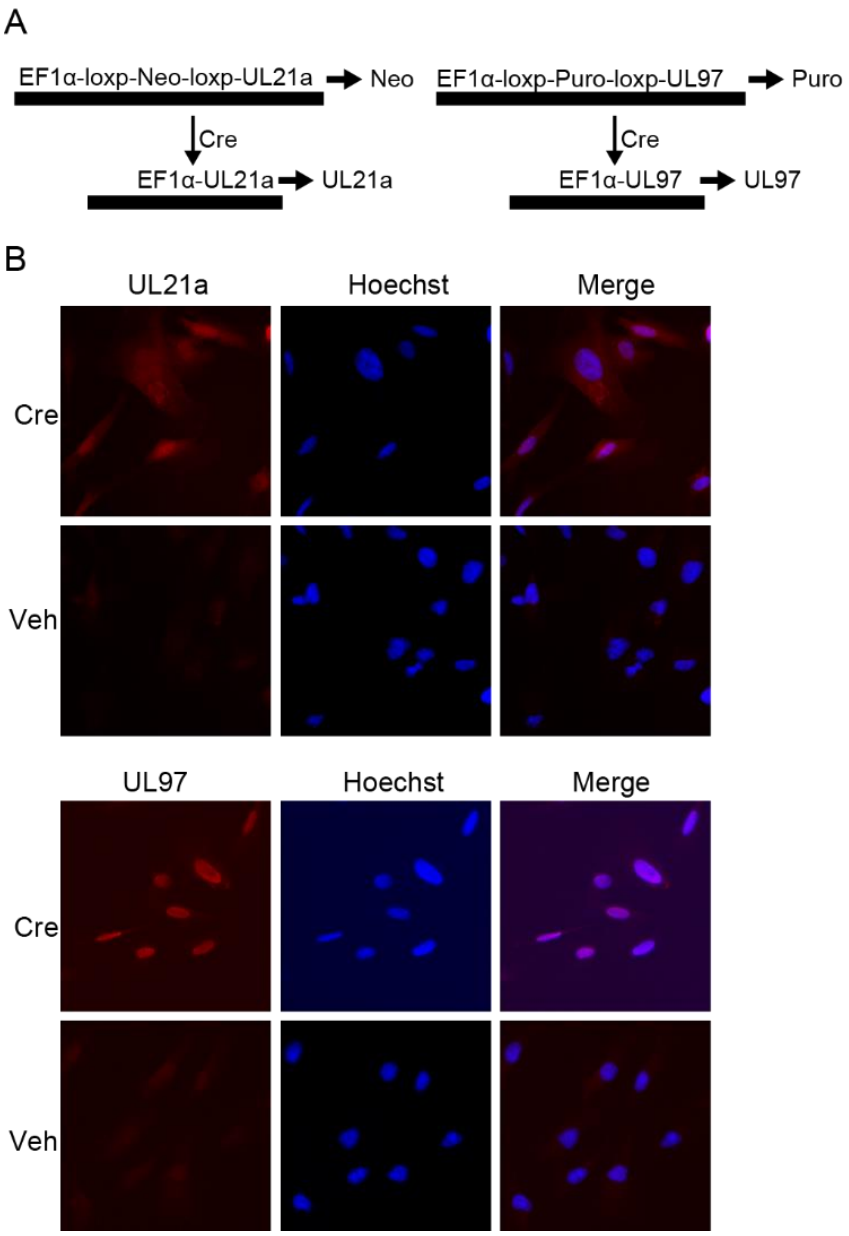

Figure 3-1. Creation of UL21a and UL97-expressing fibroblasts.
(A) EF1α promoter drives constitutive expression of a loxp-flanked selectable marker (Neomycin for UL21a and Puromycin for UL97.) Cre recombinase induces expression of viral proteins via removal of selection marker. (B) Cell-permeable cre induces expression of UL21a and UL97. Complementing cell lines plated on slips were treated with Cre and harvested 72 h later. Slips were stained with UL97 and UL21a antibodies and counterstained with Hoechst to visualize nuclei.

UL21a but not UL97 is sufficient to promote APC/C substrate accumulation in the absence of infection.

To study the effects of UL97 and UL21a, alone or together, on the APC/C and substrate accumulation, we added cell-permeable Cre to HF-21a, HF-97, and HF-21a/97 after they were confluence-synchronized in G_0/G_1. Cell-permeable Cre was also added to control confluence-synchronized HFs that did not express either protein. After 12 h of treatment, cells were washed twice with PBS and growth media was added. Cells were harvested 72 h after addition of Cre and processed for Western blot. For comparison, we infected HF with WT HCMV. We noted that Cre-induction of the viral proteins in these cells, as well as infection with WT virus, results in a mobility shift of Cdh1 in cells expressing UL97, consistent with its phosphorylation by this viral kinase (Fig 3-2). Upon expression of UL21a, APC/C substrates Geminin and Cdc6 accumulated. This is consistent with previous reports (51) that UL21a causes substrate accumulation. However, UL97 was not sufficient to induce this accumulation. It appears that UL97 alone, despite its ability to phosphorylate Cdh1, is not sufficient to induce APC/C substrate accumulation in confluence-synchronized HF's. Additionally, there was no synergistic effect on Geminin or Cdc6 levels from expression of both proteins. We also show that the extent of APC/C substrate accumulation corresponds with the level of UL21a expressed. In a HF-21a/97 cell line expressing lower levels of UL21a (Fig 3-2 left panel), the accumulation of substrates was less than in a second line of HF-21a/97 cells expressing more UL21a (Fig 3-2 right panel).

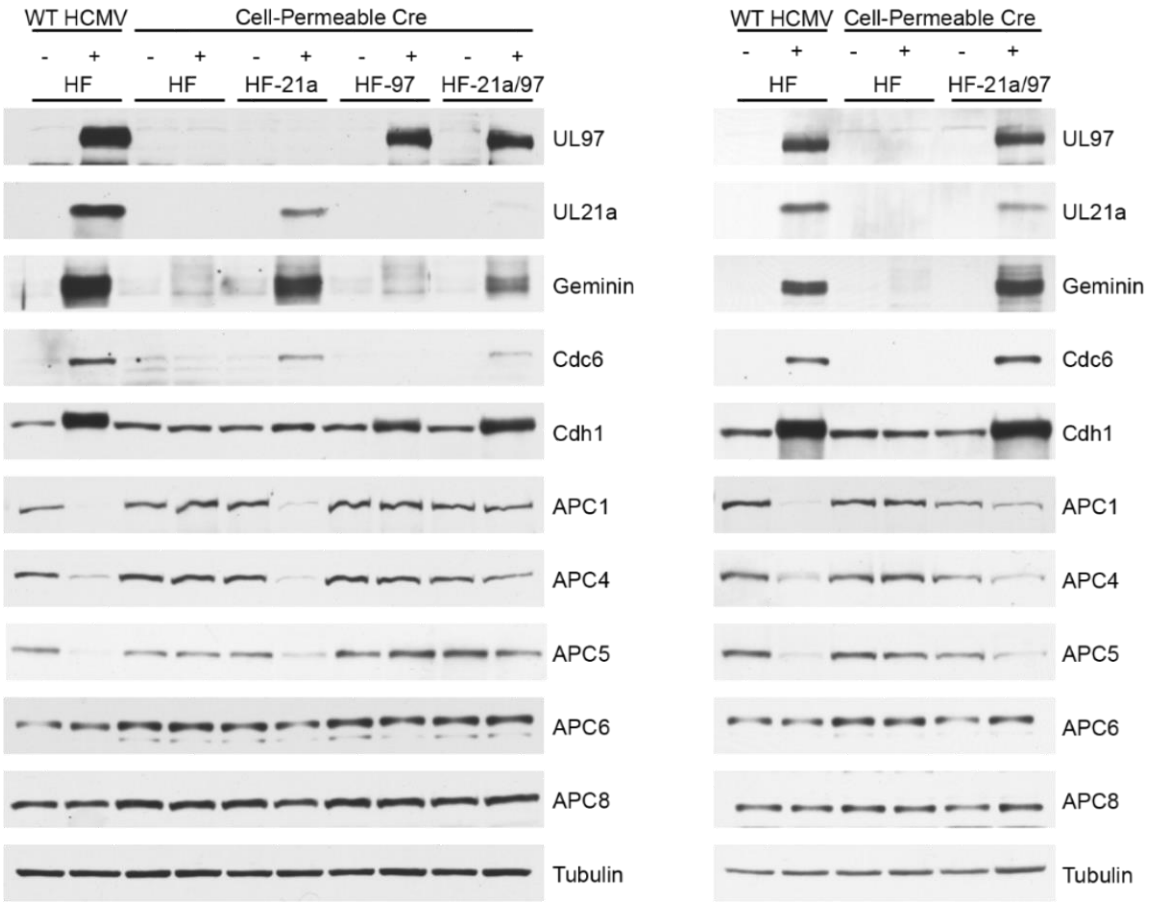

Figure 3-2. UL21a but not UL97 expressed in uninfected cells promotes accumulation of APC/C substrates.

HF, HF-21a, HF-97, and HF-21a/97 cells were treated with cell-permeable Cre to induce viral protein expression or with vehicle control for 12 h. Cells were then washed several times with PBS and maintained in HF media. Cells were harvested 72 h after beginning of Cre treatment and processed for Western blot with antibodies to UL97, UL21a, Geminin, Cdc6, Cdh1, APC1, APC4, APC5, APC6, and APC8. Tubulin serves as a loading control. HF cells were infected at MOI 3 with WT HCMV (+) and harvested 24 hpi for comparison. Shown in the left panel are HF-21a/97 cells expressing lower levels of UL21a than those in the right panel.

UL21a is necessary and sufficient for the degradation of APC4, APC5, and APC1.

To confirm that the Cre-induced expression of UL21a in the absence of other viral proteins also led to the degradation of APC4 and APC5 as previously reported (49), we assayed the lysates described above by Western blot with antibodies to APC/C subunits. Figure 3-2 shows that, as expected, the levels of APC4 and APC5 were significantly reduced during the infection with WT virus. The levels of APC4 and APC5 were also lower when UL21a was expressed alone or in the presence of UL97, and the magnitude of the decrease was dependent on the levels of UL21a. Surprisingly, we also found that in the infected cells or cells expressing UL21a there was a loss of APC1 in addition to APC4 and APC5. There was not a global degradation of APC/C subunits, as APC6 and APC8 levels did not change.

Since this result differed from that obtained previously, in which it was shown with a different antibody directed against APC1 (65, 66), that this subunit appeared to be stable and localized to the nucleus during infection (47), we confirmed the specificity of the antibody used here with siRNAs directed against the APC1 transcript and a non-targeting control siRNA (NT). As shown in Figure 3-3A (right panel), the detected band disappears in the mock-infected lane (M) upon APC1 depletion by siRNA. For comparison, we show that in cells infected with WT HCMV, the band was not present when the cells were treated with either APC1 siRNA or non-targeting control. To document that that the APC1 antibody used in this study detects a band of the correct molecular weight, we also included

a lane that contained a lysate from uninfected cells that had been immunoprecipitated with APC3 antibody (IP) (Fig 3-3A, left panel). We suspect that the previously used antibody, which was an affinity-purified rabbit antibody directed against a peptide of APC1 (aa 326-342) (65, 66), detects an alternative stable form, but we cannot confirm this as the antibody is no longer available. However, in support of the results shown here, a recent study showed by quantitative mass spectrometry that there is a 4- to 5-fold reduction in APC1 levels in infected cells (85). As further confirmation that UL21a is needed for the degradation of APC1, we infected HF cells with WT HCMV or a mutant virus lacking UL21a (ΔUL21a) and harvested the cells at 24 hpi for Western blot analysis. Figure 3-3B shows that, as was the case for APC4 and APC5, UL21a is necessary for degradation of APC1. To test whether APC1 degradation was proteasome dependent as has been shown for APC4 and APC5 (48), we induced UL21a expression in the HF-21a cells with Cre treatment in the presence or absence of the proteasome inhibitor salinosporamide A (SalA). We harvested the cells 18 h post-treatment for Western blot analysis. Figure 3-3C shows that UL21a levels increase with proteasome inhibition as expected, and APC1 and APC5 degradation is prevented.

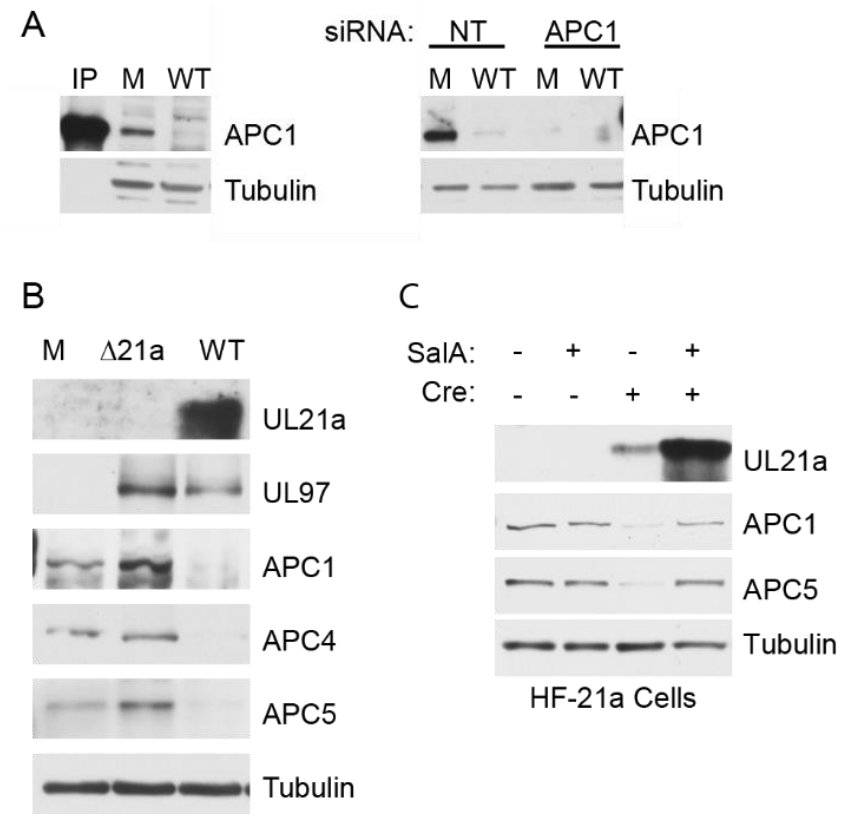

Figure 3-3. UL21a is necessary for APC1 degradation during infection.
(A) Confirmation of anti-APC1 antibody specificity. (Left panel) Cells were mock-infected or infected with WT at high MOI and harvested 24 hpi for Western blot with Ab to APC1. For molecular weight comparison, untreated HF cell lysate was immunoprecipitated with Ab to APC3 (lane IP) and run on the same gel. (Right panel) Cells were transfected with siRNA against APC1 or a non-targeting control (NT). Four days post transfection, cells were harvested and processed for Western blot. (B) HF cells were confluence-synchronized and mock infected or infected at high MOI with WT or ΔUL21a. Cells were harvested 24 hpi and processed for Western blot with antibodies to UL21a, UL97, APC1, APC4, and APC5. Tubulin serves as a loading control. (C) HF-21a cells were confluence synchronized and treated with cell-permeable Cre and/or 100nM Salinosporamide A (SalA) proteasome inhibitor as indicated. Cells were harvested 18 h post treatment and processed for Western blot with Ab to UL21a, APC1, APC5, and Tubulin.

