Characterizing the protein function, gene expression, and viral replication of chicken anemia virus

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ABSTRACT

Chicken anemia virus (CAV) is a small, non-enveloped, single-stranded DNA virus that replicates in the thymocytes and erythroblasts of young chickens, causing severe and often fatal anemia. Its circular genome encodes a single mRNA expressing three proteins: Vp1, the capsid protein, Vp2, a dual specificity phosphatase, and Vp3, a virulence factor termed apoptin. Apoptin is notable for its ability to induce p53-independent apoptosis in human cancer cells and inhibits the anaphase promoting complex/cyclosome (APC/C), causing cell cycle arrest in G2/M phase. In this thesis, we characterize the expression of the viral proteins in CAV and their role in viral replication. We confirm that apoptin is not necessary for the production of infectious viral particles, but that apoptin-knockout virus has decreased replicative efficiency and cannot induce cell cycle arrest or cell death. We show data that suggests that specific inhibition of the APC/C, but not mitotic arrest alone, rescues viral genomic replication efficiency in the absence of apoptin. We further confirm that the other CAV proteins, Vp1 and Vp2, are essential for viral reproduction and have critical roles in genomic replication. We observe differences in the temporal expression of proteins, with apoptin expressed earlier and Vp1 increasingly expressed later in infection. As we previously observed that G2/M arrest in CAV infection is correlated with global downregulation of cap-dependent translation, we are also investigating whether the CAV mRNA is capable of cap-independent translation. We propose a model of the CAV life cycle in which apoptin mediates G2/M arrest, inhibiting host translation and shifting viral infection from early to late phase.

RÉSUMÉ

Le virus de l'anémie infectieuse du poulet (CAV) est un petit virus, non-enveloppé, à ADN simple brin qui se réplique dans les thymocytes et les érythroblastes des jeunes poulets. Il provoque une anémie grave qui est souvent mortelle. Son génome circulaire se transcrit qu'en un seul ARNm qui code pour trois protéines : Vp1: la seule protéine de la capside, Vp2: une phosphatase à double spécificité, et Vp3: un facteur de virulence appelé apoptine. L'apoptine est remarquable pour sa capacité à induire l'apoptose indépendamment de p53, dans les cellules cancéreuses humaines, ainsi que celle d'inhiber le complexe promoteur de l'anaphase/cyclosome (APC/C) causant l'arrêt du cycle cellulaire en phase G2/M. Dans cette thèse, nous caractérisons l'expression des protéines virales de CAV et leur rôle dans la réplication virale. Nous confirmons que l'apoptine n'est pas nécessaire pour la production de particules virales infectieuses, mais, qu'en l'absence d'apoptine, l'efficacité de la réplication du virus est diminuée et que celui-ci ne peut pas induire l'arrêt du cycle cellulaire ou l'apoptose. Nos données suggèrent que l'inhibition spécifique de l'APC/C, et non seulement l'arrêt mitotique, peut rétablir l'efficacité de la réplication du génome viral en l'absence d'apoptine. De plus, nous confirmons que les autres protéines de CAV: Vp1 et Vp2, sont nécessaires pour la reproduction du virus et jouent un rôle essentiel dans la réplication du génome viral. Nous avons aussi remarqué des différences dans l'expression temporelle des protéines où l'apoptine s'exprime plus tôt que Vp1 durant l'infection. Comme nous l'avons précédemment noté, l'arrêt du cycle cellulaire en phase G2/M pendant une infection causée par CAV corrèle avec la diminution globale de la traduction dépendante de la coiffe. Maintenant, nous cherchons à savoir si l'ARNm de CAV peut être traduit d'une façon indépendante de la coiffe. Nous proposons donc un modèle de réplication pour CAV dans lequel l'apoptine médie un arrêt en G2/M pour inhiber la traduction de l'hôte, ce qui permet de faire passer l'infection de la phase précoce vers la phase tardive.

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It feels like an impossible task to find the right words to articulate and capture all the contributions of the people that have helped and enabled me to do the work presented in this thesis. I owe my sincerest thanks and deepest gratitude to those who have supported me, in no small number of ways, in this work and in my time at McGill.

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The staff at the Flow Cytometry Core Facility were instrumental in providing training and technical assistance with my work using the flow cytometer. Dr. Francis Robert from the Pelletier Lab generously provided advice, reagents, and assistance with the experiments for IRES evaluation. The anti-apoptin antibody used in Western blots was kindly provided by Dr. Mahvash Tavassoli of King's College London.

The Teodoro Lab has become a home to me. Thank you all for creating such an inviting, friendly, and supportive space for both scientific learning and personal growth. I am lucky to have a wonderful community of colleagues to work with, something that has never been clearer

to me than this year, working remotely or in shifts and missing our lab's energy every day. I must give special thanks to several lab members in particular for their tremendous assistance.

Although Isabelle Gamache already knows this and would tell it herself, I would like to voice my affirmation that she is "the best research assistant in the world". Thank you for your guidance in experimental protocols, your advice in troubleshooting, your sharing of institutional knowledge, your connections across the McGill community, and the immeasurable work you do in organizing the lab to make it a productive, functional, safe, and enjoyable workspace.

Much of the work in this thesis would not have been possible without the help of Yingke Liang, an incredibly talented and wise undergraduate student whom I worked with throughout my Master's. Thank you for being a second brain, my extra set of hands, and so generous with your time. I am grateful for our ability to work together, your eagerness to do good work and your adaptability in putting up with my ever-changing schedule and ever-running thoughts.

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In addition, I want to thank former lab members David Sharon and Gabriel Barragán Bravo, both of whom I did not get to overlap with during my time in the lab, but who worked on

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I am immensely grateful for the financial support I received from the National Science and Engineering Research Council of Canada both as an undergraduate and graduate student working on this project. I am also thankful to the Goodman Cancer Research Centre for the Canderel Travel Award that allowed me to attend my first scientific conference in 2019 and present my work to a wider audience for the first time.

To the friends that have kept me going – Yael Ripstein, Sean Jiang, Chris Wong, Kelsey Capiendo, Jade Milaney, Patricia Luk – thank you for the endless love and encouragement. How lucky I am to have found such gems of humans in this life. Thank you for reminding me to take breaks, filling me with joy and dealing with every version of me, even my least favourite ones.

Nothing I can achieve is possible without the support of my family. Mom, Dad, Jess – thank you for the unconditional love and for believing in me more than I usually do myself.

This thesis and the work presented in it were completed on unceded Indigenous lands, on traditional territory of the Kanien'kehá:ka. These lands and waters have long served many First Peoples, including the Kanien'kehá:ka of the Haudenosaunee Confederacy, the Huron/Wendat, Abenaki, and Anishinaabeg. As a Settler on this land, I am working to recognize the past and present Indigenous communities that make my life and work in Tiohtià:ke (Montreal) possible. I am cognizant of my privilege in being able to write and submit this thesis amidst the COVID-19 pandemic and during ongoing fights for racial justice happening across the continent and world.

This thesis is dedicated in loving memory of Tom Zhou. Thank you for growing a love of science so great that it could be shared with all those around you.

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PREFACE AND AUTHOR CONTRIBUTIONS

This thesis is prepared in compliance with the guidelines for thesis preparation outlined by Graduate and Postdoctoral Studies at McGill University.

Parts of the introduction in this thesis (Section 1.0) are adapted from a review article published in *Pathogens* **2020**, *9*(4), 294, entitled "The role of apoptin in chicken anemia virus replication", for which I am the first author. The other authors are undergraduate lab student Yingke Liang and my thesis supervisor Dr. Jose Teodoro. Figure 1 and an earlier version of Figure 3 were previously published in this review.

Figure 2 is an adapted schematic from a previous figure published by our lab in a review article written by Linda Smolders and Dr. Jose Teodoro, titled "Targeting the anaphase promoting complex: common pathways for viral infection and cancer therapy", published in *Expert Opinion on Therapeutic Targets* **2011**, *15*(6), 767-680.

As the author of this thesis, I performed all the work presented in it, with some notable help as detailed here. Much of the work included in Figures 4, 5, 6, 7 and 9 was done with the assistance of undergraduate trainee Yingke Liang who worked alongside me on this project from May 2019 to April 2020. The mutagenesis for the creation of the apoptin-knockout CAV (Figure 5A) was done by former lab student David Sharon, and experiments characterizing CAVΔApo virus and its rescue through APC/C inhibition were first performed by him, although these experiments were fully repeated in the preparation of this thesis (thus generating original data for each figure). All starting bicistronic luciferase constructs were provided by the lab of Dr. Jerry Pelletier (McGill University), along with Krebs extract, translation mix, and salt mix for the *in vitro* translation luciferase assay. Former lab member Gabriel Barragán Bravo was the first to create a set of bicistronic luciferase reporters for this project, though they were not ultimately

used in this thesis for the *in vitro* assays testing for IRES activity. The anti-Vp1 antibody was a custom antibody previously purified by Gabriel Barragán Bravo while the anti-apoptin antibody was provided by the lab of Dr. Mahvash Tavassoli (King's College London). Julien Leconte of the Flow Cytometry Core Facility assisted with flow cytometer set up and gating strategy.

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LIST OF ABBREVIATIONS

4E-BP1	eIF4E binding protein 1
APC/C	anaphase promoting complex/cyclosome
BFDV	beak and feather disease virus
CAV	chicken anemia virus
Cdc20	cell division cycle 20
Cdh1	Cdc20 homology 1
Cdk1	cyclin-dependent kinase 1
Cdk2	cyclin-dependent kinase 2
Chk1	checkpoint kinase 1
Chk2	checkpoint kinase 2
DED	death effector domain
DEDAF	death effector domain-associated factor
DDR	DNA damage response
DMSO	dimethyl sulfoxide
eIF	eukaryotic initiation factor
FADD	Fas-activated death domain
FBS	fetal bovine serum
GEF	guanine exchange factor
HBV	hepatitis B virus
HCV	hepatitis C virus
HCMV	human cytomegalovirus
HGyV	human gyrovirus
Hip-1	Huntingtin-interacting protein 1
Hippi	Hip-1 protein interactor
HIV-1	human immunodeficiency virus 1
HPV	human papillomavirus
HSV-1	herpes simplex virus 1
HTLV-1	human T lymphotrophic virus 1
IR	isoleucine-arginine
IRES	internal ribosomal entry site

MCC	mitotic checkpoint complex
MCS	multiple cloning site
MDV	Marek's disease virus
mTORC1	mechanistic target of rapamycin complex 1
NES	nuclear export signal
NLS	nuclear localization signal
ORF	open reading frame
ORFV	open reading frame virus
PABP	poly-A binding protein
PACR	poxvirus APC/cyclosome regulator
PBS	phosphate buffered saline
PCV-1	porcine circovirus 1
PCV-2	porcine circovirus 2
PKA	protein kinase A
PKC	protein kinase C
PKR	protein kinase R
Plk1	Polo-like kinase
PML	promyelocytic leukemia
PVDF	polyvinylidene fluoride
qPCR	quantitative polymerase chain reaction
RCR	rolling circle replication
RF	replicative form
SAC	spindle assembly checkpoint
SCF	Skp cullin F-box
TAME	tosyl-arginine methyl ester
TBS-T	Tris-buffered saline-Tween
TK	thymidine kinase
TTV	torque teno virus

1.0 INTRODUCTION

1.1 Chicken anemia virus

Chicken anemia virus (CAV), the causative agent of chicken infectious anemia, is the type species of the genus Gyrovirus, and until 2011 was the only Gyrovirus member identified (1, 2). CAV was first isolated by Yuasa et al. in 1979 for its ability to cause disease in chickens (2). In young chicks, the disease is generally lethal, as CAV targets and destroys the rapidly-dividing cortical thymocytes and erythroblasts, resulting in severe anemia, fatty degeneration of the red bone marrow, atrophy of the thymus and bursa of Fabricius, and liver enlargement (2, 3). In adult birds, CAV infection can rather result in a chronic, subclinical infection (3).

Due to its ability to induce immune dysfunction, CAV infection results in high mortality especially in combination with other avian pathogens, such as Marek's disease virus, infectious bursal disease virus, adenovirus, reovirus, or reticuloendotheliosis virus (4-6). As a result, CAV causes major loss in the poultry industry and is an economically important pathogen.

1.1.1 CAV structure

CAV is a single-stranded DNA virus classified within the family *Anelloviridae* in the genus Gyrovirus (7, 8). Its non-enveloped capsid is about 25 nm in diameter and contains a circular DNA genome of 2.3 kb in size, with a promoter region containing numerous 21-bp direct repeats (8). The CAV genome contained in the viral particle is that of the negative-sense strand, and is amplified via rolling circle replication of a double-stranded replicative form (RF) intermediate (9-11). CAV produces a single, 2.1 kb polyadenylated polycistronic transcript with three overlapping open reading frames encoding the viral proteins, Vp1, Vp2, and Vp3 (9, 12). Viral translation is presumed to be regulated by a leaky scanning mechanism (3).

Vp1 is the largest viral protein, measuring 51.6 kDa in size, and is the only structural protein, forming the icosahedral viral capsid (9, 13). The C-terminus of Vp1 contains a highly conserved rolling circle replication (RCR) motif, and Vp1 has been shown to co-precipitate with the viral genome, suggesting that Vp1 may play a role in genomic replication (10, 11, 14). Vp2 is a 24 kDa dual-specificity protein phosphatase that has been shown to interact with and regulate the activity of Vp3 (15, 16). Vp2 is likely to have a multifunctional role, as it is believed to be involved in viral replication via recruitment of cellular DNA replication machinery as well as in virion assembly (15, 17). Although Vp1 is the only protein found in the capsid, both Vp1 and Vp2 must be simultaneously expressed for formation of CAV-specific neutralizing antibody, suggesting that Vp2 acts as a scaffold for the assembly of the viral capsid (18, 19). Vp3 is a 13.6 kDa virulence factor, also termed apoptin, and is the viral protein responsible for inducing apoptosis in susceptible cells (12). Of the three viral proteins, apoptin is the most well-characterized and most studied as it is also capable of causing apoptosis selectively in cancer cells while leaving normal cells unharmed (20).

1.1.2. CAV infection of transformed cells

Early studies of CAV noted that the virus preferentially infected and killed transformed cells (21). CAV infection was found to be enhanced by co-infection with oncogenic viruses that induce DNA replication such as Marek's disease virus (MDV) (21). In addition, CAV and the ability of Vp3 to induce apoptosis became of particular interest when it was discovered that the effects were selective for tumour cells when introduced into non-chicken (non-natural host) cells, including human cancer cell lines (20).

Since the natural target of CAV in chickens are rapidly dividing cells, CAV has likely evolved mechanisms for replication in the division phase of the cell cycle. Whereas other DNA

viruses such as adenovirus and papillomavirus often induce S phase in the host cell, likely to promote DNA replication including that of the viral genome, CAV induces cell cycle arrest in the G2/M phase (22-24). This interaction with the cell cycle might suggest a rationale for CAV's preferential infection of transformed or otherwise rapidly-dividing cells, which are more likely to be undergoing DNA synthesis (20, 21, 25).

1.2 Apoptin

The CAV Vp3 apoptin mediates the G2/M cell cycle arrest and subsequent apoptosis in an infected host cell (24). Apoptin is also known to induce apoptosis in over 70 human cancer cell lines in a p53-independent manner (20, 26). Given the prevalence of mutations in p53 in over half of all human cancers, apoptin's tumour-cell killing ability has generated significant interest for its potential in anticancer therapy (27, 28). Hence, it has been investigated extensively in a wide range of human tumour cells, both *in vitro* and *in vivo* in mice, including melanoma, hepatoma, osteosarcoma, lung carcinoma, breast, and prostate cancers (27, 28). Additionally, the potential of apoptin as a gene therapy has been tested using an adenoviral vector and has been seen to reduce tumours in mice without significant side effects, showing promise (29, 30). Characterization of apoptin as a cancer therapeutic continues to be widely researched and reviewed (31, 32). In contrast, the role of apoptin in the CAV life cycle remains largely speculative. Thus, a deeper understanding of apoptin's functions in CAV replication and its interactions with cellular targets during infection is still needed to develop our comprehension of Gyrovirus virology (25).

1.2.1 Structure of apoptin

Apoptin is 121 amino acids in length and has a mass of 13.6 kDa (12). It does not possess any significant sequence or structure homology to any known host or viral proteins. The

structure of apoptin has not yet been solved, largely due to the tendency of apoptin to form multimeric aggregates both *in vitro* and *in vivo*, as well as the lack of secondary structure within aggregated apoptin (33). However, using homology modeling, Panigrahi et al. (2012) succeeded in constructing a 3D-model of monomeric apoptin, through the combination of peptide sequences from numerous proteins with regions similar to those in apoptin (34).

Although there are limited structural models, the structure of apoptin has been studied extensively through the mutation of various residues, in domains deemed to be important for cellular localization, protein–protein interactions, and apoptotic function (Figure 1) (25).

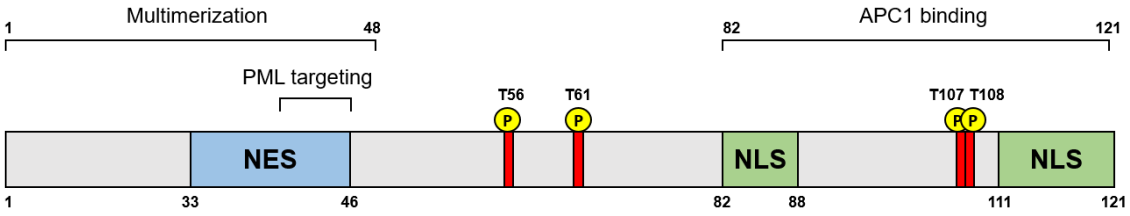


Figure 1. Schematic of the chicken anemia virus Vp3 protein, apoptin. A leucine-rich nuclear export signal (NES) is identified (blue) in the N-terminus from amino acids 33-46, and a bipartite nuclear localization signal (NLS) is pictured (green) in the C-terminus from amino acids 82-88 and 111-121. A region from amino acids 1-48 containing a multimerization domain and sequences in the NES that are important for targeting to promyelocytic leukemia (PML) bodies are indicated with brackets. The APC1 binding domain from amino acids 82-121 in the C-terminus is also shown. Threonine residues at positions 56, 61, 107, and 108 are labelled (red) as important phosphorylation sites for regulation (25).

Apoptin contains a bipartite C-terminal nuclear localization signal (NLS) from residues 82-88 and 111-121, which permits its translocation to the nucleus in a cell-type-specific manner; namely, it exhibits nuclear localization in transformed cells while remaining in the cytoplasm in non-transformed cells (35, 36). Mutation of either of these regions inhibits nuclear localization and consequently abolishes cell-type specific activity (36). Interestingly, the particular NLS sequence of apoptin is important to the protein's cell-type-specific function, as the region overlaps with the APC1 interaction domain (24, 36). Replacement of the apoptin NLS with that of another virus, the SV40 Large T NLS, preserves cell-specific localization, but not apoptotic activity (36). Therefore, the nuclear localization of apoptin, while necessary, is insufficient for its activity.

Apoptin also possesses a leucine-rich Crm1-dependent nuclear export signal (NES) in the N-terminus, from amino acids 33-46, which permits nucleocytoplasmic shuttling (35, 36). Mutation in this N-terminal region removes the cell-type specificity of apoptin's localization, wherein the mutated apoptin localizes to the nucleus in both transformed and non-transformed cells (36). Though assumed to be a canonical Crm1-dependent NES, replacement of the apoptin NES with a prototypical, well-established NES of another protein, human immunodeficiency virus 1 (HIV-1) Rev NES, abolishes cell type-specific localization of apoptin, resulting in diffuse cytoplasmic localization of apoptin in both transformed and non-transformed cells (36). This suggests that the specific sequence of the apoptin NES is critical to the cell-specific function of apoptin's nuclear export.

The NES also has a partial sequence overlap with the apoptin multimerization domain found in the N-terminus (amino acids 1-48), which contributes to the observed phenomenon of apoptin aggregation *in vitro* and *in vivo* (33, 36). In cells where apoptin is localized to the

nucleus, aggregation of apoptin via this domain obscures the NES, preventing nuclear export (12, 35). In this scenario, apoptin remains in the nucleus through its C-terminal NLS (35).

The apoptin NES region has additionally been shown to target apoptin and the associated anaphase promoting complex/cyclosome (APC/C), a ubiquitin ligase complex important in mitosis, to promyelocytic leukemia (PML) bodies within the nucleus (36). Targeting to PML bodies is typically regulated by sumoylation of lysine residues, which has been confirmed for apoptin (37). However, the precise lysine residues that are sumoylated in apoptin have not been identified, though residues in the leucine-rich sequence of the NES are required for targeting the PML bodies (37). Apoptin has been shown to bind directly to PML protein, although PML is not required for apoptotic activity (37). The PML bodies are the target of many cellular and viral sumoylated or otherwise post-translationally modified proteins, and have established roles in tumour suppression, senescence, and most critically in apoptosis (36, 38-40). As many other DNA viruses have been found to interact with PML bodies, this association suggests that localization to nuclear PML bodies might play a role in promoting efficient viral replication (41). Furthermore, apoptin's targeting of the APC/C to PML bodies suggests modification of the APC/C's mitotic functions and inhibition of its activity, which could serve to facilitate apoptotic programming (36, 42). (More on the APC/C in Section 1.3.)

1.2.2 Localization and regulation of apoptin

The most studied function of apoptin is its ability to induce apoptosis. This effect is dependent on apoptin's translocation to the nucleus, which occurs only in transformed cells (24). It has been established that cellular localization of apoptin is regulated by phosphorylation on various sites in the N- and C-terminal regions of the protein (43-45). The first to be identified and subsequently most well-characterized is threonine-108 (T108), whose phosphorylation,

though not essential, plays a role in the efficiency of apoptin activity and its cell type-specific localization (43, 45). The adjacent T107 was later found to also be phosphorylated, and in the event of T108 dephosphorylation, T107 serves as a compensatory phosphorylation site and results in a similar phenotype to T108-phosphorylated apoptin (46, 47). Previous work in our lab has identified additional phosphorylation sites of importance at T56 and T61, in the N-terminal region (44). Mutation of either of these threonines to alanine, significantly impairs nuclear localization of apoptin and consequently attenuates viral production and cytopathic effect (44).

Several kinases have been proposed to phosphorylate apoptin through a variety of mechanisms. Upon induction of apoptosis by apoptin, protein kinase B/Akt translocates to the nucleus where it serves to promote apoptosis, contrary to its normal pro-survival function, via cyclin-dependent kinase 2 (Cdk2) as an effector (48). Another cell cycle regulatory kinase, cyclin-dependent kinase 1 (Cdk1), has also been implicated in apoptin function, as Cdk1 knockdown results in impaired ability of apoptin to localize and induce apoptosis in transformed cells (49). Additionally, protein kinase C (PKCβ) has also been shown to mediate the phosphorylation state and nuclear localization of apoptin in tumour cells (50).