The APC/C degradation phenotype induced by HCMV can be mimicked by disruption of the APC8-APC1-APC4-APC5 interaction via siRNA-mediated depletion of individual APC/C subunits.

The specific APC/C subunit(s) that UL21a interacts with to mediate the degradation of the platform subunits is unknown. To further investigate the mechanism of APC/C subunit degradation by UL21a, we proceeded to test the hypothesis that UL21a might first target one of the platform subunits and that depletion of this subunit might prevent the degradation of the other two subunits. To address this, we first transfected HFs with siRNA against APC1 or APC5. Unexpectedly, we found that knocking down either of these subunits was sufficient to cause the degradation of APC1, APC4, and APC5 in uninfected cells. These experiments were repeated multiple times and representative results are shown in Fig 3-4. There was not global degradation of subunits, as the levels of two other APC/C subunits in the TPR subcomplex, APC6 and APC8, were not affected by the depletion of APC1 or APC5. To rule out off-target effects of the siRNA, we performed real-time RT-PCR on these samples and found that the siRNAs against APC1 and APC5 did not affect the RNA levels of the other subunit (Fig 3-4B).

We extended this experiment to include knockdown of the other APC/C subunit in the platform subcomplex, APC4, and three APC/C subunits in the TPR subcomplex, APC3, APC6, and APC8 (Fig 3-4C). We were surprised to find that knockdown of APC8 is sufficient to cause a reduction of APC1, APC4, and APC5, and knockdown of any one of the group of APC1, APC4, and APC5 causes reduction of the other three, but not of APC8. This was of interest since the recently

solved structure of the human APC/C places APC1 next to APC8 (8). APC6 levels were not affected by depletion of these subunits, and depletion of APC6, albeit incomplete did not significantly affect the levels of the other subunits. In the case of APC3, we sometimes observed that its depletion modestly affected the levels of some of the other subunits.

This unexpected result may underlie the cellular mechanism by which the APC/C subunits are degraded during the infection and demonstrates that UL21a may need only to disrupt the complex in order to effect APC1, APC4 and APC5 degradation.

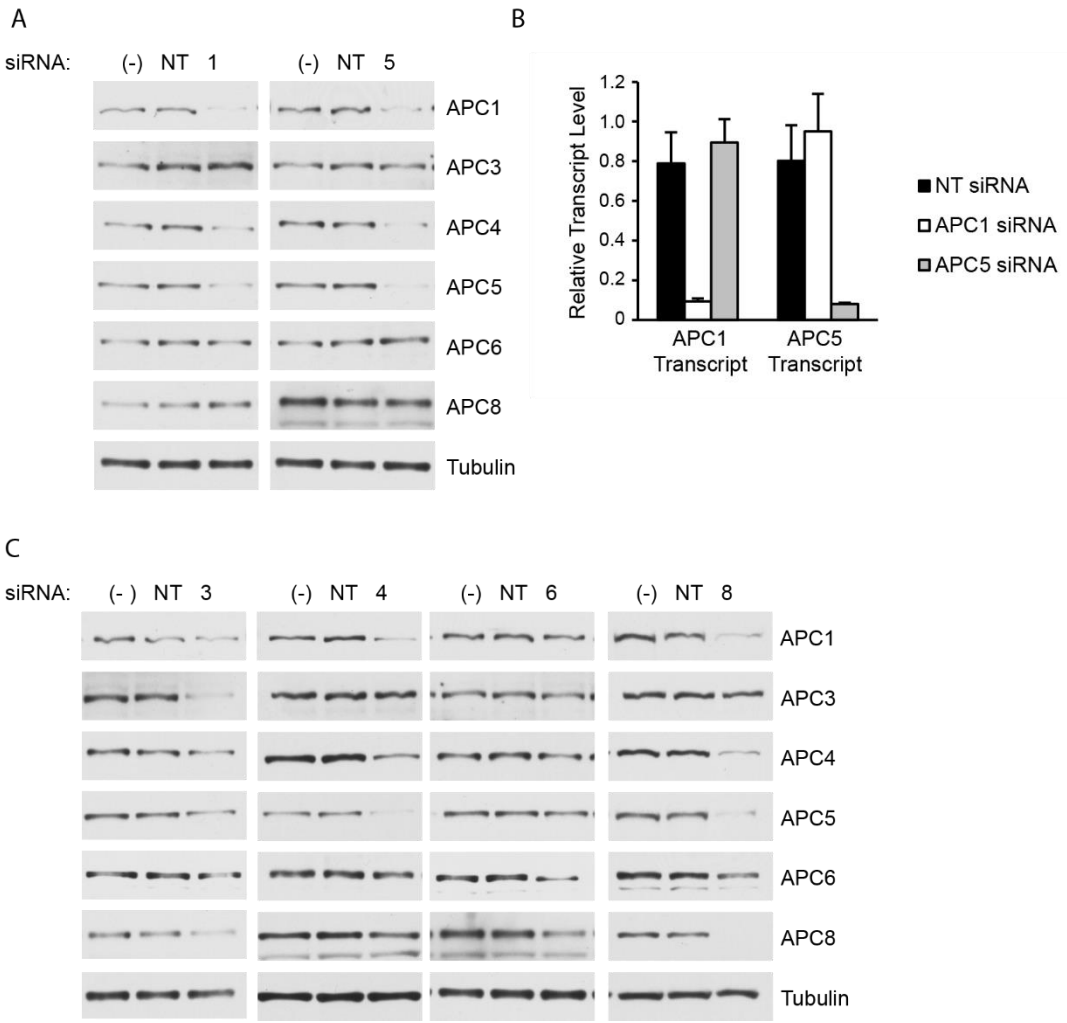

Figure 3-4. Knockdown of either APC1, APC4, APC5, or APC8 causes the degradation of APC1, 4, and 5 in uninfected cells.
(A) HF cells were transfected with siRNAs against APC1 (1), APC5 (5), non-targeting control (NT), or left untransfected (-). Cells were harvested 96 h post transfection and processed for Western blot with antibodies to APC1, APC4, APC5, APC6, and APC8. (B) RNA was extracted from the samples in (A) and real-time RT-PCR was performed to detect levels of APC1 and APC5 transcript. Transcript levels were normalized to Tubulin transcript. Error bars indicate standard deviation of three replicates of a single experiment. (C) HF cells were transfected with siRNA against APC3 (3), APC4 (4), APC6 (6), APC8 (8), non-targeting (NT) control, or were left untransfected (-). 96 h later the cells were harvested and processed for Western blot as in (A).

A double mutant ΔUL21a/UL97 is growth compromised compared to ΔUL21a and WT.

Previously, it was shown that a mutant virus that contained point mutations in UL21a that still allowed degradation of APC4 and APC5 seemed to have no growth defect, but was severely growth-impaired if a second mutation that eliminated expression of UL97 was introduced (49). A second UL21a mutant virus that contained point mutations in UL21a that abrogated its ability to degrade APC4 and APC5 also replicated like WT, but was even more severely growth-impaired than the first mutant when the UL97 mutation was introduced. One difficulty in interpreting these experiments was that the mutant viruses were not propagated on complementing cell lines and replication of the mutant viruses was analyzed in a multi-step growth assay over 21 days. We therefore proceeded to adapt the method we previously used for complementation of IE2 mutant virus growth (82). We constructed a mutant virus that contained a deletion of both UL21a and UL97, designated ΔUL21a/UL97 (Fig 3-5A), and propagated it on the Cre-inducible cell line HF-21a/97 to facilitate growth and avoid compensatory mutations. BAC recombinant clones were monitored by PCR and restriction digest and FIGE (Fig 3-5B). Compared to WT, the appearance of a 1.4 kbp fragment was diagnostic for deletion of UL21a, and the disappearance of the 13.5 kbp fragment indicated deletion of UL97 (Fig 3-5B). Since the WT, ΔUL21a, and double mutant viruses express Cre under the direction of the IE promoter, there is induction of both UL21a and UL97 in the HF-21a/97 cells upon infection. The virus stock and viral

supernatants produced in experiments were also titered on HF-21a/97 in order to accurately determine amounts of infectious virus produced.

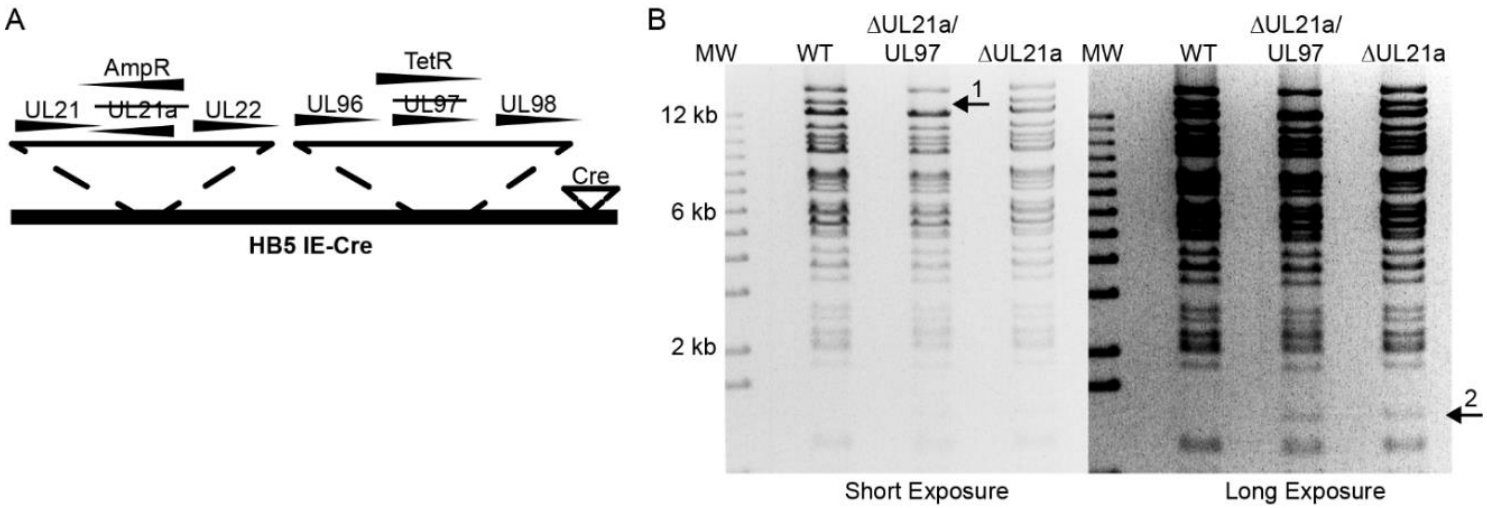

Figure 3-5. Creation of HCMV UL21a/UL97 double mutant.
(A) Diagram of BAC recombinants. UL21a alone or UL21a and UL97 open reading frames in the Ad169 BAC HB5-IE-Cre/1.2-Flp (24) (referred to here as WT) were replaced with Ampicillin (UL21a) and Tetracyclin (UL97) resistance genes respectively and propagated on the HF-21a/97 complementing cells. (B) BamHI restriction digest of BAC recombinants and MW (molecular weight marker) were resolved on an agarose gel. Arrows 1 and 2 indicate band changes predicted for mutation of UL97 and UL21a respectively.

We characterized the growth kinetics of Δ21a/97 by infecting confluence-synchronized HFs with Δ21a/97, Δ21a, or WT at a high MOI (MOI 4) and collected cell pellets and cell supernatant at several times post-infection. Viral titers were determined by plaque assay on HF-21a/97 cells. We found the double Δ21a/97 mutant to be severely growth-defective, releasing 2-3 logs less virus than WT and 10- to 50-fold less than Δ21a (Fig 3-6A). The titer of the single Δ21a mutant virus was approximately 5-fold lower than WT. Western blot analysis of viral proteins demonstrated that the double mutant is deficient in production of late proteins (Fig 3-6B) but not early or IE proteins. While there was little difference in IE2 86 protein and early protein UL57, there was a significant decrease in the levels of pp28 and the late IE2 protein p40 in the double mutant infected cells and only a modest decrease in the late protein levels in the Δ21-infected cells.