Though initial hypotheses proposed that T108 phosphorylation on apoptin is essential for its activity, this was later disproved – T108 phosphorylation is not required for apoptin's cell type-specific localization (43, 51, 52). Instead, nuclear localization of apoptin occurs only during activation of the DNA damage response (DDR) (52). This has been confirmed by two models of DDR activation – a cellular model, using Bub1 mitotic checkpoint kinase knockdown, and a chemical model, using bleomycin (52). Further work from our lab has confirmed that DDR regulates apoptin nuclear localization and its apoptotic effect, specifically through checkpoint kinases Chk1 and Chk2 (44). Inhibition of Chk1/2 in transformed cells results in cytoplasmic

accumulation of apoptin, impairing its activity (44). Chk1/2 have been found to regulate apoptin localization through phosphorylation of threonines T56 and T61, but not T108 (44).

These findings in the context of the virus offers insight into how full activation of apoptin activity might occur. In addition to the regulation occurring via phosphorylation of apoptin in transformed cells, apoptin necessarily translocates to the nucleus upon activation of the DDR, which can also be triggered by viral replication of its single-stranded DNA genome in non-transformed infected host cells (35, 44, 53). DDR signaling events would lead to an abundance of activated Chk1/2 and phosphorylated apoptin, allowing for apoptin's translocation to the nucleus, productive viral replication, and death of the host cell (44).

As apoptin possesses nucleocytoplasmic shuttling ability, its dephosphorylation must also be a regulated process. Based on observations of Vp2 and T108-phosphorylated apoptin co-localizing in the cell and that of Vp2 association to apoptin in pulldown assays, the putative phosphatase of the T108 on apoptin is the virus-encoded Vp2 dual-specificity phosphatase (16). Vp2 dephosphorylation of T108 appears to downregulate the apoptin-induced cytopathic effect but does not completely abolish it, and this function seems to play a role in modulating the CAV infection process (16). It remains to be determined whether other phosphorylation sites on apoptin are similarly regulated by Vp2 or whether other phosphatases might be involved, and to what extent the various phosphorylation sites, kinases, and phosphatases exhibit control over apoptin's function and activity.

1.2.3 Mechanisms of apoptosis by apoptin

Arguably, apoptin is most notable for its ability to induce apoptosis specifically in tumour cells specifically independently of p53 (20). However, details on the interactions and mechanisms of the signaling pathways used by apoptin to cause cell death still remain unclear.

Cell death by apoptosis is mediated by intracellular cysteine proteases called caspases, which are activated by two main signaling pathways, the extrinsic death receptor pathway and the intrinsic mitochondrial pathway (27, 54, 55). The extrinsic pathway involves ligand binding to death receptors, followed by the recruitment of Fas-activated death domain (FADD) and activation of caspase-8, leading to the formation of the death inducing signaling complex (54-56). In contrast, the intrinsic mitochondrial pathway is characterized by release of cytochrome *c* from the mitochondria into the cytosol, enabling formation of the apoptosome and initiating activation of caspase-9 (54, 55). Both apoptotic pathways converge on activation of the downstream effector caspases to carry out cell death by degrading cellular components (55).

In the case of apoptosis of cancer cells induced by expression of apoptin, the release of cytochrome *c* and activation of caspase-9 have been observed (57). This indicates that apoptin mediates cell death via the intrinsic mitochondrial pathway of apoptosis (57). One proposed mechanism involves Nur77, a nuclear receptor that appears to be phosphorylated upon apoptin introduction (possibly by Akt), translocating it from the nucleus to the cytoplasm (27, 56). Nur77 in the cytoplasm promotes conformational changes in the Bcl-2 family of proteins, converting them from anti-apoptotic protectors to pro-apoptotic factors (58). Pro-apoptotic Bcl-2 promotes targeting of Nur77 to the mitochondria, inducing subsequent cytochrome *c* release and apoptosis via the mitochondrial pathway (58). Meanwhile, the apoptotic activity of apoptin is retained in cells deficient in either FADD or caspase-8, suggesting that apoptin can act independently of the extrinsic death receptor pathway (56).

1.2.4 Cellular interactions of apoptin

Although apoptin appears to activate the intrinsic mitochondrial death pathway, apoptin interacts with many cellular proteins, including both Bcl10 and FADD (27). Colocalization of

apoptin with FADD and Bcl10 suggests involvement in the extrinsic death receptor pathway as well (51). FADD is an adaptor protein that binds to death receptor Fas upon its stimulation by the Fas ligand to recruit procaspases 8 and 10 for the death inducing signaling complex, while Bcl10 induces apoptosis via a caspase recruitment domain (59). Bcl10 also activates NF-κB, which has been shown to be important in several cell death pathways, including the Fas-mediated extrinsic pathway and cell death induced by certain viral infections (60-62).

Moreover, apoptin interacts with death effector domain-associated factor (DEDAF), a protein that associates with pro-apoptotic proteins containing death effector domains (DED) (63). In particular, DEDAF interacts with the DED in FADD and procaspases 8 and 10 to activate downstream caspases and induce cell death (64). DEDAF is predominantly localized in the nucleus and interacts with apoptin only in tumour cells, but not in normal cells, which aligns with the circumstances in which apoptin would localize to the nucleus and induce apoptosis (63).

Another cellular target of apoptin is Hippi, the protein interactor of Huntingtin-interacting protein 1 (Hip-1), which together can induce apoptosis through activation of caspase-8 (65, 66). In normal (untransformed) cells, the C-terminal half of Hippi can bind the multimerization domain in apoptin and colocalize in the cytoplasm, rather than forming the Hip-1-Hippi apoptotic complex (65). In contrast, Hippi and apoptin remain separately localized in cancer cells, and no interaction is detected between the two proteins as apoptin translocates to the nucleus while Hippi remains cytoplasmic (65). Thus, the apoptin-Hippi interaction appears to help suppress apoptosis in normal cells by preventing apoptin translocation to the nucleus and subsequent caspase activation (65).

Furthermore, in cells where apoptin localizes in the nucleus, apoptin binds to the APC1 scaffolding subunit of the APC/C, inhibiting the complex (24). Although a lack of structural data

on apoptin makes it difficult to suggest specific molecular interactions, various residues in the C-terminal domain, including K116, R117, and R118, as well as the NLS have been identified as essential for APC1 binding (24, 36). As the APC/C regulates progression through mitosis, including control of entry into anaphase, its inhibition stalls the cell cycle in the G2/M phase of the cell cycle (24). Typically, as G2 phase is the last phase of the cell cycle before mitosis, it contains final checkpoints for DNA damage detection and genomic integrity before a cell undergoes division (67). As a result, cell cycle arrest in G2/M occurs if this checkpoint is activated, triggering a corresponding cellular response until the problem has been resolved (68). As a protective mechanism, if the cell stays in G2/M phase for a prolonged period, apoptosis tends to occur, a feature that is often exploited by tumour-targeting chemical compounds (69-73). Thus, virally induced G2/M cell cycle arrest by apoptin may also serve to facilitate cell death via mitotic catastrophe. In addition, stalling in G2/M creates a favourable environment for viral replication (discussed in Section 1.4).

1.3 The anaphase promoting complex/cyclosome

The anaphase promoting complex/cyclosome (APC/C) is a large, multimeric protein complex that is a RING-family E3 ubiquitin ligase, catalyzing the addition of ubiquitin from E2 ubiquitin-conjugating enzymes to its substrates and targeting them for degradation by the proteasome (74, 75). Together with the SCF (Skp, Cullin, F-box) complex, another ubiquitin ligase, the APC/C is one of two central regulators of the cell cycle (74, 75). The APC/C in particular plays a critical role in the progression through the fundamental cellular process of mitosis (74). The APC/C has many important targets through mitosis, including cyclins and other cell cycle regulators, with its primary substrate being securin, an inhibitor of the enzyme separase (75). Upon ubiquitination of securin targeting it for degradation, separase is released to

its active form in which it cleaves the cohesin rings holding sister chromatids together in the metaphase plate (75). The degradation of the cohesin complex allows for the balanced separation and distribution of genomic material to the daughter cells, transitioning a dividing cell from mitotic metaphase into anaphase, thus giving the APC/C its name (75). In addition to crucial activities in the progression to anaphase, the APC/C also plays a role in the entry and exit from mitosis and the maintenance of G1 phase by targeting cell cycle proteins for degradation (75).

1.3.1 Structure of the APC/C

The APC/C is a 1.5 MDa protein complex consisting of at least 11 subunits with various scaffolding, catalytic, and substrate recognition functions (Figure 2) (74, 75). Many subunits of the APC/C exhibit homology between species and the structure of the APC/C is highly conserved, especially in the catalytic and substrate recognition modules (76). The largest subunit is the APC1, which bridges the catalytic and regulatory subcomplexes, thereby acting as the main scaffolding protein of the complex (76). Only two subunits, the APC2 and APC11, are catalytic modules, containing the cullin subunit and RING domain subunit, respectively; and these associate with the targeted substrate in ubiquitin ligation (76). The APC10, together with a coactivator, either Cdc20 (cell division cycle 20) or Cdh1 (Cdc20 homology 1), forms the substrate recognition module (76).

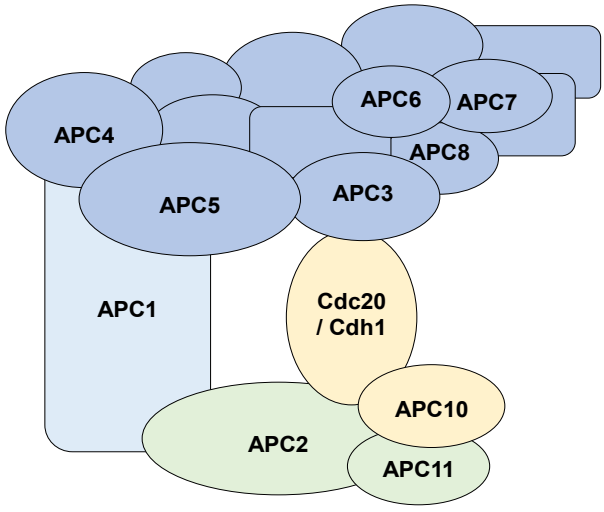


Figure 2. Schematic of the structure of the anaphase promoting complex. The APC1 scaffolding subunit (light blue) bridges the regulatory subcomplex (blue) and the catalytic subunits (green), which associate with the substrate recognition module containing the coactivator Cdc20 or Cdh1 and target substrates for ubiquitination. (76, 77)

1.3.2 Regulation of the APC/C

Activation and specific activity of the APC/C relies on association with two structurally-related specific coactivators, depending on the stage of the cell cycle – Cdc20 during mitosis and Cdh1 during G1 phase (75). Binding of the coactivator to the APC/C regulates its activity, as APC/C^{Cdc20} allows for identification of mitotic cyclins and securin as ubiquitination targets, while APC/C^{Cdh1} substrates instead include Cdc20 (leading to APC/C^{Cdc20} inactivation) and others essential for S phase entry and DNA replication (75, 76, 78). Both coactivators associate with the APC/C through three conserved sequence motifs – the C box and the KILR tetrapeptide motif, both in the N-terminus, as well as the IR dipeptide tail, found in the C-terminus (76, 79).

Meanwhile, substrate recognition by the coactivators bound to the APC/C is mediated through a C-terminal WD40 β-propeller domain found in both Cdc20 and Cdh1 (79, 80). Most APC/C substrates contain at least a D (destruction) box motif (consensus sequence RxxLxxxxN) or a KEN box motif (consensus sequence KENxxxN) which is recognized by the WD40 domain (76, 79). As the APC/C^{Cdc20} preferentially recognizes D box motifs and the APC/C^{Cdh1} recognizes KEN box motifs, many APC/C substrates may have both consensus sequences if targeted by both coactivator-bound forms of the APC/C (76, 80).