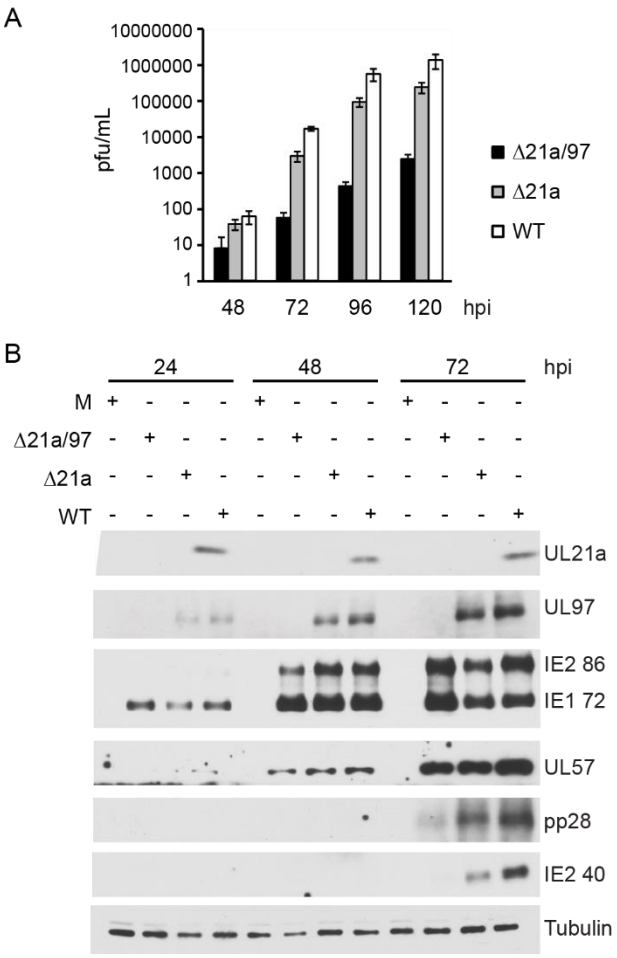

Figure 3-6. ΔUL21a/UL97 is growth-defective and deficient in late protein production.

HF cells were confluence synchronized and mock infected (M) or infected with ΔUL21a/UL97, ΔUL21a, or WT at high MOI. (A) Supernatants were harvested 48, 72, 96, and 120 hpi and titered on HF-21a/97s. Shown are representative results of at least 2 independent experiments. Error bars indicate standard deviation. (B) Cells were harvested 24, 48, and 72 hpi and processed for Western blot with antibodies to UL21a, UL97, IE proteins (IE2 86/IE1 72), early protein (UL57), and late proteins (pp28 and IE2 40).

UL21a and UL97 contribute to, but are not essential for, accumulation of APC/C substrates in infected cells.

We next tested the ability of Δ21a/97 to promote accumulation of APC/C substrates. Cells were infected at high MOI with Δ21a/97, Δ21a, or WT and harvested at 24, 48, and 72 hpi for Western blot analysis. Lysates were probed for Cdh1 and several APC/C substrates. We found that at 24 hpi, there is a significant defect in accumulation of APC/C substrates in cells infected with either mutant. At later times, however, APC/C substrates accumulate in cells infected with both mutants, but with reduced efficiency for the Δ21a/97 double mutant compared to either Δ21a or to WT (Fig 3-7). These data indicate that other viral proteins or cellular proteins induced during the infection can contribute to blocking APC/C function in the absence of UL21a and UL97. As expected, in cells infected with the double mutant, Cdh1 fails to exhibit the change in migration that results from phosphorylation by UL97.

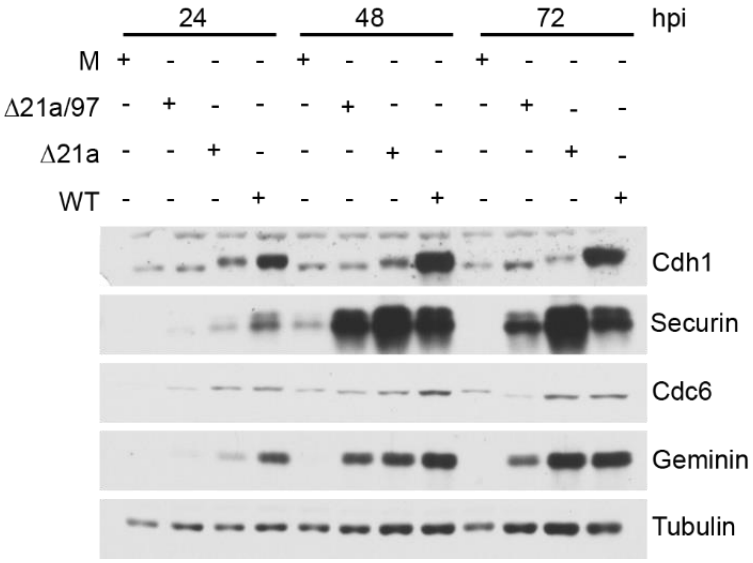

Figure 3-7. Cells infected with ΔUL21a/UL97 show a delay in accumulation of APC/C substrates compared to cells infected with WT and ΔUL21a.
HF cells were confluence synchronized and mock infected (M) or infected with ΔUL21a/UL97, ΔUL21a, or WT a MOI of 4. Cells were harvested 24, 48, and 72 hpi and processed for Western blot with antibodies to Cdh1, Securin, Geminin, and Cdc6.

The relative contribution of exogenously expressed UL97 and UL21a to complementation of the growth of double mutant ΔUL21a/UL97 is MOI-dependent.

Having compared the influence of UL21a and UL97 on APC/C substrate levels in the context of the infection, we sought to characterize the relative contribution of each protein for growth of the double mutant Δ21a/97 during a single cycle HCMV infection at high and at low MOI. This was accomplished by comparing the Δ21a/97 mutant infection on HF relative to HF-21a, HF-97, and HF-21a/97 complementing cells where UL21a and/or UL97 was provided exogenously upon expression of Cre by the mutant virus. All cells were confluence-synchronized in G_0/G_1 and then infected at a low MOI of 0.1 or a high MOI of 3. At 96 hpi, cell pellets were harvested for Western blot analysis. Supernatant was collected at various times pi to determine the titer of infectious virus produced.

Virion production was monitored by titering supernatants on HF-21a/97's to provide complementing function. At both high and low MOI (Fig 3-8A and B), infection on HF-21a's resulted in higher titers of virus relative to infection of HFs, with a greater increase in titer when mutant virus was grown on HF-97's at high MOI. At low MOI, there was a delay in the production of virus on HF-21a's, but at the last time point collected, the amount of virus produced on HF-21a's was comparable to that produced on HF-97's. Infection of cells expressing both proteins (HF-21a/97) with the double mutant resulted in still higher titers than infection of HF-97's at low MOI, but the difference was less at high MOI. Cell pellets from these experiments harvested at 96 hpi were processed for Western blot and

probed for pp28, IE2 86, IE2 60, and IE2 40. Representative blots are shown in Fig 3-8C and Fig 3-8D.

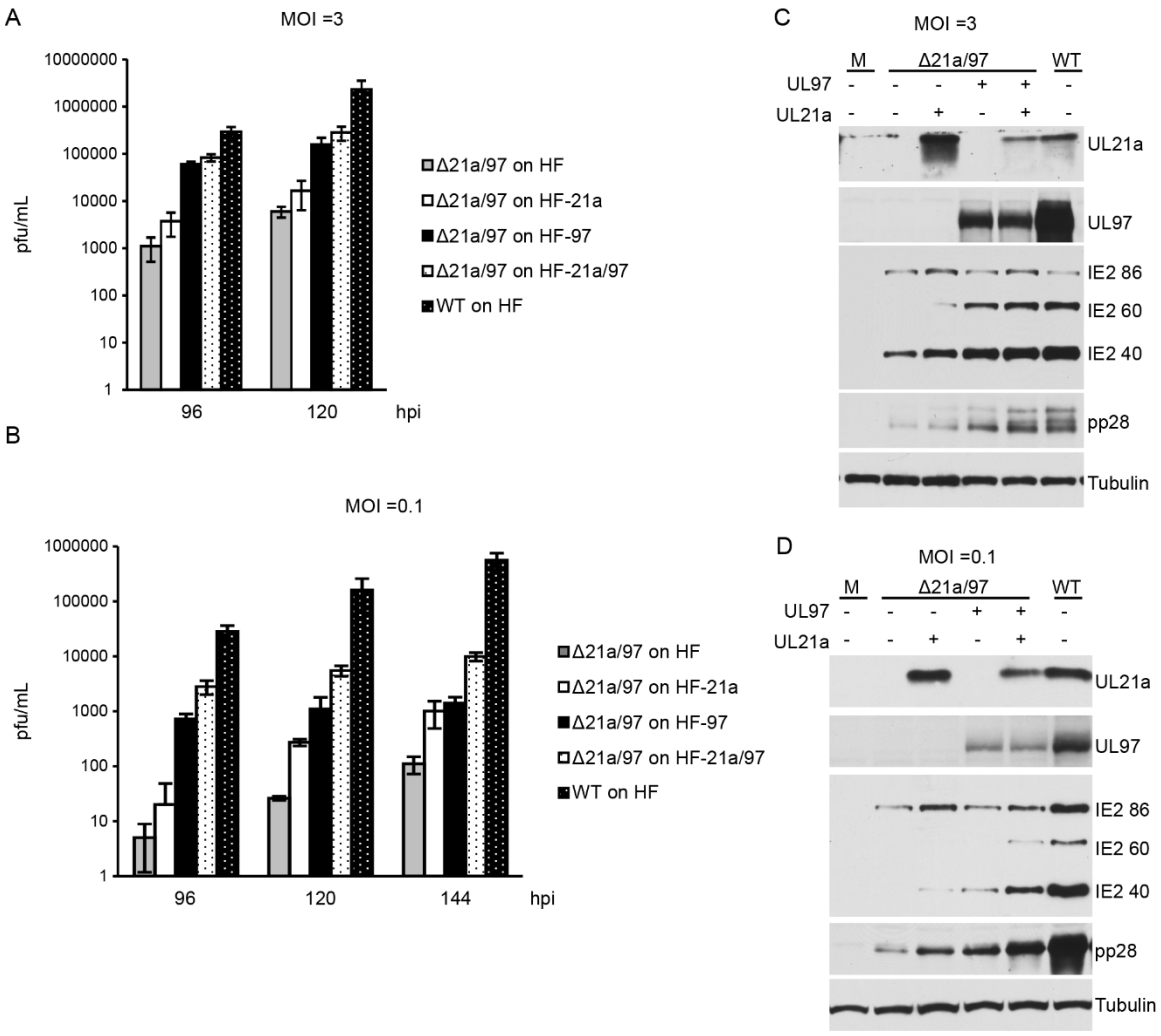

Figure 3-8. Relative requirement for exogenous expression of UL97 and UL21a in complementing the growth of double mutant ΔUL21a/UL97 is MOI-dependent.
HF cells or the cells lines HF-21a, HF-97, and HF-21a/97 were confluence synchronized and infected at MOI 3 (A, C) or MOI 0.1 (B, D). Supernatants were collected and titered on HF-21a/97s (A, B), and cells were harvested 96 hpi and processed for Western blot with antibodies to UL21a, UL97, IE2 proteins (IE2 86, IE2 60, IE2 40) and pp28 (C, D). Shown are representative results from 2-3 independent experiments. Error bars indicate standard deviation.

We observed that at high MOI, infection of HF-97's with the double mutant led to higher levels of the late proteins, pp28, IE2 60, and IE2 40, than infection of HF-21a's, consistent with the titer results (Fig 3-8C). Likewise the results of the Western blot were consistent with the titer at low MOI, with infection on UL21a-expressing and UL97-expressing cells showing similar increases in late protein levels (Fig 3-8D). At both high and low MOI, infecting HF-21a/97's led to the greatest increase in late protein expression compared to double mutant infection of HF's.

Exogenous inactivation of the APC/C during double mutant infection results in increased in late protein expression and viral titers.

As both UL97 and UL21a perform other functions during the infection apart from phosphorylating Cdh1 and mediating degradation of APC/C subunits, respectively, we were interested in determining the degree of rescue of the double mutant's growth-deficiency that results from inactivation of the APC/C. To decrease the activity of the APC/C, we used siRNAs to deplete APC5 and APC8. A non-targeting siRNA was used as a control. Growth of the double mutant on the various siRNA treated cells was assessed by measuring late viral protein accumulation and viral titers. Efficient knockdown of both APC5 and APC8 took several days, resulting in the cells coming to confluence (and thus a G_0/G_1 state) prior to infection. Since one function of UL21a is to degrade Cyclin A2, we wanted to ensure that this would not be a confounding variable in the experiment. To document that the siRNA-treated cells are not cycling and do not express significant levels of Cyclin A2, we harvested the cells at 96h post-transfection and

probed for Cyclin A2 along with APC/C subunits 5 and 8 (Fig 3-9A). We show that under these conditions, confluent siRNA-treated cells have only low levels of Cyclin A2, in contrast to the very high levels found in asynchronous cycling non-treated cells (Cyc) and in si-RNA treated cells that were reseeded at low density to allow the cells to enter G_1 and then harvested 24h later when they entered S phase of the cell cycle.