Phosphorylation of several core subunits of the APC/C (1, 3, 6, 7, 8) also contributes to regulating the temporal substrate specificity of the APC/C, and is specifically required for Cdc20

binding to the complex (81). In contrast, phosphorylation of the Cdh1 adaptor protein inhibits its binding to the APC/C (78). Several kinases have been shown to phosphorylate the APC/C – cyclin-dependent kinase 1 (Cdk1) and Polo-like kinase (Plk1) act in synergy to promote APC/C activity, while protein kinase A (PKA) phosphorylates the complex in an inhibitory manner (82).

As the APC/C^{Cdc20} has a crucial role in mediating entry into anaphase, it must be well-regulated to ensure the balanced separation of sister chromatids into daughter cells. Prior to its activity in targeting securin for degradation and allowing the progression from metaphase to anaphase, the APC/C is inhibited by the spindle assembly checkpoint (SAC) (83). The mitotic checkpoint complex (MCC), composed of the proteins Mad2, BubR1, and Bub3, inhibits Cdc20 and prevents its binding to the APC/C for subsequent activity (83-85). Inhibition through the SAC continues until all kinetochores of chromosomes are properly attached to spindles (83, 86).

1.3.3 Role of the APC/C in the cell cycle

As previously discussed, the primary function of the APC/C is to regulate the metaphase-to-anaphase transition through ubiquitination of securin, targeting it for degradation (75). In addition, the APC/C is responsible for the degradation of cyclin B, which is required for mitotic progression, as well as many other important substrates throughout the cell cycle (75). Starting at the prometaphase-to-metaphase transition, the APC/C^{Cdc20} is activated to induce degradation of cyclin A and Nek2 (74, 75). As progression through metaphase occurs, the Cdc20-activated complex is responsible for the degradation of cyclin B, Xkid, and securin, while after entry into anaphase, the APC/C^{Cdc20} targets kinesins Kip1 and Cin8 (74). All these proteins continue to be targeted for degradation throughout mitotic exit until the end of G1 phase, but by the Cdh1-bound APC/C, which also ubiquitinates other proteins, including Cdc20, Plk1, Aurora-A, Cdc6, and geminin in late mitosis and early G1 (74).

1.3.4 Viral inhibition of the APC/C

CAV is one of several viruses known to inhibit the APC/C, which is unsurprising given its functions in cell cycle regulation; however, this poses an interesting problem for the life cycle and replication of CAV and other viruses that induce G2/M arrest (87). Viruses including adenovirus, human T lymphotrophic virus 1 (HTLV-1), human papillomavirus (HPV), open reading frame virus (ORFV), human cytomegalovirus (HCMV), and hepatitis B virus (HBV), along with CAV, have been found to interact with the APC/C to disrupt its activity (87, 88). These viruses modulate APC/C functions through a wide range of mechanisms, supporting the notion that such viruses have convergently evolved these similarities (88). For example, while CAV apoptin binds to APC1 and dissociates the APC/C, the HPV E2 protein binds directly to coactivators Cdh1 or Cdc20, HCMV pUL97 phosphorylates Cdh1 to inhibit its binding to the APC/C, and the ORFV poxvirus APC/cyclosome regulator (PACR) protein mimics and displaces the APC11 subunit (88). Some viral proteins may activate APC/C activity as a form of deregulation, such as adenovirus E4orf4 (which can activate or inhibit the complex) and HTLV-1 Tax (88). In the case of HBV, the X protein binds to BubR1, disrupting the SAC complex and releasing Cdc20 for premature binding to and activation of the APC/C (88).

In many of the instances listed above, modulation of the APC/C has been shown to be required for efficient viral replication; however, it is still unclear why viruses such as CAV inhibit the APC/C and how cell cycle disruption may facilitate viral processes. For viruses that inhibit the APC/C^{Cdh1}, proteins that disrupt the APC/C may serve to induce S phase early by preventing the maintenance of G1 phase, allowing for an increase in some factors needed for viral and cellular replication, such as thymidine kinases (TK) (87). For example, while other members of the poxvirus family encode their own TK, ORFV does not, and inhibiting the

APC/C^{Cdh1} may assist the virus in making use of the enhanced cellular TK (89). However, induction of S phase alone may be a redundant function for these viral proteins, as ORFV, HPV, and HCMV all encode oncoproteins that stimulate S phase induction (89-91). In the case of CAV, apoptin inhibits the Cdc20-bound APC/C instead and arrests the cell in G2/M phase, seemingly not needing the cellular factors expressed in S phase to replicate its viral genome (87). This may be due to the fact that CAV target cells (i.e. thymocytes and erythroblasts) are already rapidly-dividing cells independent of cell cycle manipulation (21).

One explanation for viral inhibition of the APC/C^{Cdc20} proposes that cell cycle arrest in G2/M leads to cell death via mitotic catastrophe, which can promote viral egress (24, 92). Indeed, apoptosis is a suitable method of effective viral propagation for non-enveloped viruses such as CAV (92, 93). In the case of apoptin, inhibition of the APC/C and its other functions likely play a critical role in the CAV life cycle as well, since the protein already promotes the induction of apoptosis in several ways aside from mitotic arrest (27).

Another explanation for viral induction of mitotic arrest may be tied to translation, as cellular translation is downregulated in G2/M phase compared to interphase (94). This could favour the virus and its replication while it manipulates and interferes with host cell translation (discussed in Section 1.4.5).

1.4 Translation

Translation is the critical, multistep process of the cell in which mRNA transcripts are synthesized into proteins (95). It is a complex, highly regulated and energy-consuming process (95). Translation is composed of three phases: initiation, elongation, and termination. During initiation, the 80S ribosome is assembled at a methionine AUG start codon and the first tRNA adds the first amino acid, an initiator methionine (95, 96). Translational elongation follows,

during which the ribosome scans the mRNA by 3-nucleotide codons, adding the corresponding amino acids to the growing polypeptide chain using tRNA carriers (96, 97). Finally, termination of translation occurs when stop codons are recognized by terminator proteins and release factors are recruited to destabilize the ribosome and mRNA interaction (97). The translational machinery can then be recycled to other transcripts (97).

1.4.1 Initiation of translation

The canonical translation initiation mechanism is dependent on the 5' cap, a 7-methyl guanosine capped at the 5' end of all mature eukaryotic mRNAs (96). The eukaryotic initiation factor (eIF) 4E binds to the 5' cap and associates with other factors in the eIF4F complex to initiate translation (95, 96). The eIF4F complex consists of the eIF4E cap-binding protein, the eIF4G scaffolding protein, and the eIF4A ATP-dependent RNA helicase (95, 98). As the scaffolding factor, eIF4G interacts with the poly-A binding protein (PABP), which associates with the 3' polyadenylated tail of mature mRNAs, thus circularizing the mRNA and bringing the 5' capped end and 3' tail end in close proximity (98). eIF4G also helps to recruit helicase eIF4A and its cofactor eIF4B, which together unwind secondary structures in the mRNA to allow for complex assembly and codon scanning (95, 98). Together, the eIF4F complex interacts with eIF3, another translation initiation factor that is associated with the 40S ribosomal subunit (98). The initiator tRNA ($tRNA_i^{Met}$) is brought to the initiation complex by eIF2-GTP, and hydrolysis of GTP stimulates the dissociation of several factors from the small ribosomal subunit to allow for association of the 60S large subunit and assembly of the complete 80S ribosome (95, 98).

The vast majority of cellular mRNA translation, possibly 95-97%, is cap-dependent, with the remainder of transcripts being translated by alternative, cap-independent mechanisms (99).

1.4.2 Cellular regulation of translation initiation

Translational control regulates the rate of protein synthesis, with most of it occurring at the initiation step by converging on the initiation factors, especially those in the eIF4F cap-binding complex (95). In fact, eIF4E, the cap-binding protein, is generally considered the rate-limiting step in translation and is one of the main regulatory points of translational control (95).

A family of eIF4E-binding proteins (4E-BPs) regulate the interaction between eIF4E and eIF4G (95). 4E-BP phosphorylation controls affinity of binding to eIF4E, which interferes with eIF4F complex association (95). Hypo-phosphorylated 4E-BP binds with high affinity to 4E, while hyper-phosphorylation prevents binding; therefore, phosphorylation status of 4E-BPs is a major mechanism of controlling 4E availability to translate cap-dependent mRNAs (95).

In addition to eIF4E sequestration, availability of other initiation factors may also be targeted. Commonly observed under stress conditions, the eIF2-GTP-$tRNA_i^{Met}$ complex responsible for delivering the initiator tRNA can be prevented from re-assembling (98). Upon GTP hydrolysis after recognition of a start codon, eIF2-GDP is typically recycled by a guanine exchange factor (GEF) back to eIF2-GTP (98, 100). However, phosphorylation of the eIF2α subunit inhibits the GEF with high affinity and attenuates protein synthesis initiation (100).

1.4.3 Inhibition of translation during mitosis

With cells expending more energy on translation than any other activity, it is understandable that translation is inhibited during cell division, another energetically consuming and carefully regulated essential process (95, 101). In G2/M phase, cap-dependent translation is highly downregulated, to approximately 25% the rate of translation of cells in interphase (94).

Several mechanisms have been implicated in the repression of translation in mitosis and make use of eIF control of translational regulation as outlined above. For instance, during

mitosis, 4E-BP1 is de-phosphorylated, which leads to increased binding to eIF4E and displacement of the cap-binding complex interaction (101, 102). In contrast, hyper-phosphorylation of 4E-BP1 at the end of mitosis corresponds with its release from eIF4E and restimulation of cap-dependent translation as cells resume G1 phase (103).

Mechanistic target of rapamycin complex 1 (mTORC1) is a master growth regulator and a primary controller of protein synthesis that senses many environmental factors, including growth factors, nutrient availability, energy levels, and cellular stress (104). As a serine/threonine kinase, mTORC1 regulates translation in mitosis through several direct and indirect phosphorylation events, affecting factors such as eIF4G, eIF4B, and 4E-BP1 (104). Through control of 4E-BP1, mTORC1 regulates the availability of eIF4E, while eIF4G phosphorylation induces differences in binding sensitivity to other initiation factors (105, 106). Another well-characterized target of mTORC1 is ribosomal kinase S6K, which phosphorylates eIF4B to control its recruitment to the translation pre-initiation complex (107).

In mitosis, eIF4B function is also regulated through a mechanism that requires tumour suppressor 14-3-3σ, which typically binds to and sequesters cell cycle regulators such as cyclin B1 after DNA damage to inhibit cell cycle progression (108). 14-3-3σ binding of eIF4B prevents its association to the cap-binding complex and inhibits translation initiation efficiency (108).

Cyclin B is active in early mitosis and is degraded by the APC/C in the progression to anaphase, and also plays a role in downregulating translation at the start of cell division through phosphorylation in complex with Cdk1 (74). Cyclin B/Cdk1 together phosphorylate both eIF4A and eIF4G to reduce their binding affinity for each other, decreasing the association of the factors with mRNA and inhibiting mitotic translation (109).

1.4.4 Non-canonical cap-independent translation

Besides canonical cap-dependent translation, non-canonical mechanisms of translation, generally cap-independent, exist to promote translation initiation of mRNAs that are enhanced when global translation is otherwise repressed via stress, viral infection or cell division (110).

The best-characterized cap-independent translational mechanisms make use of an internal ribosomal entry site (IRES) that is capable of recruiting the 40S ribosomal subunit to a start codon independent of eIF4F (111, 112). IRESs are not defined nucleotide sequences but regions of structure in the mRNA and thus must be confirmed experimentally (112, 113). IRESs are found in many viruses as well as some host mRNAs, especially those involved in the cellular stress response, and contain a high degree of variability and diversity with respect to sequence, secondary structure, and requirement of eukaryotic initiation factors (112, 114).