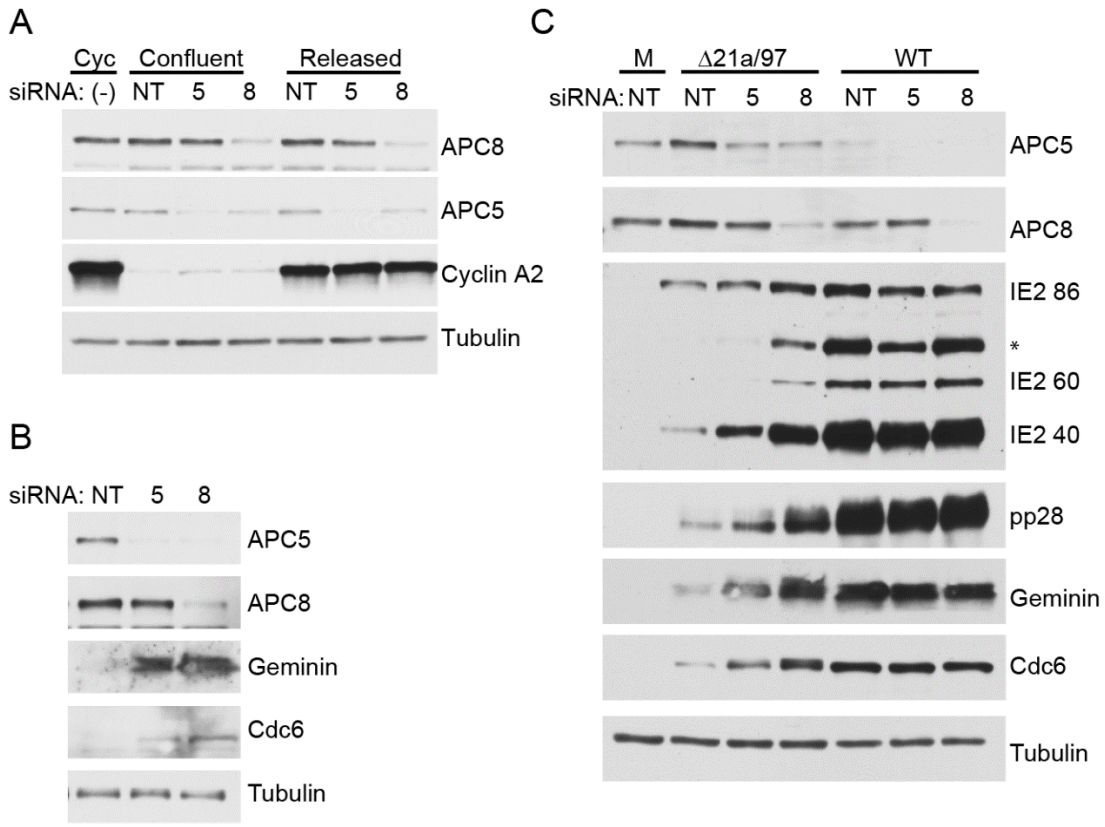

Figure 3-9. Knockdown of APC5 or APC8 results in increased ΔUL21a/UL97 growth.

(A) HF cells were transfected with siRNA against APC5 (5), APC8 (8), non-targeting control (NT), or were untreated (-). At 72 h after transfection, some cells were replated at lower density to release them into G_1 phase (Released) while other cells were maintained at confluence (Confluent). The untreated cells were maintained in a cycling state (Cyc) by passaging them several times prior to harvest. At 96 h post-transfection, cells were harvested and processed for Western blot with antibodies against APC5, APC8, Cyclin A2, and Tubulin. (B,C) HF cells were transfected with siRNA as in panel A. At 96 h after transfection, cells were mock-infected or infected at a MOI of 0.5 with ΔUL21a/UL97 or WT. Cells were harvested 96 hpi and processed for Western blot with antibodies to APC5, APC8, Geminin, and Cdc6 (B), and antibodies to IE2 proteins (IE2 86, IE2 60, IE2 40) and pp28 (C). Asterisk indicates another possible form of IE observed with current lot of antibody.

In Figures 3-9B and 3-9C, cells were mock-infected (Fig 3-9B) or infected (Fig 3-9C) with Δ21a/97 or WT at a MOI of 0.5 four days after transfection of siRNA. In the uninfected cells depleted of the APC/C subunits (Fig 3-9B), there was accumulation of substrates, with slightly greater accumulation in cells treated with siRNA to APC8, probably due to the fact that depletion of APC8 also resulted in degradation of APC1, APC4, and APC5. It should be noted that in accord with the results shown above, depletion of APC8 also resulted in depletion of APC5. Supernatant was collected at various times post-infection, and the cells were harvested at 96 hpi for Western blot. Membranes were probed for APC5 and APC8 to ensure maintenance of protein knockdown late in the infection and for viral late proteins (Fig 3-9C). Western blot analysis showed that the knockdown of both APC/C proteins was maintained until at least 96 hpi and that APC/C subunit knockdown resulted in a greater accumulation of APC/C substrates in cells infected with the double mutant. Knockdown of APC5, and to a greater extent knockdown of APC8, also results in increased production of late proteins. Supernatants were assayed for viral production by titering on the complementing HF-21a/97 cells. Shown are the results of two independent experiments. In accord with the levels of viral late proteins observed, viral titers were significantly increased by depletion of APC5, with a greater increase observed in cells depleted of APC8 (Figure 3-10).

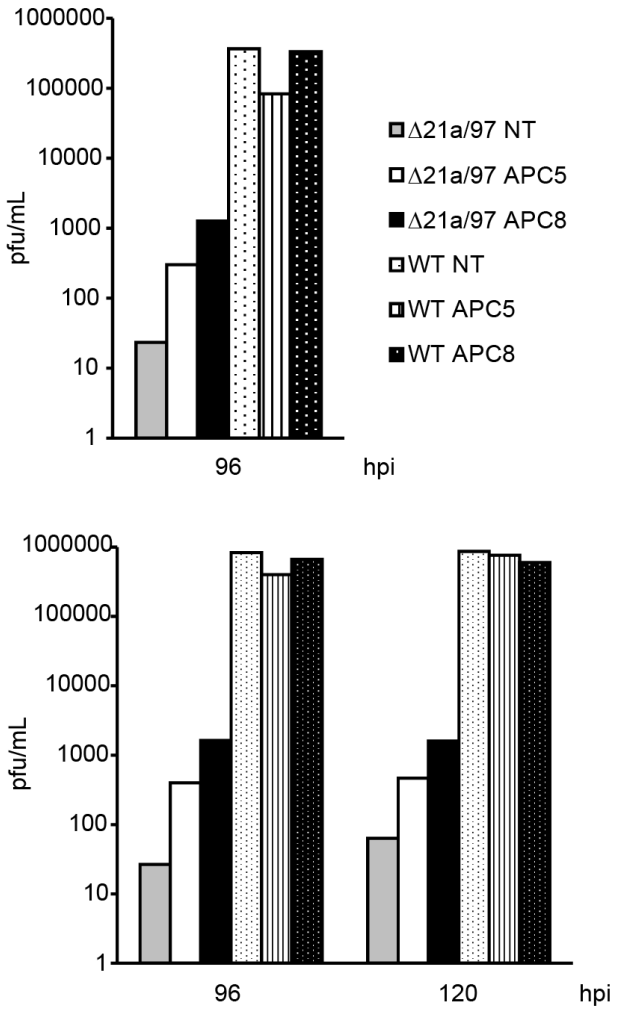

Figure 3-10. Knockdown of APC5 or APC8 results in increased ΔUL21a/UL97 growth.

HF cells were transfected with siRNA against APC5 (5), APC8 (8), non-targeting control (NT), or were untreated (-). Cell supernatants were harvested at the indicated time points and titered on HF-21a/97s. Shown are two independent experiments.

A comparison of Figure 3-8B with Figure 3-9D shows that the increase in viral titer when mutant virus was grown on cells expressing UL21a, relative to HF, was comparable to the increase when mutant virus was grown on HF cells that were depleted of APC5 or APC8. While the mutant was still growth-debilitated relative to WT, depletion of APC/C subunits was able to increase viral growth in a single cycle infection. Growth of WT virus was not affected by knockdown of either protein.

Discussion

In this study, we discovered that APC1 is an additional target of degradation by HCMV protein UL21a. Particularly noteworthy is our finding that recapitulation of the pattern of APC/C subunit degradation induced by HCMV occurs in uninfected cells upon depletion of either APC1, APC4, APC5, or APC8. Finally, we show that when complementing cells are infected at low MOI with a mutant virus that lacks both UL97 and UL21a (Δ21a/97), there is a delay in the production of infectious virus on cells expressing UL21a relative to UL97, but the final titer and level of late proteins are comparable. Growth of the double mutant on cells expressing both proteins is further enhanced. In contrast, when complementing cells are infected at high MOI with the double mutant, UL97 provides a greater increase in accumulation of late viral proteins and production of infectious virus than UL21a, and there is only a modest further increase when the virus is grown on cells that express both proteins. These results are consistent with those of Prichard et al. (86) who showed that the complementation of a UL97 deletion virus by exogenous UL97 was more efficient at high MOI.

Our results also confirm the studies by Fehr et al. (49) that showed UL21a could disable the APC/C and is responsible for the degradation of APC4 and APC5. In addition, we find that UL21a expression leads to APC1 degradation. In support of our antibody-based observation, the reduction in APC1 levels during HCMV infection was recently confirmed by quantitative mass spectrometry (85). We also show that UL97 expression in uninfected cells does not cause accumulation of APC/C substrates despite phosphorylation of Cdh1. The

expressed UL97 protein was properly localized to the nucleus, but it is possible that the phosphorylation of Cdh1 by UL97 is incomplete. Phosphorylation of Cdh1 leaves the APC/C itself intact, so it may be that a relatively small amount of unphosphorylated Cdh1 would be sufficient to co-activate the APC/C.

In the course of experiments to determine the mechanism of APC/C subunit degradation, we tested the hypothesis that depletion of one of the platform subunits might prevent UL21a-mediated degradation of the other subunits. We were surprised to see that knocking down any one of the three platform subunits led to the degradation of others in the absence of infection. Moreover depletion of APC8, a member of the TPR complex, also led to degradation of all of the platform subunits, but APC8 was not degraded either by UL21a or by depletion of a platform subunit. To our knowledge, this phenomenon has not been described previously. However, the result is not totally unexpected since it was previously shown that the binding of APC1, APC4, APC5, and APC8 is interdependent in vitro (80), and the recently solved structure of the human APC/C places APC1 next to APC8 (8). This process does confound the interpretation of RNAi experiments intended to delineate the sufficiency of any of these individual subunits in this and other viral or cellular events.

It is currently unclear whether the subunits are degraded by some active process or whether they are degraded as part of normal APC/C turnover, with newly synthesized subunits being recognized as improperly-folded proteins if they do not associate correctly. Our studies show that there is a clear cellular process in place for decreasing the levels of the APC/C subunits APC1, APC4, and APC5,

but not APC3, APC6, and APC8 if the APC/C becomes destabilized. These results suggest that UL21a may need only to disrupt the association of the APC/C subunits, and a cellular process will then mediate the degradation of subunits APC1, APC4, and APC5. To date, our time course experiments have been unable to distinguish whether degradation of the subunits precedes or follows release from the rest of the complex, but it is possible that the use of new high-resolution imaging tools may help answer the question.

Our studies also show that the double mutant ΔUL21a/UL97 is growth defective compared to both ΔUL21a and WT with respect to late protein expression and production of infectious virus. As is the case with some other HCMV mutants, the growth defect of the double mutant is MOI dependent and much greater at low MOI than high MOI. Interestingly, APC/C substrates accumulated, albeit with a delay in cells infected with either ΔUL21a or ΔUL21a/UL97. Thus, even in the absence of UL21a (in the presence or absence of UL97), APC/C substrates begin to accumulate later in the infection. This may be due to a yet to be discovered viral or induced cellular protein that can disable APC/C function. Alternatively, the infection may activate some cellular mechanism that normally operates during S/G_2 phase to inactivate the APC/C and allow substrate accumulation. Regardless of mechanism, further inactivation of the APC/C by depletion of APC/C subunits APC5 and APC8 during ΔUL21a/UL97 infection leads to a growth benefit in a single cycle of infection.

Previously, a double mutant virus that contained a UL97 deletion and UL21a point mutations that rendered UL21a incapable of interacting with the APC/C was

shown to be more growth-impaired than a double mutant that contained a UL97 deletion and UL21a point mutations where UL21a was still able to degrade APC4 and APC5 (49), suggesting that inactivation of the APC/C was important in the absence of UL97. One difficulty in interpreting these experiments was that the mutant viruses were not propagated on complementing cell lines and replication of the mutant viruses was analyzed in a multi-step growth assay over 21 days where the cells continued to proceed through the cell cycle. They were also conducted in MRC5 fibroblasts, where the growth defect of an UL21a deletion virus is significantly greater than that in HFs. In our studies, we constructed a double mutant virus ΔUL21a/UL97 that was unable to express any UL21a and UL97. By growing the double mutant virus on complementing HFs, we were able to avoid the complication of any second site mutations that might change viral growth. We were also able to show directly in a single cycle growth experiment in cells that were confluence-synchronized and maintained in G_0/G_1 that the increase in viral titer in cells expressing UL21a was comparable to that in cells in which the APC/C was inactivated by depletion of APC/C subunits.

The benefit resulting from knocking down APC5 and APC8 for ΔUL21a/UL97 replication is impressive considering that during the infection both UL21a and UL97 possess other functions that are independent of the APC/C. UL97 in particular is multi-functional. It phosphorylates several viral proteins, as well as itself, and a number of cellular proteins, including Rb, Lamins A/C, p32, and eukaryotic elongation factor 1 delta (54, 55, 57, 87-95). Studies of UL97 deletion mutants indicate a role for this protein in viral DNA synthesis, DNA

encapsidation, capsid maturation, nuclear egress, and cytoplasmic secondary envelopment (53, 54, 93, 96, 97), although the extent of the defects appears to depend on the culture conditions. Which of these functions are being complemented by UL21a or by inactivation of the APC/C remains to be determined.

It has been reported that UL21a, in addition to inactivating the APC/C, functions at a later time to promote viral DNA replication and late gene expression (52). One recently discovered function of UL21a is associated with proteasome-dependent degradation of Cyclin A2, and the domain responsible for interaction with Cyclin A2 is separate from that which interacts with the APC/C (43). It was reported that Cyclin A2 degradation was necessary for efficient multi-step replication in MRC5 cells, and siRNA-mediated depletion of Cyclin A2 removed the growth defect seen in an UL21a deletion mutant. However, these experiments were done at low MOI in MRC5 cells progressing through the cell cycle and viral titers were assayed after virus had undergone multiple cycles of replication. Additionally, cells were infected in S phase when Cyclin A2 levels are very high. Recent work by Eifler et al examined UL21a-mediated Cyclin A2 degradation in confluence-synchronized HEL cells (44). They found that degradation of Cyclin A2 by UL21a was required to prevent mitotic catastrophe as in the absence of UL21a's Cyclin A2 degradation function, infected cells were not prevented from entering mitosis, and mitotic aberrations and cell death followed. This appeared to be specific to the Cyclin A2 degradation locus of UL21a: RXL2. While the UL21a null mutant also induced accumulation of Cyclin A2, a similar level of mitotic

catastrophe and cell death did not take place. Correspondingly, the increase in Cyclin A2 in cells infected with the UL21a RXL2 point mutant was much greater than that seen during infection with the UL21a null. In agreement with this work, we also see a small increase in Cyclin A2 levels during infection with the UL21a null mutant and the UL21a/UL97 double mutant, but this increase is very modest in comparison to the levels observed in cycling HF cells.