IRESs were first identified in viruses, and are especially prevalent in RNA viruses (111, 112). Viral IRESs are rich in secondary structure which provides a scaffolding for the recruitment of the ribosomal subunits (115). Viral IRESs are classified by their varying needs for translation initiation factors, as well as their secondary structure and the relative location of the start codon (115). IRESs in viral mRNAs confer advantages to viruses which often trigger host stress responses, such as through the protein kinase R (PKR) pathway of innate antiviral immunity that serves to repress translation by a variety of mechanisms such as eIF4A deactivation and eIF2α phosphorylation (110). In cases of host translational shutdown, viruses can thus continue to express their own proteins despite antiviral responses at the protein synthesis level (110).

Cellular mRNAs that contain functional IRES elements are typically those that encode critical regulatory proteins that must remain actively translated during G2/M, when protein

synthesis is inhibited (95, 116). IRES-mediated initiation is often unaffected by changes in eIF4E, eIF4B, and eIF2α activity (116). Cellular IRESs are often more diverse and generally unclassified, including very different sequences and structures for mRNAs encoding proteins in the DNA damage response, stress responses, cell division processes, and cell survival pathways (114, 117). Some cellular IRESs may be used by mRNAs in a cap-independent manner only under specific sets of conditions, such as in the G2/M phase of the cell cycle, while their translation in G1/S phase remains canonical and cap-dependent (118).

1.4.5 Viral manipulation of host cell translation

All viruses hijack the host translational machinery in order to reproduce, as they do not carry their own protein biosynthesis machinery (110). As a result, viruses have evolved many mechanisms to favour optimal synthesis of their viral proteins over the translation of cellular mRNAs (110). Many viruses therefore use non-canonical mechanisms such that the synthesis of cellular proteins can be repressed via canonical translational mechanisms while their viral mRNAs can still be effectively translated (110).

Some viruses manipulate host translation through interference with cellular mRNAs. For example, influenza recognizes and cleaves the 5' cap from cellular mRNAs to subvert it for viral protein synthesis, using the 5' capped cleaved end as a primer for viral transcripts and thus making use of the associated host translational machinery (119). Such a mechanism also leaves host mRNAs vulnerable to degradation by endonucleases, as without a 5' cap, the transcripts are relatively unstable and less likely to be translated (120). In addition, adenoviruses interfere with host mRNA through a block in nuclear export, while others such as herpes simplex virus 1 (HSV-1) inhibit splicing of cellular mRNAs (121, 122).

Disruption of eIF activity is also used by some viruses, such as poliovirus, whose 2A protease cleaves eIF4G to prevent the translation of capped mRNAs relying on canonical translation (123). Meanwhile, poliovirus itself contains an IRES, so it is able to continue its own mRNA translation upon eIF4G cleavage (123). Positive-sense RNA viruses including the picornaviruses and caliciviruses encode a VPg protein that associates with the 5' leader of viral mRNA and binds eIF4E – essentially acting as a viral protein mimic of the 5' cap on cellular mRNAs (110, 124) . Several viral families, including adenoviruses, herpesviruses, poxviruses, and picornaviruses modulate the phosphorylation status of 4E-BPs, affecting the affinity of eIF4E to eIF4G for cap-dependent translation (110).

Another interesting circumstance presented by some viruses includes the induction of G2/M phase as a means of suppressing host translation. HIV-1 induces a G2/M arrest through inhibition of Cdk1, preventing the activation of the APC/C^{Cdc20} and inhibiting cellular translation (125). HIV-1 appears to synthesize its proteins through both a cap-dependent and IRES-driven mechanism, the latter of which is preferentially active in G2/M phase (126, 127). For viruses that can synthesize their proteins in G2/M phase, there exists an advantage as the antiviral response appears to be inhibited in G2/M, thus greatly enhancing the efficiency of viral replication and secondary infection (126). This may provide an immunological rationale for why these viruses favour cell cycle arrest if they are translationally competent in mitosis (126).

1.5 Apoptin and the CAV life cycle

CAV encodes only three proteins, suggesting that the virus must be very efficient, and that each protein is likely multifunctional, in order to aid and facilitate viral replication. Though apoptin is the best studied of the three proteins for its apoptotic functions, its roles in viral reproduction are not particularly well-characterized. In studying the molecular biology of CAV,

it is important to examine the protein functions in the context of natural infection to understand the purpose of their evolution and host interactions (25).

Electron microscopy of CAV in the cell shows that it largely accumulates in the nuclei of infected cells (128). Vp1, the capsid protein, contains both NLS and NES sequences, providing evidence that CAV is assembled in the nucleus (13, 129). Vp1 is known to interact with Vp2, which also has distinct sites for interaction with apoptin – suggesting that Vp2 might link the two proteins and have a role in scaffolding (16, 130, 131). Apoptin interaction with APC1, leading to the inhibition and disassembly of the APC/C and host cell G2/M arrest, is postulated to be the purpose of apoptin's nucleocytoplasmic shuttling activity, allowing targeting of cytoplasmically localized APC/C to PML bodies in the nucleus (36, 132). This could serve to facilitate the viral life cycle, as the sequestration and inhibition of the APC/C along with induced mitotic arrest might trigger apoptotic signaling or play other functional roles that favour CAV replication. In fact, CAV particles have been observed in apoptotic bodies of infected dying cells that are absorbed by neighbouring epithelial cells, so it can be deduced that the cytopathic effect of apoptin is important to viral propagation (93). This observation supports the notion that induction of apoptosis is a means of viral egress that can be used by non-enveloped viruses such as CAV.

Combining what is known, our lab has developed a working model for CAV infection and viral propagation (Figure 3) (25). Upon entry of the virus into a cell, its ssDNA genome enters the nucleus and is transcribed. Then, as mRNA translation occurs, apoptin is made, and in transformed cells or cells with activated DDR, apoptin binds the APC/C, localizes to the nucleus and induces G2/M arrest, which promotes efficient viral replication. As canonical cap-dependent translation is inhibited in G2/M, the virus may differentially regulate translation of its own mRNA to favour its continued protein synthesis upon cell cycle arrest. As the Vp1 and Vp2

proteins are also made and accumulate, they facilitate viral particle assembly in the nucleus. Apoptosis of the host cell then promotes viral exit and dispersal, and CAV sequestered into apoptotic bodies may be phagocytosed by neighboring cells, providing a means of entry and continued infection (25).

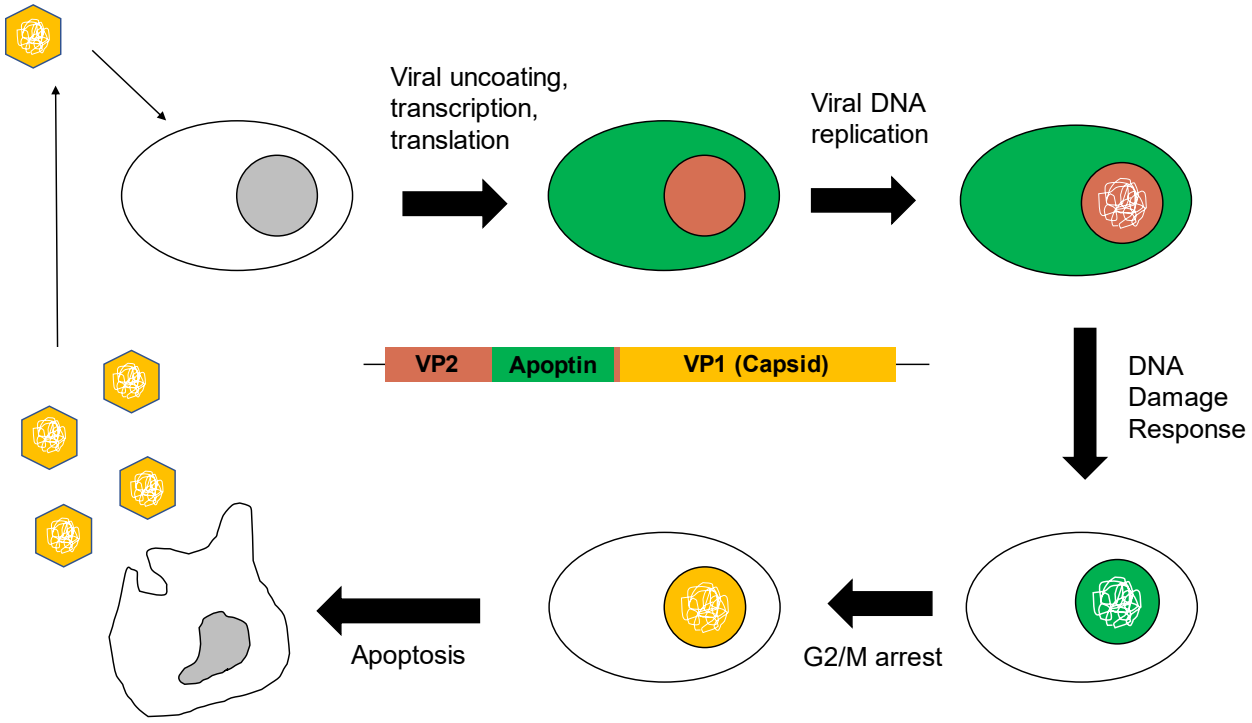


Figure 3. Schematic diagram showing the proposed model of the CAV life cycle. Entry of the virus into the cell is followed by viral uncoating, transcription, and translation. The putative replicase, Vp2 (pink), would localize to the nucleus to initiate viral DNA replication while apoptin (green) is initially localized in the cytoplasm. As viral DNA accumulates and triggers the DNA damage response, apoptin is phosphorylated and localizes to the nucleus, where it aggregates and sequesters the APC/C. G2/M phase arrest is induced while viral capsid protein (yellow) and viral genome continue to be synthesized. Virions (yellow hexagons) are packaged and apoptosis is induced to facilitate viral egress and spread (25).

1.6 Similar viruses and viral proteins

When seeking to understand the CAV viral life cycle and its viral proteins' functions, it is also valuable to examine related viral families and mechanistically similar viral proteins, as any similarities can provide insight into the purpose of such mechanisms. In particular, proteins found in members of the anellovirus and circovirus families have been observed or hypothesized to have similar functions to apoptin. Comparison with other single-stranded DNA viruses that encode apoptin-like proteins suggests that the role of CAV apoptin in viral replication and pathogenesis is likely critical, and that the interactions with host-cell factors may be conserved within related viral families (25).

1.6.1 Anelloviridae

CAV was reclassified in 2016 to the *Anelloviridae*, due to closer similarities than to its previous viral family of *Circoviridae* (7). The type virus of the anelloviruses, torque teno virus (TTV), is widespread in the human population, replicating in liver cells, though its pathogenic effects remain unclear (133). TTV and CAV share comparable genomic organizations, with both harboring negative-sense single-stranded DNA genomes containing overlapping open reading frames (ORFs) (134, 135). CAV and TTV share several conserved motifs between their Vp1 and ORF1, respectively, as well as another highly conserved motif between the CAV Vp2 and TTV ORF2 (135). The functions of each of these proteins are also similar – the TTV ORF1 and CAV Vp1 are structural proteins in the capsid, the TTV ORF2 and CAV Vp2 both have phosphatase activity, and most notably, the TTV ORF3, like apoptin, has been shown to have apoptotic activity in certain cancer cell lines (133). In a study by Prasetyo et al. (2009) where cells were transfected with an apoptin-knockout clone of CAV, DNA replication was impaired but rescued not only by the supplementation of apoptin, but also by TTV ORF3 (136). These results suggest

a close-relatedness of CAV and TTV and their Vp3/ORF3 proteins, as they not only share similarities in terms of apoptotic oncolytic activity, but likely also in viral replication (136).