Interestingly, Cyclin A2 is a substrate of the APC/C, and inactivation of the APC/C should lead to accumulation of Cyclin A2. However, we previously showed that Cyclin A2 transcription is inhibited during the infection, and if the virus is grown in confluence-synchronized cells, which remain in G_0/G_1, there is very little Cyclin A2 until later times in the infection (39). We show here that Cyclin A2 levels remain very low in our APC/C subunit depletion experiment when cells are maintained in a G_0/G_1 state (Fig 3-9.), and thus the levels are negligible at the time of infection compared to the levels in asynchronous cycling cells. It is only when cells are infected in S phase, where there are already high levels of Cyclin A2, that it might be necessary for UL21a to degrade the Cyclin A2 for the infection to initiate. The situation is likely more complicated, as it has been reported that the HCMV tegument protein pp150 also binds to Cyclin A2, and this binding between pp150 on the incoming particle and Cyclin A2 is responsible for the block in initiation of HCMV IE gene expression when cells are infected in S phase (42, 98). We cannot exclude that Cyclin A2 increases contribute to the growth-deficiency of our double mutant. However, direct comparison to the above-mentioned studies is difficult due to the difference in cell type, as different cells types may respond differently to

a given level of Cyclin A2. Another possibility includes yet to be discovered functions of UL21a that are required for efficient viral DNA synthesis and late gene expression, and this is likely cell type dependent (e.g. there is greater impairment of replication of UL21a deletion mutants in MRC5 cells than in HF's).

It is still to be determined what the functional relevance of APC/C inactivation is in HCMV infection in vivo. However, the fact that inactivation of the APC/C by HCMV is seen not only in fibroblasts, but also aortic endothelial cells and neural progenitors (unpublished results) suggest that APC/C inactivation serves some additional role in vivo that is undetectable in a tissue culture system. Experiments are in progress to determine more precisely the molecular mechanisms governing disassembly of the APC/C and degradation of specific subunits in the presence of UL21a.

Acknowledgements

Chapter 3, in full, is a reprint of the material as it appears in Studies on the Contribution of Human Cytomegalovirus UL21a and UL97 to Viral Growth and Inactivation of the Anaphase-Promoting Complex/Cyclosome (APC/C) E3 Ubiquitin Ligase Reveal a Unique Cellular Mechanism for Downmodulation of the APC/C Subunits APC1, APC4, and APC5. Journal of Virology, 2015. The dissertation author was the primary investigator and author of this paper.

We thank Don Coen for the UL97 antibody, Dong Yu for the UL21a antibody, and Bradley Moore for salinosporamide A. We especially want to thank Jean-Philippe Belzile for valuable advice and other members of the Spector laboratory for helpful discussions. This work was supported by NIH grants R01CA034729 and R21AI083991 to D.H.S. and training grant T32DK007541.

CHAPTER 4

The APC/C-Cdh1 interaction and the APC/C-UL21a interaction are mutually exclusive.

Introduction

One of the remaining unknowns concerning the UL21a-APC/C interaction is the UL21a binding site. It was previously reported that APC8 is required for interaction of UL21a and the APC/C, based on depletion of APC8 by siRNA (49). However, the results presented in Chapter 3 show that knocking down APC8 will result in the loss of APC1, 4, and 5, which are other possible binding sites. This different interpretation suggests that an intact bridge subcomplex (APC1, 4, and 5 in contact with APC8) is required for UL21a to bind to the APC/C.

Identifying a binding site on a large multisubunit protein complex in cells is problematic, as any overexpressed subunit may associate with others of the endogenous complex and confound the results. To overcome this difficulty, a GST-UL21a fusion protein was used as bait for in vitro translated APC/C subunits. While no subunit tested, alone or in combination, showed a convincing interaction with GST-UL21a (unpublished), it was noticed that Cdh1 was not among the proteins interacting with GST-UL21a when it was incubated with cell lysate. Recent publication of a detailed structure of the APC/C with and without Cdh1 bound reveals a conformation shift involving the orientation of the catalytic core and bridge subunits in relation to the TPR subunits, and it is possible that

the inability of UL21a to co-precipitate Cdh1 along with the APC/C may reveal further information about the interaction and binding site.

Materials and Methods

Cloning and constructs

Wild type UL21a was amplified from the HB5 BAC. Primers added a 5' BamHI and 3' XhoI site for ligation into pcDNA3. Primers used were 5'-GAT CGG ATC CAT GGG AGG TAG CCC TGT TCC-3' and 5' GAT CCT CGA GTT AAA ACT GGT CCC AAT GTT CTT C-3'. The fragment was ligated into pcDNA3 to make pcDNA3-UL21a. The UL21a ORF was inserted downstream of GST in EcoRI-linearized pGEX4T-1 by ligation-independent cloning using the InFusion kit (Stratagene) using the primers InFusionForward: 5' GGA TCC CCG GAA TTC ATG GGA GGT AGC CCT GTT CC-3' and Reverse: 5'-TCA ACC CGG GAA TTC CTC GAG TTA AAA CTG GTC CCA ATG-3'. to make pGEX-UL21a. Integrity of the construct was confirmed by sequencing. Point mutants were created that were deficient in interaction with the APC/C (mutation of amino acids proline-arginine at position109-110 to two alanines) or with Cyclin A, as a control (mutation of amino acids arginine-arginine-leucine at positions 42-44 to alanine-arginine-alanine). Mutation was carried out using the Quikchange kit following manufacturer's instructions (Agilent Technologies) on pcDNA3-UL21a using primers previously reported (43, 49), then similarly inserting into pGEX4T-1 to create pGEX-UL21aPR-AA and pGEX-UL21aRRL-ARA.

Expression and purification of GST proteins

pGEX-4T1, pGEX-UL21a, pGEX-UL21aPR-AA, and pGEX-UL21aRRL-ARA were transformed into BL21 chemically competent bacteria. Protein expression was induced with 0.1 mM IPTG, and cultures were pelleted and lysed

in NET-N buffer (50mM Tris-Cl pH8, 150mM NaCl, 1mM EDTA, 0.5%NP40.) Uninduced pGEX-4T1 culture was also pelleted and lysed and used to equalize volumes during GST-pulldown. Clarified lysates were aliquoted and stored at -80C. Glutathione beads (GE Healthcare) were washed with NET-N and incubated with equal µg amounts of GST protein lysate (normalized to equal volumes using uninduced GST lysate). Beads were washed and used immediately.

GST pulldown

HF cell pellets were lysed in HF lysis buffer (20 mM Tric-HCL pH8, 150 mM NaCl, 5 mM $MgCl_2$, 0.2% NP40, 5% glycerol, + 1 mM DTT, and protease/phosphatase inhibitor cocktail (Thermo Scientific)) and dounced on ice. GST-protein-bound glutathione beads prepared as detailed above were washed three times with HFF lysis buffer, then incubated with HFF lysates for two hours. Beads were washed with lysis buffer and interacting proteins were released by boiling in reducing Laemmli buffer. Pre and post-pulldown samples were stored for Western blot analysis.

Immunoprecipitaton

A monoclonal antibody to APC3 was crosslinked to Protein-G Dynabeads (Life Technologies) per manufacturer's instructions. HF lysates prepared as indicated above were rotated with beads 2 hours at 4 degrees. Beads were washed with lysis buffer and co-immunoprecipitating proteins were eluted by boiling in reducing Laemmli buffer.

Results

APC/C co-precipitates with GST-UL21a, but Cdh1 does not.

In order to determine which APC/C subunit(s) carries the UL21a binding site, UL21a was tagged with GST and purified on glutathione beads. GST was expressed alone as a control. Two point mutants of UL21a were also tagged with GST as controls. The first, UL21a PR109AA, was described to lack the ability to bind to the APC/C or cause degradation of APC4 and 5 (49) and is used as a negative control. The second, UL21a RRL42ARA, is able to interact with and inhibit the APC/C, but it cannot bind or cause degradation of cyclin A (43). During validation of these recombinant UL21a proteins, they were incubated with HF cell lysates to ensure that they would interact with the endogenous APC/C as reported. HF cells were allowed to come to confluence and maintained for 3 days to establish G1/G0. Cells were harvested and lysed in NP40 lysis buffer. Lysates were incubated with beads bound with GST-UL21a WT, GST-UL21a PR109AA, GST-UL21a RRL42ARA, or GST alone. Bound proteins were eluted by boiling in Laemmli buffer and assayed by Western blot (Figure 4-1). The result was unexpected. While APC/C subunits interacted with GST-UL21a and GST-UL21a RRL42ARA but not GST or GST-UL21a PR109AA, the coactivator Cdh1 did not interact.

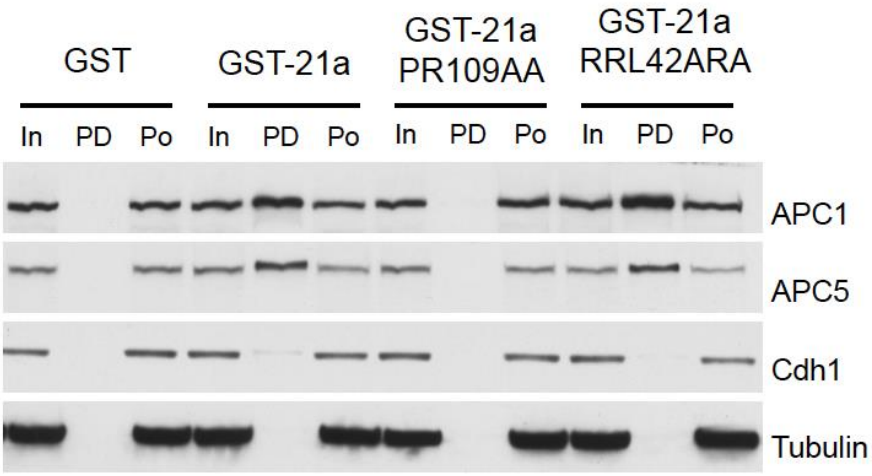

Figure 4-1. GST-UL21a does not interact with Cdh1
Confluence-synchronized HFs were harvested by trypsinization and lysed. Lysates were exposed to GST, GST-UL21a, or GST-UL21a point mutants immobilized on Glutathione beads. Adherent proteins were eluted after washing by boiling in Laemmli buffer and analyzed by Western blot. Input (In) and post (Po) lanes represent 10% of pulldown (PD) lanes.

The lack of Cdh1 binding is not due to absence from APC/C.

To rule out the possibility that Cdh1 does not bind simply because it is not interacting with the APC/C under these lysis conditions, a sequential pulldown was performed (Figure 4-2A). Confluence synchronized HF cells were lysed as above and incubated with beads bound to GST-UL21a. GST-UL21a PR109AA is used as a negative control. After incubation with the beads, they were spun and supernatants were transferred to new tubes containing anti-APC3 antibody immobilized on beads in order to immunoprecipitate the APC/C remaining in the lysate after exposure to UL21a beads. As before, GST-UL21a precipitates APC/C subunits, but not Cdh1. However, the APC3 IP demonstrates that all Cdh1 present in the lysate is bound to the APC/C (Figure 4-2B). An APC3 immunoprecipitation performed side-by-side with the first round of GST pulldowns bound a comparable amount of APC/C and Cdh1 as the IP sequentially following the GST pulldown (Fig 4-2C), indicating that the APC/C leftover from the GST-UL21a pulldown was bound with Cdh1. As expected the UL21a PR109AA point mutant did not pull down any APC/C subunits. One further observation is that simple interaction of UL21a with the APC/C is insufficient in vitro to cause APC1 degradation.

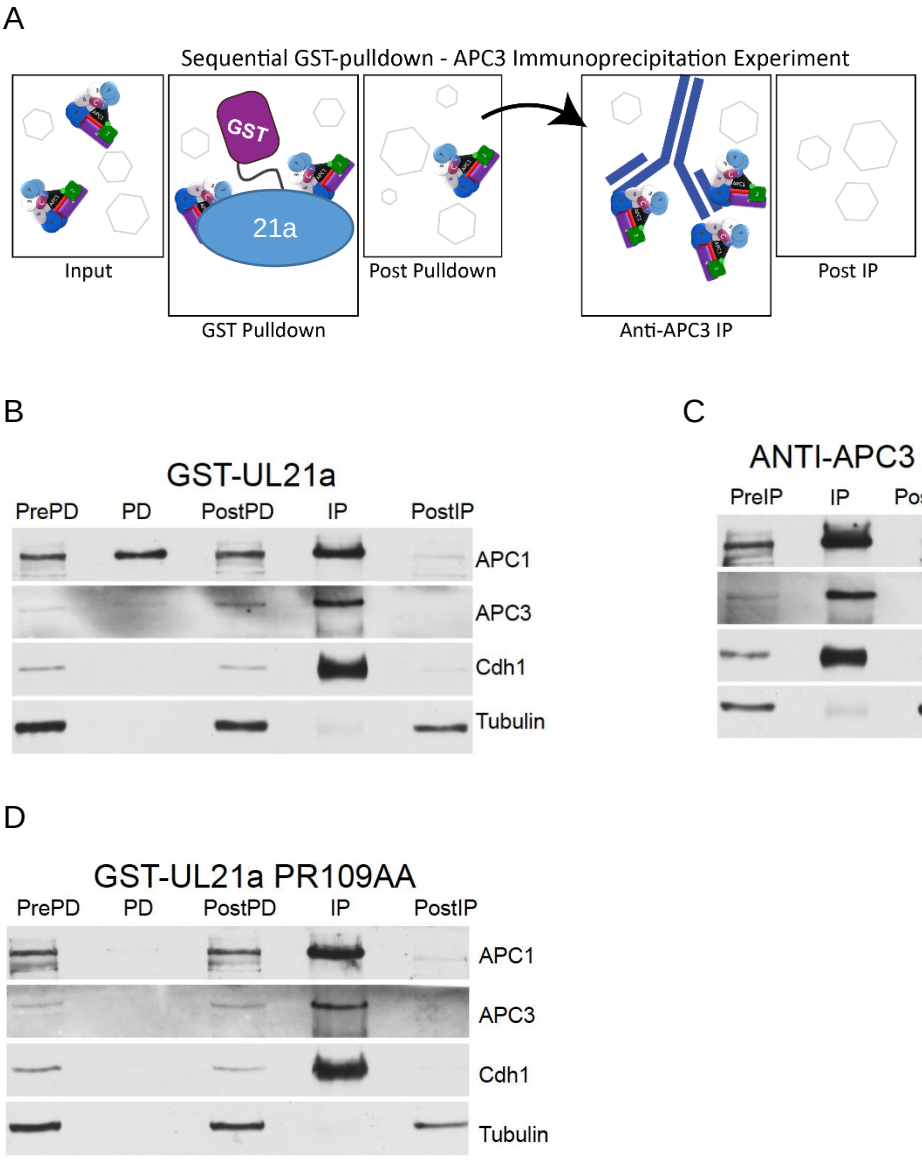

Figure 4-2. Lack of Cdh1 binding to UL21a is not due to failure of Cdh1 to associate with the APC/C in pulldown conditions.
(A) Diagram of experiment. Confluence-synchronized HFs were harvested by trypsinization and lysed. Lysates were exposed to (B) GST-UL21a or (D) GST-UL21a PR109AA immobilized on Glutathione beads. Flowthrough was then exposed to anti-APC3 antibody immobilized on Protein G magnetic beads. Adherent proteins were eluted after washing by boiling in Laemmli buffer. (C) The lysates from (B) were directly incubated with anti-APC3 beads. Adherent proteins were eluted after wash via boiling in Laemmli buffer and analyzed by Western blot. Inputs and Posts represent 10% of Pulldowns and IPs.