A more recently discovered virus of the Gyrovirus genus, human gyrovirus (HGyV), is a more closely related virus to CAV, with similar genomic structure encoding three proteins, Vp1, Vp2, and Vp3 (1). Similar to apoptin, studies on the HGyV Vp3 protein have shown that the protein also induces G2/M arrest and apoptosis in transformed cells (137, 138). HGyV Vp3 displays similar subcellular localization patterns to apoptin, wherein it translocates to the nucleus in cancer cells but remains predominantly in the cytosol in non-transformed cells (137). Therefore, it appears that HGyV Vp3 may retain the mechanism of tumour cell-specific activity seen with apoptin and provides support for an apoptin-like protein playing a conserved critical role in the natural viral life cycle of gyroviruses, as well as other anelloviruses (25).

1.6.2 Circoviridae

CAV does not exhibit a close phylogenetic relationship to the circoviruses and studies have demonstrated that gyroviruses such as CAV are not structurally related to circoviruses (13, 135). However, despite CAV having been reclassified away from the *Circoviridae*, there are some notable similarities in terms of viral protein functions and the viral life cycle, which might emphasize the importance of these viral mechanisms. The Circovirus genus of the *Circoviridae* family contains important avian and porcine pathogens, including beak and feather disease virus (BFDV), and porcine circovirus 2 (PCV-2) (139). In contrast to CAV and the gyroviruses, circoviruses have a circular genome with an ambisense organization, containing at least two identifiable ORFs, a conserved replication-associated protein (Rep), and a capsid protein (Cp) (139). In addition to the Rep- and Cp-encoding ORFs, circoviruses may express other proteins –

for example, PCV-1 and PCV-2 are known to encode a third and fourth protein, ORF3 and ORF4, with apoptotic activity and potential anti-apoptotic functions, respectively (140-143).

In the case of PCV-2, a circovirus that causes postweaning multisystemic wasting syndrome in pigs, the ORF3 has been shown to induce apoptosis through a caspase-8 dependent pathway (142). Though PCV-2 ORF3 has been observed to induce apoptosis in melanoma cells, both *in vitro* and in a mouse model, demonstrating potential as a cancer therapeutic, the effect on tumour reduction is less dramatic than that of apoptin (29, 144). Additionally, PCV-2 ORF3 does not cause G2/M arrest in infected cells and similar apoptotic effects were observed in both non-transformed and transformed cells, suggesting that PCV-2 ORF3 does not share the tumour cell-specific killing of apoptin (142, 144). Although differences in the tumour selectivity of PCV-2 ORF3 and CAV apoptin exist, ORF3 has interestingly been demonstrated to play a role in the systemic dissemination of PCV-2 infection, expediting viral spread (145). These features support the notion that apoptin-like proteins appear to aid in the viral life cycle of CAV-related viruses and lend themselves to efficient viral replication.

Another porcine circovirus, PCV-1, also encodes a viral protein with apparent tumour-selective apoptotic properties (141). While the apoptotic activity of apoptin seems directly linked to its nuclear localization and occurs only in transformed cells, PCV-1 ORF3 appears to localize in the cytoplasm in both normal and transformed cells, but displays apoptosis-inducing activity only in transformed cells (141). Unlike PCV-2, PCV-1 is non-pathogenic and ubiquitous in pigs; however, PCV-1 ORF3 exhibited induction of apoptosis in more types of cells than PCV-2 ORF3 (146). Assuming homology in viral protein functions due to their similarity in structure, these findings suggest that the ORF3 of porcine circoviruses is not the sole determinant of pathogenicity in pigs and might instead point to a functional role during viral replication (25).

1.7 Research rationale and objectives

Despite a significant body of literature on CAV and its viral protein apoptin, many aspects of the molecular biology how the virus replicates remain unclear. Past work in our lab has shown that apoptin's inhibition of the APC/C mediates G2/M arrest, and recent work by a former graduate student has confirmed that CAV infection induces translational repression in the host cell (24, 147). Prior to the work presented in this thesis, results from previous work in our lab has suggested that the Vp1 capsid of CAV may be regulated by a region of high secondary structure upstream of it in the viral mRNA. In a ^{35}S-labelled autoradiography blot wherein global translation was seen to be inhibited at 24 hours after CAV infection, the appearance of a 52-kDa protein believed to be Vp1 was observed despite significantly reduced cellular translation (147). As a result, we hypothesize that G2/M arrest caused by apoptin shifts viral gene expression from an early to late phase, wherein early viral proteins help to induce cell cycle arrest, and late viral proteins favour viral production and egress. The research done in this thesis aims to understand the role of the viral proteins in CAV, particularly that of apoptin, and how their interactions with the cell cycle contribute to viral replication.

CAV remains an important agricultural pathogen and garners much interest for its potential in human health. The results of this study could provide novel mechanistic insights into viral replication and gene expression strategy of chicken anemia virus and other gyroviruses.

2.0 MATERIALS AND METHODS

2.1 Maintenance of cell culture

MDCC MSB-1 cells (CLS Cell Lines Service), a chicken lymphoblastoid cell line immortalized by MDV, were cultured in RPMI-1640 (Wisent) supplemented with 10% fetal bovine serum (FBS) and 50 ug/mL Gentamicin. Cells were incubated at 41°C under 5% CO_2 and split every 2-3 days to maintain density between 0.3-1.5x10^6 cells/mL.

2.2 Transfection and virus production

CAV virus was produced through transfection of double-stranded RF CAV genomes into MDCC MSB-1 cells. A pIC20H-CAV plasmid containing the CAV genome between EcoRI restriction sites in a pIC20H vector was amplified in DH5α *Escherichia coli*. Purified plasmid was digested by EcoRI to linearize the CAV viral genome, which was purified by agarose gel. Linear CAV was recircularized with T4 DNA ligase (NEB) at very low concentration (1 ng/uL) to ensure intramolecular ligation. Circular CAV was recovered using a silica spin column and following the PCR cleanup instructions from the GenepHlow Gel/PCR Kit (Geneaid). Circular RF CAV was visualized and confirmed by agarose gel before transfection.

The same methods were used to create transfection DNA for CAV lacking expression of each of its proteins (CAVΔVp1, CAVΔVp2, and CAVΔApo). The CAV sequences with mutated ORF start codons (as described in Section 2.3 and reference (147)) were similarly excised using EcoRI from their respective pIC20H plasmids, purified, and recircularized to create double-stranded circular genomes for transfection.

For transfection, 900 ug of CAV genome was electroporated into 2.0x10^6 cells MSB-1 cells using the AMAXA Nucleofector II system and reagent Kit T (Lonza). Cells were added to growth media at density of 3.0x10^5 cells/mL to recover for 1 hour before being washed three

times in phosphate buffered saline (PBS) to remove any residual RF CAV that did not enter into cells. Cells were allowed to grow for 3 days, with a 140 uL sample removed for viral genome quantification every 24 hours. After 72 hours, cells were lysed by three freeze-thaw cycles and virus released into the media was filtered through a 0.45 um membrane (Corning) to collect cell-free viral supernatants. Another sample for viral genome quantification was taken before virus was frozen and stored at -80^{o}C until use for infection.

2.3 Site-directed mutagenesis of CAV mutants

Single point mutations were generated in the pIC20H-CAV plasmid to create CAV genomes lacking expression of Vp1 or Vp2 (CAVΔVp1 and CAVΔVp2) by altering a base in the corresponding AUG start codon. Due to complex secondary structures in the CAV sequences, a short region of the CAV genome surrounding the intended mutation site was first excised using restriction enzymes and ligated into pBluescript II SK(-) (pSK). Following mutagenesis, the mutated sequences were removed from the intermediary plasmid and cloned into the original pIC20H-CAV vector to create pIC20H-CAVΔVp1 and pIC20H-CAVΔVp2.

To create pIC20H-CAVΔVp1, a 615 bp fragment containing the Vp1 start codon was first excised by PstI and NheI (blunted with Pfu polymerase) and ligated by T4 DNA ligase into pSK digested with PstI and SmaI. The resulting pSK-Vp1 plasmid was used as 50 ng of template DNA in a mutagenesis reaction mixture using KAPA HiFi DNA polymerase (Kapa Biosystems). The forward primer GAAGGCGTATAAGACTGTAAG<u>G</u>TGGCAAGACGAGCTCGCAG (mutation base A→G underlined) and its complementary reverse primer CTGCGAGCTCGTCTTGCCAC<u>C</u>TTACAGTCTTATACGCCTTC (mutation base underlined) were used to eliminate the start codon in Vp1 without interfering with the overlapping arginine codon of Vp2 in the +1 reading frame. The reaction mixture was assembled according to

manufacturer instructions and mutagenesis was performed in a T100 Thermal Cycler (Bio-Rad) with the following program: initial denaturation for 3 minutes at 95ºC, 12 cycles of 20s at 98ºC, 20s at 55ºC, and 4 minutes at 72ºC, and a final extension for 5 minutes at 72ºC.

For pIC20H-CAVΔVp2, a similar strategy was used but for the Vp2-containing region of CAV, accordingly. The pIC20H-CAV and pSK plasmids were both digested with restriction enzymes EcoRV and PstI to insert a 594 bp region of CAV including the Vp2 start codon into the pSK vector using T4 ligase to generate pSK-Vp2. Following the same reaction program but with GTATACGCAAGGCGGTCCGGGTGGATACACGGGAACGGCGGACAAC (forward) and TTGTCCGCCGTTCCCGTGTATCCACCCGGACCGCCTTGCGTATAC (reverse) primers (mutation bases underlined), site-directed mutagenesis was used to alter the Vp2 start codon.

Following the mutagenesis reaction, DpnI (NEB) was used to digest the template DNA and transformation into DH5α *E. coli* was used to amplify the mutated DNA. Sanger sequencing (Génome Québec) was performed to validate successful point mutation at the intended site before the CAV fragment was reinserted into the pIC20H-CAV plasmid to create pIC20H-CAVΔVp1 and pIC20H-CAVΔVp2. The Vp1 mutant region was excised from the mutation plasmid and inserted into pIC20H-CAV with BsmI and StuI, while the Vp2 mutant region was excised from its mutation plasmid and inserted into pIC20H-CAV with BsrGI and BlpI, through ligation with T4 ligase. Following re-amplification in DH5α, sequencing was used to confirm the integrity of the DNA and RF genomes were prepared for transfection as detailed above.

2.4 Viral genome quantification by qPCR

DNA was extracted from samples of virus-containing MSB-1 cells and filtered cell-free viral supernatants using the QIAAmp Viral RNA Mini Kit (QIAGEN) according to manufacturer instructions. Isolated DNA was digested with DpnI to remove any remaining bacterially

amplified RF CAV from electroporation. Viral genomes were quantified by real-time quantitative polymerase chain reaction (qPCR) based on a standard curve generated by known dilutions of pIC20H-CAV DNA, using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and a CFX Connect Real-Time PCR System (Bio-Rad). Primers for qPCR were matched to a sequence of the CAV genome and designed such that the amplicon spanned a DpnI restriction site (forward: ATGACCCTGCAAGACATGGG; reverse: CTTTTTGCCACCGGTTCTGG). The cycling program was as follows: initial denaturation for 2 minutes at 95ºC, 40 cycles of 10s at 95ºC, 20s at 60ºC, and 10s at 72ºC, followed by a melt curve analysis.

2.5 CAV infection of chicken cells

One day prior to infection, MDCC MSB-1 cells were seeded at $3.0x10^5$ cells/mL. Aliquots of up to 5 mL of virus-laden media of at least $1.0x10^9$ viral genomes/mL were thawed and used to resuspend 10 million MSB-1 cells for each infection. Cells were incubated with virus (or media for mock infection) for 1 hour at 41ºC before viral suspension was removed and cells were washed 3 times in PBS. Cells were resuspended at $3.0x10^5$ cells/mL in their regular media.

2.6 Nocodazole treatment of CAVΔApo-infected cells

MSB-1 cells were infected with CAVΔApo following the infection procedure described above. At 12 hours post-infection, Nocodazole in dimethyl sulfoxide (DMSO) was added to the CAVΔApo-infected cells' growth media at a final concentration of 25 ng/mL (or 80 nM). An equivalent volume of DMSO was also added to mock-infected cells as a vehicle control.

2.7 proTAME treatment of CAVΔApo-infected cells

CAVΔApo virus was used to infect MSB-1 cells as previously described. Immediately following infection, pro-N-4-tosyl-L-arginine methyl ester (proTAME) (Boston Biochem) in DMSO or DMSO only (vehicle) was added to culture media to a final concentration of 10 uM.

2.8 Cell counts and viability

Cells were counted using a hemocytometer and percent cell viability was determined by trypan blue staining (1:10 dye:cells) under light microscopy.

2.9 Cell cycle analysis by flow cytometry

Samples of at least $1.0x10^6$ cells were removed from culture flasks, washed twice in PBS, and fixed in cold 70% ethanol. Fixed cells were stored at -20ºC for up to 2 weeks. Prior to analysis, cells were rehydrated in PBS and stained with propidium iodide (PI) solution (100 ug/mL PI, 0.2 mg/mL RNase A, 0.6% Nonidet P-40 in PBS). Samples were either incubated for 1 hour at 37ºC or overnight at 4ºC. Flow cytometry was performed using LSRFortessa (BD Biosciences) 488 nm laser to determine DNA content and analyzed with Flowing Software 2.5.1.

2.10 Western blot

An equivalent volume of MSB-1 cells was taken at each time point from the culture flask, washed twice in PBS, and lysed in 1X Laemmli Buffer. DNA was sheared using a 23-gauge needle (BD PrecisionGlide) and a 1 cc syringe (BD). Cell lysates were boiled and protein was quantified by the Pierce 660 nm Protein Assay (Thermo Scientific) in microplate format using a Varioskan LUX Multimode Microplate Reader (Thermo Scientific). After protein concentration determination, 25 ug of each sample was loaded and separated in a 1.5 mm 12% TGX Stain-Free polyacrylamide gel (Bio-Rad). Stain-free gel was activated using the ChemiDoc XRS+ (Bio-Rad) stain-free enabled UV transilluminator (Bio-Rad) with Image Lab 6.0.1. Transfer to methanol-treated low-fluorescent polyvinylidene fluoride (PVDF) membrane was performed using the Trans-Blot Turbo Transfer System (Bio-Rad) and total protein density was visualized with the ChemiDoc XRS+ UV transilluminator.

Membranes were blocked using 5% skim milk in Tris-buffered saline 0.5% Tween-20 (TBS-T). Primary antibodies were 1:500 rabbit polyclonal anti-Vp1 (purified by previous lab members from EZBiolab custom antibody rabbit serum) in 3% milk 3% bovine serum albumin in TBS-T, and 1:1000 rabbit polyclonal anti-apoptin (previously described in Jiang et al. (2010), obtained from the Tavassoli lab) in 5% milk TBS-T (50). The secondary antibody was 1:5000 goat anti-rabbit HRP (Jackson labs, lot #87768) in 5% milk TBS-T. Blots were developed using Clarity Western ECL substrate (Bio-Rad) and imaged using the ChemiDoc XRS+ and Image Lab 6.0.1 software.

2.11 Cloning of bicistronic luciferase constructs

A suspected IRES region from bases 491-852 of the CAV genome was amplified by *Taq* polymerase using FW primer GTAAACCGGTCGCTCTCCAAGAAGAT (AgeI restriction site underlined) and RV primer GATCGCTAGCCTTACAGTCTTATACGCCTTC (NheI restriction site underlined). This sequence was inserted into the multiple cloning site (MCS) of a pKS/FF/MCS/Ren plasmid kindly provided by the Pelletier lab, containing a T3 RNA promotor and the Firefly luciferase gene followed by the *Renilla* luciferase gene with an intercistronic MCS. This construct containing the CAV sequence insertion was called pKS/FF/CAV852/Ren.

Another construct, pKS/FF/CAV896/Ren, was cloned to contain a larger region of the suspected IRES, from bases 491-896 of the CAV genome, in order to extend into the Vp1 coding sequence, while ensuring that the Vp1 start codon was kept in frame with the *Renilla* start codon. A new RV primer GTAAGCTAGCAAGGCGTAAAATCGGC (NheI site underlined) and the same FW primer were used, and the insert was cloned into the same site as described above.

A third construct, pKS/FF/HCV/Ren was cloned in order to generate a positive control, using the known IRES from hepatitis C virus (HCV) (148). Primers were designed to amplify a

region of the HCV genome from base 14-381 flanked by restriction sites for PacI and BlpI. The FW primer was GCAGTTAATTAATGGGGGCGACACTCCAC (PacI site underlined) and the RV primer was GCCCGGCTTAGCGTTACGTTTGTTTTTTTTTTGAG (BlpI site underlined). The HCV IRES was amplified and inserted into the MCS in the empty dual luciferase vector, ensuring that the start codon found in the HCV IRES was kept in frame with that of the *Renilla* luciferase. The template for the HCV IRES was another bicistronic luciferase construct previously cloned by the Pelletier lab, pSP/$(CAG)_{33}$/FF/HCV/Ren (149). All cloning was confirmed by Sanger sequencing.

2.12 Cloning of CAV mRNA-expressing construct

The pKS/FF/MCS/Ren plasmid (described above) used as the backbone for luciferase construct cloning and reporter testing was also used for expressing the CAV mRNA under a T3 RNA promotor. A 2238 nt region of the CAV sequence containing the complete polycistronic transcript from NruI to EcoRI (blunted with T4 DNA polymerase) was inserted into the pKS transcription vector with the luciferase genes removed by digestion with EcoRV and NruI. Fragments were purified by agarose gel electrophoresis and extraction using the GenepHlow Gel/PCR Kit and ligated by T4 DNA ligase. Correct insert orientation was screened for using digestion by restriction enzyme BamHI and confirmed by Sanger sequencing.

2.13 In vitro transcription

Plasmid DNA was first linearized by restriction enzyme BamHI (for bicistronic luciferase constructs) or NotI (for CAV mRNA expression construct) and purified by phenol-chloroform extraction and Sephadex G50 Superfine beads (GE Healthcare). To generate mRNA, T3 RNA polymerase (NEB) was added to digested plasmid in the presence of ribonucleotide triphosphates (Thermo Scientific), anti-reverse cap analog (NEB) and RNase inhibitor (NEB), and incubated at

37ºC for 3-4 hours. Remaining plasmid DNA was removed by DNase I (Thermo Scientific) and RNA was again purified by phenol-chloroform extraction and G50 beads. Quality of RNA was confirmed on 1% agarose gel and visualized with the AlphaImager (Alpha Innotech).

2 .14 In vitro translation

In vitro translation of luciferase reporter mRNA was performed in Krebs extracts prepared by and obtained from the lab or Dr. Jerry Pelletier, a Krebs ascites-derived cell-free extract developed for similar translation assays (149). *In vitro* transcribed mRNA was added to Krebs extract in salt mix (to final concentrations 5 mM KOAc, 75 uM $MgCl_2$, 25 uM spermidine) and translation mix (to final concentrations 1.25 mM HEPES pH 7.3, 0.1 mM ATP, 0.02 mM GTP, 0.02 mM CTP, 0.02 mM UTP, 1 mM creatine phosphate, 2 uM 19 amino acids without methionine) along with 14 mg/mL creatine phosphokinase and methionine at final concentration of 40 uM. Reactions of 10 uL were incubated at 30ºC for 1 hour and 5 uL was removed and used for the luciferase activity assay.

2.15 Dual luciferase reporter assay

Firefly luciferase and *Renilla* luciferase were assayed using addition of Firefly reagent (25 mM glycylglycine, 15 mM K_xPO_4 pH 8.0, 4 mM EGTA, 2 mM ATP, 1 mM DTT, 15 mM $MgSO_4$, 0.1 mM coenzyme A, 75 uM Luciferin) and *Renilla* substrate (1.1 M NaCl, 2.2 mM Na_2EDTA, 0.22 M K_xPO_4 pH 5.1, 0.44 mg/mL BSA, 1.3 mM NaN_3, 1.43 uM Coelenterazine). Luciferase activity measurements were taken using a Lumat LB 9507 luminometer (Berthold).

2.16 Statistical analysis

Data was compiled and computed in Microsoft Excel and GraphPad Prism 8.0.2. A maximum p-value of 0.05 was used to determine statistical significance or not significant (n.s.).

3.0 RESULTS

3.1 Temporal expression of Vp1 and apoptin in CAV-infected MSB-1 cells

To first explore our hypothesis that viral proteins playing different roles in virus replication would be expressed at different times during infection, we investigated the temporal expression of Vp1 (the viral capsid) and Vp3 (apoptin). MDCC MSB-1 cells were infected with a maximal titre (500 viral genomes per cell) of wild-type CAV to ensure complete synchronous infection, and samples collected every 4 hours over 48 hours were examined for protein expression. A mock infection (no virus introduced) was performed alongside the CAV infection and its samples used as a control. Western blot analysis of Vp1 (52 kDa) and apoptin (14 kDa) with their respective antibodies detected expression of viral proteins only in the CAV-infected samples, with apoptin presence appearing earlier, starting at 16 hours after infection and peaking at 24 hours after infection (Figure 4A). Compared to apoptin, Vp1 expression was delayed, first appearing at 20 hours after infection and increasing in the later hours of infection. Flow cytometry analysis of propidium iodide-stained cells confirmed that at 24 hours after infection, the vast majority of CAV-infected MSB-1 cells are arrested in G2/M phase, while mock-infected cells remain primarily in interphase (Figure 4B). Cell viability over the course of viral infection was relatively high up until 24 hours, after which dramatic cell death of CAV-infected cells was observed, with a resulting cell viability of less than 5% at 48 hours post-infection (Figure 4C). Together these results suggest that apoptin expression occurs earlier during infection and peaks when the virus induces cell cycle arrest in G2/M phase (at 24 hours), after which translation of the capsid protein, needed for packaging, increases until cell death and viral egress. This understanding aligns with our hypothesized model of viral infection wherein an “early” and “late” phase of viral translation occurs to facilitate virus production and replication.