Discussion

In this study we constructed a recombinant UL21a and demonstrated that the protein and its point mutants behave in vitro as has been previously reported in mammalian cells with respect to APC/C interaction. Two models have emerged to explain the mutual exclusivity of the interactions between APC/C, Cdh1, and UL21a. In the first, UL21a is selectively co-precipitating an APC/C which does not contain Cdh1, and in the second, Cdh1 is somehow removed from the APC/C in the process of interacting with UL21a. These address the possibly competitive nature of the binding between APC/C, Cdh1, and UL21a. It would be possible to distinguish between these models by depleting or overexpressing Cdh1 prior to the GST-UL21a pulldowns. Also, establishing binding curves between APC/C and UL21a with increasing amount of added recombinant Cdh1 would determine if Cdh1 prevents the binding of UL21a to APC/C.

The original goal of this line of experimentation was to find the binding site of UL21a on the APC/C. However, it is not possible to make conclusions about the UL21a binding site from these data. It would be tempting at first glance to suggest that the mutual exclusivity of Cdh1 and UL21a interacting with the APC/C points to a shared binding site. This is not necessarily true because the presence or absence of Cdh1 alters the confirmation of the APC/C, (8) and it is possible that this shift exposes a binding site that is not the Cdh1 binding site. Further work is required to distinguish between the two presented models.

During infection, Cdh1 accumulates to high levels if UL21a is present, but it remains to be seen if it is performing some function in the infection. Preliminary

studies from our lab have not shown drastic differences in the progression of the wildtype infection when Cdh1 is depleted by siRNA but in some experiments has demonstrated a mild detriment to the UL21a deletion mutant (Unpublished). Cdh1 has functions in addition to activating the APC/C. Cdh1 was reported to compete with E2F1 to bind hyperphosphorylated Rb (99). As Rb is phosphorylated in infected cells by UL97, it is possible that the excess Cdh1 could be interacting with it, leading to greater activation of E2F transcriptional targets.

CHAPTER 5

CONCLUSION

This work has expanded both our understanding of the HCMV-host cell interaction and of the APC/C itself. To the field of HCMV research, this work demonstrates that UL21a causes proteasome-dependent degradation of an additional subunit (APC1), and it emphasizes the importance of inactivation of the APC/C to the viral infection by showing that growth of the severely debilitated ΔUL21a/UL97 double mutant can be improved by knockdown of APC/C subunits. This work independently confirms the finding of the identity of the HCMV APC/C inactivating protein UL21a and expands the understanding of the roles played by the two proteins reported to have inactivated the APC/C. It shows that contrary to expectation, expression of viral kinase UL97 alone in G0 synchronized cells does not induce APC/C substrate accumulation, even when it successfully causes Cdh1 phosphorylation. However, restoring UL97 expression to the double mutant infection provides greater rescue than restoring UL21a or restoring APC/C inactivation by siRNA-mediated depletion (Chapter 3, (100)). It can be concluded that the benefit provided by UL97 must be due to its functions other than APC/C inactivation. Similarly, exogenously removing another UL97 target, Rb, does not compensate for UL97's role in lamin phosphorylation and capsid nuclear egress, (101) and replacement of UL97 with the Rb-degrading E7 protein from Human Papillomavirus strain 16 results in an incomplete rescue of ΔUL97 deletion mutant (102).

To the field of Anaphase Promoting Complex research, this work demonstrates for the first time that the protein levels of APC/C subunits 1, 4, 5, and 8 are interdependent in cells. In vitro, association of subunits 1, 4, 5, and 8 is interdependent (103) and absence of any one of these prevents the association of the others. However, it appears that in cells this association is necessary for the stability of each of these subunits, but not the entire complex. Other studies have included knockdown of APC/C subunits. Most do not probe for other subunits, but some show data which agree with our conclusions. In all cases, a subunit or subunits were knocked down to determine that it was or was not necessary for the biological phenomenon of interest. Knockdown studies have addressed APC1(68), APC2 (104, 105), APC3 (105), APC5 (106, 107), APC7 (106), APC8 (49), APC11 (104), and cdc26/APC12 (108). The study by Mo et al showing APC1 knockdown partially supports our results. There is very slight reduction in APC4 levels during their APC1 knockdown but no loss of apparent binding to APC3. However, APC1 was not probed for in their immunoprecipitation to ensure that it was completely absent. Data from our lab has shown that during an incomplete APC/C subunit knockdown, the remaining subunit still co-immunoprecipitates with APC3 (unpublished). Studies in which APC2 or APC3 are knocked down did not show levels of other subunits. The study by Ahlskog et al (105) indirectly showed that depletion of APC2 and 3 did not affect the other. Similarly, we showed that APC3 knockdown did not reduce levels of other subunits (Chapter 3). The study by Ho et al (106) in which APC5 and APC7 were was knocked down, other subunits were not probed for but conclusions were drawn about the ability of APC5 to inhibit

IL-17 signaling based on the knockdown experiment. The APC11 knockdown study by Kelly et al (104) also did not show levels of other subunits. In the study by Masuda et al (108), Cdc26/APC12 depletion did not appear to reduce levels of APC6 or APC4, as APC/C was purified from APC12-depleted lysates via APC4 IP. One study by Sedgwick et al (109) depleted APC/C subunits 2, 3, 6, 8, and 10 from HeLa cells to examine the subunit requirements for binding the protein Nek2A. Their data show various fluctuations in APC/C subunits, including a very small decrease in APC4 when APC8 is depleted, but unfortunately no loading control was shown, as the experiment was not designed to compare APC/C subunit levels in the lysate, only in immunoprecipitations. APC1 and 5 were not probed for. Finally, in the study by Fehr et al naming UL21a as the protein which degrades APC4 and 5, shRNA against APC8 showed convincing depletion of APC4 and APC5 in uninfected cells (49) in agreement with our results. APC1 was not assayed by Western blot, and knockdowns of other subunits were not performed. Caution must be taken in making conclusions about the necessity of APC/C subunits in a biological process. The authors discovering UL21a argued that APC8 was necessary for APC4 and 5 degradation by UL21a, but in light of the data presented by this work, APC4 and 5 degradation are instead a consequence of APC8 knockdown.

The mechanism of this APC/C subunit down-modulation is not known, but possibilities include active degradation, reduced stability of nascent APC/C subunits due to improper folding without the necessary binding partners, or reduced rate of synthesis of subunits when one or more is missing. The distinction

could be made by assaying the protein half-lives of the APC/C subunits during subunit knockdown by siRNA. Determining whether depletion of these subunits is proteasome dependent would be problematic, as adding proteasome inhibitor could interfere with the knockdown itself, though this has not been tested in our lab. Only one report was found reporting changes in the levels of APC/C subunits, showing that levels of APC5 drop as K562 cells (a leukemia cell line) is induced to differentiate into megakaryocytes (110). In that study, levels of other APC/C were not assayed.

Several further questions remain. First, what is the site of UL21a binding and mechanism of action. Secondly, what role in viral pathogenesis is played by APC/C inactivation during host infection? The first question was addressed but not resolved by a binding screen using GST-UL21a as bait for in vitro translated APC/C subunits. The results of the assay were inconclusive, as none of the tested subunits showed a convincing affinity for GST-UL21a (Unpublished). It is possible that one of the untested subunits may have been a binding partner, as only APC1, 4, 5, 8, and 11 were tested. It is also possible that UL21a recognizes a shape formed by the junction between subunits, that individual subunits translated in vitro do not fold correctly, or that there is a cellular intermediate necessary for the interaction.

The nature of APC/C subunit degradation by UL21a is also unclear. Subunits are protected by proteasome inhibitor during infection (48) and when UL21a is expressed in uninfected cells (Chapter 3). However, whether the subunits are being degraded in a ubiquitin-dependent fashion is unknown. It was

reported that UL21a degradation itself is proteasome-dependent but ubiquitin-independent (49) . It is possible that the proteasome-mediated degradation of the APC/C subunits and of UL21a is not a conventional degradation. Karen Tran showed in her 2010 publication that proteasome subunits relocalize during viral infections (111). Specifically, proteasome subunit Rpn2, which possesses PC (proteasome/cyclosome) repeat domains found in APC1, (112) localizes to viral replication centers. It is possible that an uncharacterized interaction between the APC/C and proteasome takes place during infection that might explain the down-modulation of the majority of the complex.

The second unanswered question involves the role in viral pathogenesis of APC/C inactivation. APC/C may be inactivated to promote accumulation of a substrate which would benefit the infection. To date the APC/C has over 60 substrates. Two likely candidate substrates, Geminin and PFKFB3, were knocked down by siRNA during infection, but knockdown had no effect on the progression of the infection (Unpublished). Geminin seemed a likely choice because its accumulation would lead to inhibition of cellular DNA synthesis, and its increase in levels upon infection is one of the most drastic of all the substrates we have measured. PFKFB3 was included because the regulation of metabolism by both the APC/C and by HCMV has gained much recent attention (78, 113-118). It is also possible that an antiviral non-ligase function of an APC/C subunit is inhibited when APC1, 4, and 5 are degraded. APC5 has been reported to interact with Poly(A) binding protein to suppress IRES-mediated translation (110) and to interact with A20 to inhibit IL-17 signaling (106). Finally, it is possible that one or

more subunits is useful to the virus for some other purpose. No work to date has examined the fate of the catalytic core subunits APC2 and 11, which are not degraded during infection, nor has further work been performed on the TPR subunits, which relocalize to the cytoplasm during infection. Further studies will be required to determine whether APC/C ubiquitin ligase activity or some other aspect of the APC/C or one of its subunits is the true target of HCMV-mediated deregulation and important for pathogenesis of this virus.

There are clinical implications to inactivation of the APC/C by HCMV. An important role of the APC/C is in neuronal development and survival, and several in vitro studies have shown that APC/C is active and necessary in post-mitotic neurons (26-32). More recently studied is its requirement in vivo for proper brain development and memory formation. A recent study by Esteban et al (119) shows that developing mouse embryo brain size but not body size decreases when Cdh1 is knocked out. It is interesting to note that a typical birth defect caused by HCMV infection is microcephaly, and while it is most likely a highly multifactorial process, APC/C inhibition may play a role. The results of another study by Kuczera et al (120) showed that conditional deletion of APC2 in postmitotic excitatory forebrain neurons of adult mice caused impaired spatial memory and an increased maintenance of fear memory.

Through this work, we have broadened the fields of both virology and cell biology with novel findings relating to inactivation of the APC/C E3 ubiquiting ligase by HCMV and to the new phenomenon of APC/C subunit down-modulation upon destabilization of the APC/C complex. The immediate relevance to current studies

in cell biology lies in the inconclusiveness of any study that claims a specific role for APC/C subunits 1, 4, 5, or 8 based on a knock down experiment. The data presented here show that knock down of one may lead to the disappearance of all. Further work is required to find the cell type specificity of this event as well as the molecular mechanisms governing it.

REFERENCES

1. **Malumbres M.** 2014. Cyclin-dependent kinases. Genome Biol **15:**122.

2. **Malumbres M, Barbacid M.** 2005. Mammalian cyclin-dependent kinases. Trends in biochemical sciences **30:**630-641.

3. **McGarry TJ, Kirschner MW.** 1998. Geminin, an inhibitor of DNA replication, is degraded during mitosis. Cell **93:**1043-1053.

4. **Tada S, Li A, Maiorano D, Mechali M, Blow JJ.** 2001. Repression of origin assembly in metaphase depends on inhibition of RLF-B/Cdt1 by geminin. Nat. Cell Biol. **3:**107-113.

5. **Wohlschlegel JA, Dwyer BT, Dhar SK, Cvetic C, Walter JC, Dutta A.** 2000. Inhibition of eukaryotic DNA replication by geminin binding to Cdt1. Science **290:**2309-2312.

6. **Cardozo T, Pagano M.** 2004. The SCF ubiquitin ligase: insights into a molecular machine. Nat Rev Mol Cell Biol **5:**739-751.

7. **Sudakin V, Ganoth D, Dahan A, Heller H, Hershko J, Luca FC, Ruderman JV, Hershko A.** 1995. The cyclosome, a large complex containing cyclin-selective ubiquitin ligase activity, targets cyclins for destruction at the end of mitosis. Mol Biol Cell **6:**185-197.

8. **Chang L, Zhang Z, Yang J, McLaughlin SH, Barford D.** 2014. Molecular architecture and mechanism of the anaphase-promoting complex. Nature.

9. **Passmore LA, McCormack EA, Au SW, Paul A, Willison KR, Harper JW, Barford D.** 2003. Doc1 mediates the activity of the anaphase-promoting complex by contributing to substrate recognition. EMBO J **22:**786-796.

10. **Wu T, Merbl Y, Huo Y, Gallop JL, Tzur A, Kirschner MW.** 2010. UBE2S drives elongation of K11-linked ubiquitin chains by the anaphase-promoting complex. Proc. Natl. Acad. Sci. U. S. A. **107:**1355-1360.

11. **Rape M.** 2010. Assembly of k11-linked ubiquitin chains by the anaphase-promoting complex. Subcell Biochem **54:**107-115.

12. **Glotzer M, Murray AW, Kirschner MW.** 1991. Cyclin is degraded by the ubiquitin pathway. Nature **349:**132-138.

13. **Fang G, Yu H, Kirschner MW.** 1998. Direct binding of CDC20 protein family members activates the anaphase-promoting complex in mitosis and G1. Mol Cell **2:**163-171.

14. **King RW, Glotzer M, Kirschner MW.** 1996. Mutagenic analysis of the destruction signal of mitotic cyclins and structural characterization of ubiquitinated intermediates. Mol Biol Cell **7:**1343-1357.

15. **Pfleger CM, Kirschner MW.** 2000. The KEN box: an APC recognition signal distinct from the D box targeted by Cdh1. Genes Dev **14:**655-665.

16. **Pfleger CM, Lee E, Kirschner MW.** 2001. Substrate recognition by the Cdc20 and Cdh1 components of the anaphase-promoting complex. Genes Dev **15:**2396-2407. 17. **Lu Y, Wang W, Kirschner MW.** 2015. Specificity of the anaphase-promoting complex: a single-molecule study. Science **348:**1248737.

18. **Irniger S, Nasmyth K.** 1997. The anaphase-promoting complex is required in G1 arrested yeast cells to inhibit B-type cyclin accumulation and to prevent uncontrolled entry into S-phase. J Cell Sci **110 (Pt 13):**1523-1531.

19. **Kotani S, Tanaka H, Yasuda H, Todokoro K.** 1999. Regulation of APC activity by phosphorylation and regulatory factors. J Cell Biol **146:**791-800.

20. **Hsu JY, Reimann JD, Sorensen CS, Lukas J, Jackson PK.** 2002. E2F-dependent accumulation of hEmi1 regulates S phase entry by inhibiting APC(Cdh1). Nat. Cell Biol. **4:**358-366.

21. **Wang W, Kirschner MW.** 2013. Emi1 preferentially inhibits ubiquitin chain elongation by the anaphase-promoting complex. Nat. Cell Biol. **15:**797-806.

22. **Cohen-Fix O, Peters JM, Kirschner MW, Koshland D.** 1996. Anaphase initiation in Saccharomyces cerevisiae is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. Genes Dev **10:**3081-3093.

23. **Funabiki H, Yamano H, Kumada K, Nagao K, Hunt T, Yanagida M.** 1996. Cut2 proteolysis required for sister-chromatid seperation in fission yeast. Nature **381:**438-441.

24. **Yamamoto A, Guacci V, Koshland D.** 1996. Pds1p, an inhibitor of anaphase in budding yeast, plays a critical role in the APC and checkpoint pathway(s). J Cell Biol **133:**99-110.

25. **Zou H, McGarry TJ, Bernal T, Kirschner MW.** 1999. Identification of a vertebrate sister-chromatid separation inhibitor involved in transformation and tumorigenesis. Science **285:**418-422.

26. **Gieffers C, Peters BH, Kramer ER, Dotti CG, Peters JM.** 1999. Expression of the CDH1-associated form of the anaphase-promoting

complex in postmitotic neurons. Proc. Natl. Acad. Sci. U.S.A. **96:**11317-11322.

27. **Lasorella A, Stegmuller J, Guardavaccaro D, Liu G, Carro MS, Rothschild G, de la Torre-Ubieta L, Pagano M, Bonni A, Iavarone A.** 2006. Degradation of Id2 by the anaphase-promoting complex couples cell cycle exit and axonal growth. Nature **442:**471-474.

28. **Yang Y, Kim AH, Yamada T, Wu B, Bilimoria PM, Ikeuchi Y, de la Iglesia N, Shen J, Bonni A.** 2009. A Cdc20-APC ubiquitin signaling pathway regulates presynaptic differentiation. Science **326:**575-578.

29. **Konishi Y, Stegmuller J, Matsuda T, Bonni S, Bonni A.** 2004. Cdh1-APC controls axonal growth and patterning in the mammalian brain. Science **303:**1026-1030.

30. **Stegmuller J, Huynh MA, Yuan Z, Konishi Y, Bonni A.** 2008. TGFbeta-Smad2 signaling regulates the Cdh1-APC/SnoN pathway of axonal morphogenesis. J. Neurosci. **28:**1961-1969.

31. **Stegmuller J, Konishi Y, Huynh MA, Yuan Z, Dibacco S, Bonni A.** 2006. Cell-intrinsic regulation of axonal morphogenesis by the Cdh1-APC target SnoN. Neuron **50:**389-400.

32. **Teng FY, Tang BL.** 2005. APC/C regulation of axonal growth and synaptic functions in postmitotic neurons: the Liprin-alpha connection. Cellular and molecular life sciences : CMLS **62:**1571-1578.

33. **Huang J, Ikeuchi Y, Malumbres M, Bonni A.** 2015. A Cdh1-APC/FMRP Ubiquitin Signaling Link Drives mGluR-Dependent Synaptic Plasticity in the Mammalian Brain. Neuron **86:**726-739.

34. **Spector DH.** 2015. Human cytomegalovirus riding the cell cycle. Med Microbiol Immunol **204:**409-419.

35. **Jault FM, Jault JM, Ruchti F, Fortunato EA, Clark C, Corbeil J, Richman DD, Spector DH.** 1995. Cytomegalovirus infection induces high levels of cyclins, phosphorylated Rb, and p53, leading to cell cycle arrest. J. Virol. **69:**6697-6704.

36. **Lu M, Shenk T.** 1996. Human cytomegalovirus infection inhibits cell cycle progression at multiple points, including the transition from G1 to S. J. Virol. **70:**8850-8857.

37. **Bresnahan WA, Boldogh I, Thompson EA, Albrecht T.** 1996. Human cytomegalovirus inhibits cellular DNA synthesis and arrests productively infected cells in late G1. Virology **224:**150-160.

38. **Dittmer D, Mocarski ES.** 1997. Human cytomegalovirus infection inhibits G1/S transition. J. Virol. **71:**1629-1634.

39. **Salvant BS, Fortunato EA, Spector DH.** 1998. Cell cycle dysregulation by human cytomegalovirus: influence of the cell cycle phase at the time of infection and effects on cyclin transcription. J. Virol. **72:**3729-3741.

40. **Wiebusch L, Hagemeier C.** 1999. Human cytomegalovirus 86-kilodalton IE2 protein blocks cell cycle progression in G(1). J. Virol. **73:**9274-9283.

41. **Lu M, Shenk T.** 1999. Human cytomegalovirus UL69 protein induces cells to accumulate in G1 phase of the cell cycle. J. Virol. **73:**676-683.

42. **Bogdanow B, Weisbach H, von Einem J, Straschewski S, Voigt S, Winkler M, Hagemeier C, Wiebusch L.** 2013. Human cytomegalovirus tegument protein pp150 acts as a cyclin A2-CDK-dependent sensor of the host cell cycle and differentiation state. Proc. Natl. Acad. Sci. U. S. A. **110:**17510-17515.

43. **Caffarelli N, Fehr AR, Yu D.** 2013. Cyclin A degradation by primate cytomegalovirus protein pUL21a counters its innate restriction of virus replication. PLoS Pathog. **9:**e1003825.

44. **Eifler M, Uecker R, Weisbach H, Bogdanow B, Richter E, Konig L, Vetter B, Lenac-Rovis T, Jonjic S, Neitzel H, Hagemeier C, Wiebusch L.** 2014. PUL21a-Cyclin A2 interaction is required to protect human cytomegalovirus-infected cells from the deleterious consequences of mitotic entry. PLoS Pathog. **10:**e1004514.

45. **Biswas N, Sanchez V, Spector DH.** 2003. Human cytomegalovirus infection leads to accumulation of geminin and inhibition of the licensing of cellular DNA replication. J. Virol. **77:**2369-2376.

46. **Wiebusch L, Bach M, Uecker R, Hagemeier C.** 2005. Human cytomegalovirus inactivates the G0/G1-APC/C ubiquitin ligase by Cdh1 dissociation. Cell Cycle **4:**1435-1439.

47. **Tran K, Mahr JA, Choi J, Teodoro JG, Green MR, Spector DH.** 2008. Accumulation of substrates of the anaphase-promoting complex (APC) during human cytomegalovirus infection is associated with the phosphorylation of Cdh1 and the dissociation and relocalization of APC subunits. J. Virol. **82:**529-537.

48. **Tran K, Kamil JP, Coen DM, Spector DH.** 2010. Inactivation and disassembly of the anaphase-promoting complex during human cytomegalovirus infection is associated with degradation of the APC5 and

APC4 subunits and does not require UL97-mediated phosphorylation of Cdh1. J. Virol. **84:**10832-10843.

49. **Fehr AR, Gualberto NC, Savaryn JP, Terhune SS, Yu D.** 2012. Proteasome-dependent disruption of the E3 ubiquitin ligase anaphase-promoting complex by HCMV protein pUL21a. PLoS Pathog. **8:**e1002789.

50. **Yu D, Silva MC, Shenk T.** 2003. Functional map of human cytomegalovirus AD169 defined by global mutational analysis. Proc. Natl. Acad. Sci. U.S.A. **100:**12396-12401.

51. **Fehr AR, Yu D.** 2010. Human cytomegalovirus gene UL21a encodes a short-lived cytoplasmic protein and facilitates virus replication in fibroblasts. J. Virol. **84:**291-302.

52. **Fehr AR, Yu D.** 2011. Human cytomegalovirus early protein pUL21a promotes efficient viral DNA synthesis and the late accumulation of immediate-early transcripts. J. Virol. **85:**663-674.

53. **Krosky PM, Baek MC, Coen DM.** 2003. The human cytomegalovirus UL97 protein kinase, an antiviral drug target, is required at the stage of nuclear egress. J. Virol. **77:**905-914.

54. **Hamirally S, Kamil JP, Ndassa-Colday YM, Lin AJ, Jahng WJ, Baek MC, Noton S, Silva LA, Simpson-Holley M, Knipe DM, Golan DE, Marto JA, Coen DM.** 2009. Viral mimicry of Cdc2/cyclin-dependent kinase 1 mediates disruption of nuclear lamina during human cytomegalovirus nuclear egress. PLoS Pathog. **5:**e1000275.

55. **Hume AJ, Finkel JS, Kamil JP, Coen DM, Culbertson MR, Kalejta RF.** 2008. Phosphorylation of retinoblastoma protein by viral protein with cyclin-dependent kinase function. Science **320:**797-799.

56. **Iwahori S, Hakki M, Chou S, Kalejta RF.** 2015. Molecular Determinants for the Inactivation of the Retinoblastoma Tumor Suppressor by the Viral Cyclin-dependent Kinase UL97. J. Biol. Chem.

57. **Krosky PM, Baek M-C, Jahng WJ, Barrera I, Harvey RJ, Biron KK, Coen DM, Sethna PB.** 2003. The human cytomegalovirus UL44 protein is a substrate for the UL97 protein kinase. J. Virol. **77:**7720-7727.

58. **Littler E, Stuart AD, Chee MS.** 1992. Human cytomegalovirus UL97 open reading frame encodes a protein that phosphorylates the antiviral nucleoside analogue ganciclovir. Nature **358:**160-162.

59. **Sullivan V, Talarico CL, Stanat SC, Davis M, Coen DM, Biron KK.** 1992. A protein kinase homologue controls phosphorylation of ganciclovir in human cytomegalovirus-infected cells. Nature **359:**85.

60. **Lurain NS, Chou S.** 2010. Antiviral drug resistance of human cytomegalovirus. Clin Microbiol Rev **23:**689-712.

61. **Chou S.** 2009. Diverse cytomegalovirus UL27 mutations adapt to loss of viral UL97 kinase activity under maribavir. Antimicrobial agents and chemotherapy **53:**81-85.

62. **Kamil JP, Hume AJ, Jurak I, Münger K, Kalejta RF, Coen DM.** 2009. Human papillomavirus 16 E7 inactivator of retinoblastoma family proteins complements human cytomegalovirus lacking UL97 protein kinase. Proc. Natl. Acad. Sci. U.S.A. **106:**16823-16828.

63. **Fehr AR, Yu D.** 2013. Control the host cell cycle: viral regulation of the anaphase-promoting complex. J. Virol. **87:**8818-8825.

64. **Mo M, Shahar S, Fleming SB, Mercer AA.** 2012. How viruses affect the cell cycle through manipulation of the APC/C. Trends Microbiol **20:**440-448.

65. **Heilman DW, Teodoro JG, Green MR.** 2006. Apoptin nucleocytoplasmic shuttling is required for cell type-specific localization, apoptosis, and recruitment of the anaphase-promoting complex/cyclosome to PML bodies. J. Virol. **80:**7535-7545.

66. **Teodoro JG, Heilman DW, Parker AE, Green MR.** 2004. The viral protein Apoptin associates with the anaphase-promoting complex to induce G2/M arrest and apoptosis in the absence of p53. Genes Dev. **18:**1952-1957.

67. **Mo M, Fleming SB, Mercer AA.** 2009. Cell cycle deregulation by a poxvirus partial mimic of anaphase-promoting complex subunit 11. Proc. Natl. Acad. Sci. U. S. A. **106:**19527-19532.

68. **Mo M, Fleming SB, Mercer AA.** 2010. Orf virus cell cycle regulator, PACR, competes with subunit 11 of the anaphase promoting complex for incorporation into the complex. J. Gen. Virol. **91:**3010-3015.

69. **Kuo YL, Giam CZ.** 2006. Activation of the anaphase promoting complex by HTLV-1 tax leads to senescence. EMBO J **25:**1741-1752.

70. **Liu B, Hong S, Tang Z, Yu H, Giam CZ.** 2005. HTLV-I Tax directly binds the Cdc20-associated anaphase-promoting complex and activates it ahead of schedule. Proc. Natl. Acad. Sci. U. S. A. **102:**63-68.

71. **Merling R, Chen C, Hong S, Zhang L, Liu M, Kuo YL, Giam CZ.** 2007. HTLV-1 Tax mutants that do not induce G1 arrest are disabled in activating the anaphase promoting complex. Retrovirology **4:**35.

72. **Sakaue-Sawano A, Kurokawa H, Morimura T, Hanyu A, Hama H, Osawa H, Kashiwagi S, Fukami K, Miyata T, Miyoshi H, Imamura T, Ogawa M, Masai H, Miyawaki A.** 2008. Visualizing spatiotemporal dynamics of multicellular cell-cycle progression. Cell **132:**487-498.

73. **Paladino P, Marcon E, Greenblatt J, Frappier L.** 2014. Identification of herpesvirus proteins that contribute to G1/S arrest. J. Virol. **88:**4480-4492.

74. **Qian Z, Xuan B, Hong TT, Yu D.** 2008. The full-length protein encoded by human cytomegalovirus gene UL117 is required for the proper maturation of viral replication compartments. J. Virol. **82:**3452-3465.

75. **Wiebusch L, Uecker R, Hagemeier C.** 2003. Human cytomegalovirus prevents replication licensing by inhibiting MCM loading onto chromatin. EMBO Rep **4:**42-46.

76. **Konishi Y, Stegmüller J, Matsuda T, Bonni S, Bonni A.** 2004. Cdh1-APC controls axonal growth and patterning in the mammalian brain. Science **303:**1026-1030.

77. **Almeida A, Bolaños JP, Moreno S.** 2005. Cdh1/Hct1-APC is essential for the survival of postmitotic neurons. J. Neurosci. **25:**8115-8121.

78. **Herrero-Mendez A, Almeida A, Fernandez E, Maestre C, Moncada S, Bolanos JP.** 2009. The bioenergetic and antioxidant status of neurons is controlled by continuous degradation of a key glycolytic enzyme by APC/C-Cdh1. Nat. Cell Biol. **11:**747-752.

79. **Uzunova K, Dye BT, Schutz H, Ladurner R, Petzold G, Toyoda Y, Jarvis MA, Brown NG, Poser I, Novatchkova M, Mechtler K, Hyman AA, Stark H, Schulman BA, Peters JM.** 2012. APC15 mediates CDC20 autoubiquitylation by APC/C(MCC) and disassembly of the mitotic checkpoint complex. Nat. Struct. Mol. Biol. **19:**1116-1123.

80. **Thornton BR, Ng TM, Matyskiela ME, Carroll CW, Morgan DO, Toczyski DP.** 2006. An architectural map of the anaphase-promoting complex. Genes Dev. **20:**449-460.

81. **Bellanger S, Blachon S, Mechali F, Bonne-Andrea C, Thierry F.** 2005. High-risk but not low-risk HPV E2 proteins bind to the APC activators Cdh1 and Cdc20 and cause genomic instability. Cell cycle **4:**1608-1615.

82. **Sanders RL, Clark CL, Morello CS, Spector DH.** 2008. Development of cell lines that provide tightly controlled temporal translation of the human cytomegalovirus IE2 proteins for complementation and functional analyses of growth-impaired and nonviable IE2 mutant viruses. J. Virol. **82:**7059-7077.

83. **Nolden L, Edenhofer F, Peitz M, Brustle O.** 2007. Stem cell engineering using transducible Cre recombinase. Methods Mol. Med. **140:**17-32.

84. **DuRose JB, Li J, Chien S, Spector DH.** 2012. Infection of vascular endothelial cells with human cytomegalovirus under fluid shear stress reveals preferential entry and spread of virus in flow conditions simulating atheroprone regions of the artery. J. Virol. **86:**13745-13755.

85. **Weekes MP, Tomasec P, Huttlin EL, Fielding CA, Nusinow D, Stanton RJ, Wang EC, Aicheler R, Murrell I, Wilkinson GW, Lehner PJ, Gygi SP.** 2014. Quantitative temporal viromics: an approach to investigate host-pathogen interaction. Cell **157:**1460-1472.

86. **Prichard MN, Gao N, Jairath S, Mulamba G, Krosky P, Coen DM, Parker BO, Pari GS.** 1999. A recombinant human cytomegalovirus with a large deletion in UL97 has a severe replication deficiency. J. Virol. **73:**5663-5670.

87. **Baek M-C, Krosky PM, Coen DM.** 2002. Relationship between autophosphorylation and phosphorylation of exogenous substrates by the human cytomegalovirus UL97 protein kinase. J. Virol. **76:**11943-11952.

88. **Kamil JP, Coen DM.** 2007. Human cytomegalovirus protein kinase UL97 forms a complex with the tegument phosphoprotein pp65. J. Virol. **81:**10659-10668.

89. **Marschall M, Freitag M, Suchy P, Romaker D, Kupfer R, Hanke M, Stamminger T.** 2003. The protein kinase pUL97 of human cytomegalovirus interacts with and phosphorylates the DNA polymerase processivity factor pUL44. Virology **311:**60-71.

90. **Thomas M, Rechter S, Milbradt J, Auerochs S, Muller R, Stamminger T, Marschall M.** 2009. Cytomegaloviral protein kinase pUL97 interacts with the nuclear mRNA export factor pUL69 to modulate its intranuclear localization and activity. J. Gen. Virol. **90:**567-578.

91. **Baek M-C, Krosky PM, Pearson A, Coen DM.** 2004. Phosphorylation of the RNA polymerase II carboxyl-terminal domain in human cytomegalovirus-infected cells and in vitro by the viral UL97 protein kinase. Virology **324:**184-193.

92. **Kawaguchi Y, Matsumura T, Roizman B, Hirai K.** 1999. Cellular elongation factor 1delta is modified in cells infected with representative alpha-, beta-, or gammaherpesviruses. J. Virol. **73:**4456-4460.

93. **Marschall M, Marzi A, aus dem Siepen P, Jochmann R, Kalmer M, Auerochs S, Lischka P, Leis M, Stamminger T.** 2005. Cellular p32 recruits cytomegalovirus kinase pUL97 to redistribute the nuclear lamina. J. Biol. Chem. **280:**33357-33367.

94. **Prichard MN, Sztul E, Daily SL, Perry AL, Frederick SL, Gill RB, Hartline CB, Streblow DN, Varnum SM, Smith RD, Kern ER.** 2008. Human cytomegalovirus UL97 kinase activity is required for the hyperphosphorylation of retinoblastoma protein and inhibits the formation of nuclear aggresomes. J. Virol. **82:**5054-5067.

95. **Sharma M, Bender BJ, Kamil JP, Lye MF, Pesola JM, Reim NI, Hogle JM, Coen DM.** 2014. Human Cytomegalovirus UL97 Phosphorylates the Viral Nuclear Egress Complex. J. Virol.

96. **Milbradt J, Auerochs S, Sticht H, Marschall M.** 2009. Cytomegaloviral proteins that associate with the nuclear lamina: components of a postulated nuclear egress complex. J. Gen. Virol. **90:**579-590.

97. **Wolf DG, Courcelle CT, Prichard MN, Mocarski ES.** 2001. Distinct and separate roles for herpesvirus-conserved UL97 kinase in cytomegalovirus DNA synthesis and encapsidation. Proc. Natl. Acad. Sci. U.S.A. **98:**1895-1900.

98. **Oduro JD, Uecker R, Hagemeier C, Wiebusch L.** 2012. Inhibition of human cytomegalovirus immediate-early gene expression by cyclin A2-dependent kinase activity. J. Virol. **86:**9369-9383.

99. **Gao D, Inuzuka H, Korenjak M, Tseng A, Wu T, Wan L, Kirschner M, Dyson N, Wei W.** 2009. Cdh1 regulates cell cycle through modulating the claspin/Chk1 and the Rb/E2F1 pathways. Mol Biol Cell **20:**3305-3316.

100. **Clark E, Spector DH.** 2015. Studies on the Contribution of Human Cytomegalovirus UL21a and UL97 to Viral Growth and Inactivation of the Anaphase-Promoting Complex/Cyclosome (APC/C) E3 Ubiquitin Ligase Reveal a Unique Cellular Mechanism for Downmodulation of the APC/C Subunits APC1, APC4, and APC5. J. Virol. **89:**6928-6939.

101. **Reim NI, Kamil JP, Wang D, Lin A, Sharma M, Ericsson M, Pesola JM, Golan DE, Coen DM.** 2013. Inactivation of retinoblastoma protein does not overcome the requirement for human cytomegalovirus UL97 in lamina disruption and nuclear egress. J. Virol. **87:**5019-5027.

102. **Kamil JP, Hume AJ, Jurak I, Munger K, Kalejta RF, Coen DM.** 2009. Human papillomavirus 16 E7 inactivator of retinoblastoma family proteins complements human cytomegalovirus lacking UL97 protein kinase. Proc. Natl. Acad. Sci. U. S. A. **106:**16823-16828.

103. **Schreiber A, Stengel F, Zhang Z, Enchev RI, Kong EH, Morris EP, Robinson CV, da Fonseca PC, Barford D.** 2011. Structural basis for the subunit assembly of the anaphase-promoting complex. Nature **470:**227-232.

104. **Kelly A, Wickliffe KE, Song L, Fedrigo I, Rape M.** 2014. Ubiquitin chain elongation requires E3-dependent tracking of the emerging conjugate. Mol Cell **56:**232-245.

105. **Ahlskog JK, Bjork JK, Elsing AN, Aspelin C, Kallio M, Roos-Mattjus P, Sistonen L.** 2010. Anaphase-promoting complex/cyclosome participates in the acute response to protein-damaging stress. Mol Cell Biol **30:**5608-5620.

106. **Ho AW, Garg AV, Monin L, Simpson-Abelson MR, Kinner L, Gaffen SL.** 2013. The anaphase-promoting complex protein 5 (AnapC5) associates with A20 and inhibits IL-17-mediated signal transduction. PloS one **8:**e70168.

107. **Budhavarapu VN, White ED, Mahanic CS, Chen L, Lin FT, Lin WC.** 2012. Regulation of E2F1 by APC/C Cdh1 via K11 linkage-specific ubiquitin chain formation. Cell cycle **11:**2030-2038.

108. **Masuda K, Chiyoda T, Sugiyama N, Segura-Cabrera A, Kabe Y, Ueki A, Banno K, Suematsu M, Aoki D, Ishihama Y, Saya H, Kuninaka S.** 2015. LATS1 and LATS2 phosphorylate CDC26 to modulate assembly of the tetratricopeptide repeat subcomplex of APC/C. PloS one **10:**e0118662.

109. **Sedgwick GG, Hayward DG, Di Fiore B, Pardo M, Yu L, Pines J, Nilsson J.** 2013. Mechanisms controlling the temporal degradation of Nek2A and Kif18A by the APC/C-Cdc20 complex. EMBO J **32:**303-314.

110. **Koloteva-Levine N, Pinchasi D, Pereman I, Zur A, Brandeis M, Elroy-Stein O.** 2004. The Apc5 subunit of the anaphase-promoting complex/cyclosome interacts with poly(A) binding protein and represses internal ribosome entry site-mediated translation. Mol Cell Biol **24:**3577-3587.

111. **Tran K, Mahr JA, Spector DH.** 2010. Proteasome subunits relocalize during human cytomegalovirus infection, and proteasome activity is necessary for efficient viral gene transcription. J. Virol. **84:**3079-3093.

112. **Lupas A, Baumeister W, Hofmann K.** 1997. A repetitive sequence in subunits of the 26S proteasome and 20S cyclosome (anaphase-promoting complex). Trends in biochemical sciences **22:**195-196.

113. **Vastag L, Koyuncu E, Grady SL, Shenk TE, Rabinowitz JD.** 2011. Divergent effects of human cytomegalovirus and herpes simplex virus-1 on cellular metabolism. PLoS Pathog. **7:**e1002124.

114. **Yu Y, Clippinger AJ, Alwine JC.** 2011. Viral effects on metabolism: changes in glucose and glutamine utilization during human cytomegalovirus infection. Trends Microbiol **19:**360-367.

115. **Yu Y, Clippinger AJ, Pierciey FJ, Jr., Alwine JC.** 2011. Viruses and metabolism: alterations of glucose and glutamine metabolism mediated by human cytomegalovirus. Adv Virus Res **80:**49-67.

116. **Rodriguez-Rodriguez P, Fernandez E, Almeida A, Bolanos JP.** 2012. Excitotoxic stimulus stabilizes PFKFB3 causing pentose-phosphate pathway to glycolysis switch and neurodegeneration. Cell death and differentiation **19:**1582-1589.

117. **Tudzarova S, Colombo SL, Stoeber K, Carcamo S, Williams GH, Moncada S.** 2011. Two ubiquitin ligases, APC/C-Cdh1 and SKP1-CUL1-F (SCF)-beta-TrCP, sequentially regulate glycolysis during the cell cycle. Proc. Natl. Acad. Sci. U. S. A. **108:**5278-5283.

118. **Almeida A, Bolanos JP, Moncada S.** 2010. E3 ubiquitin ligase APC/C-Cdh1 accounts for the Warburg effect by linking glycolysis to cell proliferation. Proc. Natl. Acad. Sci. U. S. A. **107:**738-741.

119. **Delgado-Esteban M, Garcia-Higuera I, Maestre C, Moreno S, Almeida A.** 2013. APC/C-Cdh1 coordinates neurogenesis and cortical size during development. Nat Commun **4:**2879.

120. **Kuczera T, Stilling RM, Hsia HE, Bahari-Javan S, Irniger S, Nasmyth K, Sananbenesi F, Fischer A.** 2011. The anaphase promoting complex is required for memory function in mice. Learning & memory (Cold Spring Harbor, N.Y.) **18:**49-57.