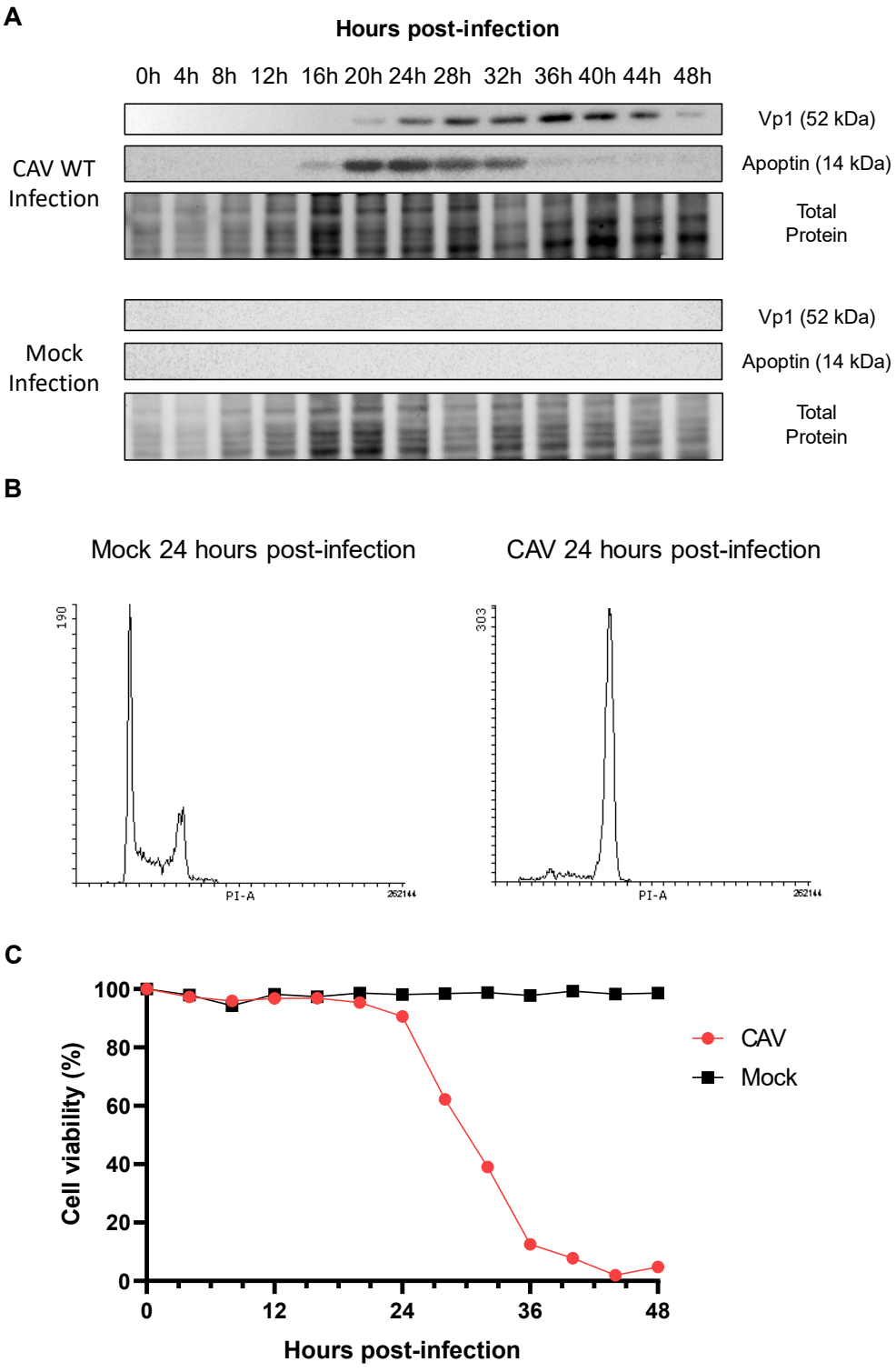


Figure 4. Vp1 is expressed later than apoptin in CAV infection, following cell cycle arrest. Wild-type CAV infection of MSB-1 cells was monitored over 48 hours post-infection. (A)

Western blot analysis of Vp1 (52 kDa) and apoptin (14 kDa) expression in samples from CAV infection (top) and mock infection (bottom) taken every 4 hours after infection. Imaging on polyvinylidene fluoride membrane visualized by Image Lab 6.0.1 using total protein density to indicate loading and for normalization. (B) Flow cytometry histogram of propidium iodide stained cells from 10 000 recorded events of mock-infected (left) and CAV-infected (right) cells at 24 hours post-infection. (C) Cell viability change measured by trypan blue dye every 4 hours for 48 hours after CAV (red circle) and mock (black square) infection.

3.2 Apoptin is not necessary for but contributes to efficiency of viral replication

Previous work in the lab completed by former student David Sharon explored the role of apoptin in viral replication by generation of an apoptin-mutant CAV (CAVΔApo) in which the start codon of Vp3 is eliminated by a single point mutation to knock out apoptin expression, while retaining the asparagine codon in the Vp2 reading frame (Figure 5A). Sharon's work confirmed that CAVΔApo does not express apoptin and demonstrated that transfection with CAVΔApo DNA is capable of generating CAVΔApo viral particles (147). To confirm results from these previous experiments as well as expand upon these findings, we performed infections with CAVΔApo virus and also observed that apoptin is not needed to generate infectious virus, though it contributes to the efficiency of viral replication.

Quantification of CAVΔApo genomes in MSB-1 cells after infection shows an increase in genome copy number, confirming that infectious viral particles containing everything needed for viral replication must exist in the cell-free supernatant harvested from viral DNA transfections. However, CAVΔApo demonstrates a decreased efficiency of viral genomic

replication, resulting in a viral genome count only about 40% that of the genome copies of the wild-type virus after 48 hours (Figure 5B). Furthermore, where wild-type CAV, expressing intact apoptin, was previously shown to induce G2/M arrest in infected cells (Figure 4B), CAVΔApo virus was not capable of arresting the cell cycle in mitosis, showing no significant differences from the mock-infected cells primarily in G1 (Figure 5C). This result is as expected since the lack of expression of apoptin in the mutant virus suggests no protein interaction with the APC/C that would serve to inhibit the cell cycle. Lack of apoptin expression in the CAVΔApo mutant virus was confirmed by Western blot at 24 hours after infection while ensuring that the Vp1 capsid was still expressed, retaining the ability to package viral particles (Figure 5D). As predicted, CAVΔApo-infected MSB-1 cells were also not observed to undergo a dramatic decrease in cell viability as seen previously with wild-type CAV infection (Figure 5E).

In a subsequent experiment, co-transfection of 1 ug of a 3xFLAG vector expressing apoptin along with the CAVΔApo mutant transfection demonstrated increased viral genomic replication over 72 hours after transfection compared to the CAVΔApo viral DNA alone (Figure 5F). This finding suggested that the impacted replication efficiency of CAVΔApo was indeed due to the lack of apoptin, and could be rescued by addition of exogenous apoptin in transfection.

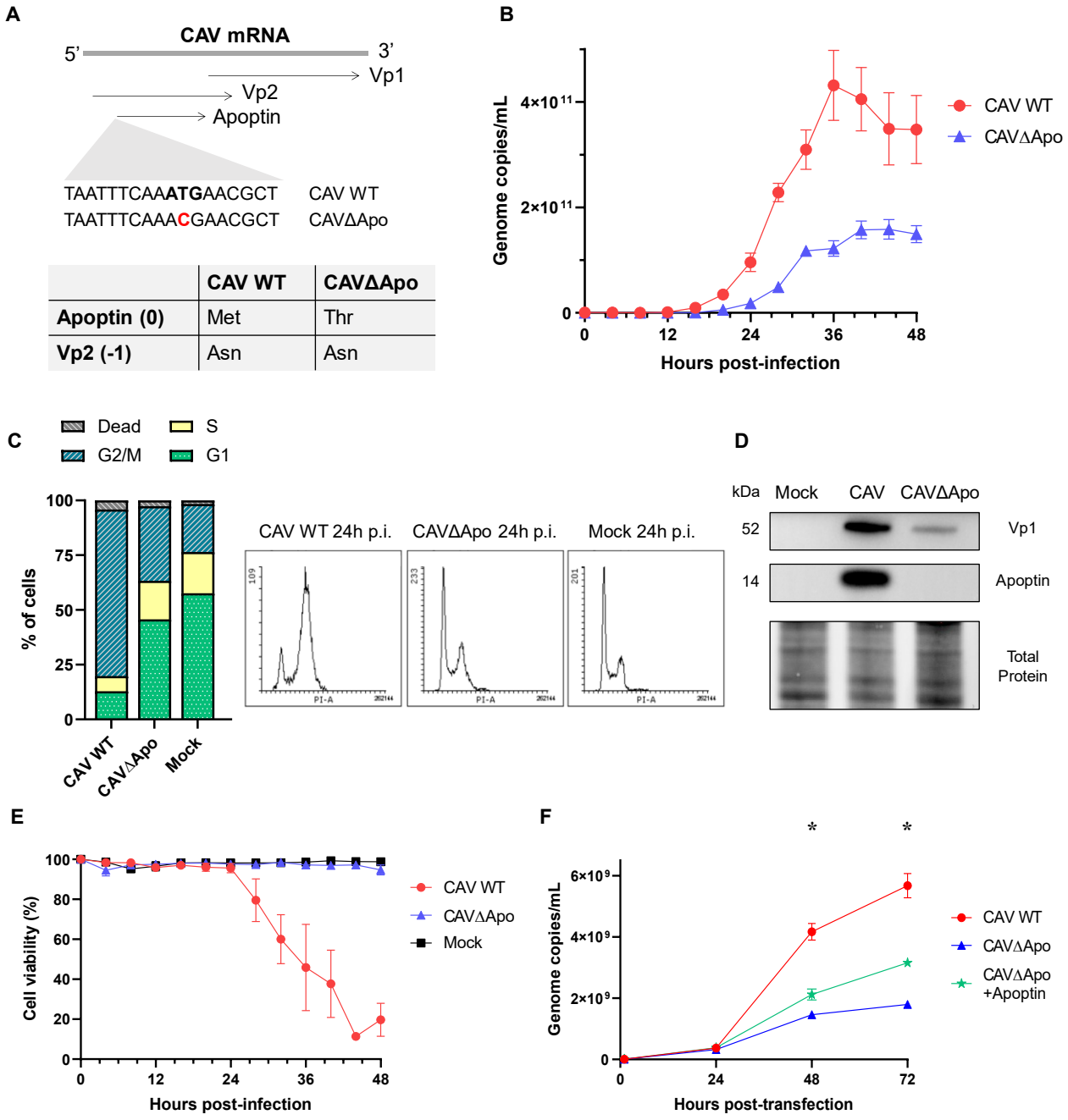


Figure 5. CAVΔApo has reduced replication efficiency and does not induce cell cycle arrest or cell death. (A) Schematic showing mutagenesis strategy used to generate CAVΔApo. A T→C point mutation disrupts the start codon of apoptin without altering the overlapping Vp2 reading frame. (B) Comparison of growth in viral genomes for 48 hours, sampled every 4 hours and quantified by qPCR, after infection with CAV (red circle) or CAVΔApo (blue

triangle) virus from cell-free transfection supernatants in MSB-1 cells. Data represent means and SEM. N=3. Student's *t* test; $p < 0.05$. (C) Quantification and histograms of flow cytometry analysis of 10 000 recorded events of propidium-iodide stained MSB-1 cells infected with CAV or CAVΔApo (compared to mock infection). Percent of dead cells were determined by trypan blue exclusion. (D) Western blot on polyvinylidene fluoride membrane of Vp1 (52 kDa) and apoptin (14 kDa) expression in samples of MSB-1 cells at 24 hours after infection with CAV or CAVΔApo. Total protein density used to indicate loading and for normalization. (E) Cell viability change measured by trypan blue dye every 4 hours for 48 hours after CAV (red circle), CAVΔApo (blue triangle) or mock (black square) infection in MSB-1 cells. Data show means and SD. N=3. Student's *t* test; $p < 0.05$, CAV to CAVΔApo or mock. n.s., CAVΔApo to mock. (F) Quantification of viral genomes by qPCR for 72 hours after transfection of MSB-1 cells with either wild-type CAV DNA, CAVΔApo DNA alone or CAVΔApo co-transfected with 1 ug of a 3xFLAG-Apoptin expression vector. Data represent means and SEM. N=3. Student's *t* test; * denotes $p < 0.05$, for all samples.

3.3 Vp1 and Vp2 are critical to viral replication

Although it is widely understood from existing literature on CAV that both other viral proteins, Vp1 and Vp2, would be critical for viral replication due to their identified roles, we sought to confirm their impacts on CAV replication as well. Vp1 is the only structural protein found in the viral capsid and thus is needed to package the viral DNA and form virions (9). Vp2 possesses multifunctional roles in virion assembly, viral replication and as a phosphatase (partially responsible for apoptin's subcellular localization), implying that it is absolutely essential for viral replication (15, 17-19). A similar mutagenesis strategy to the previously

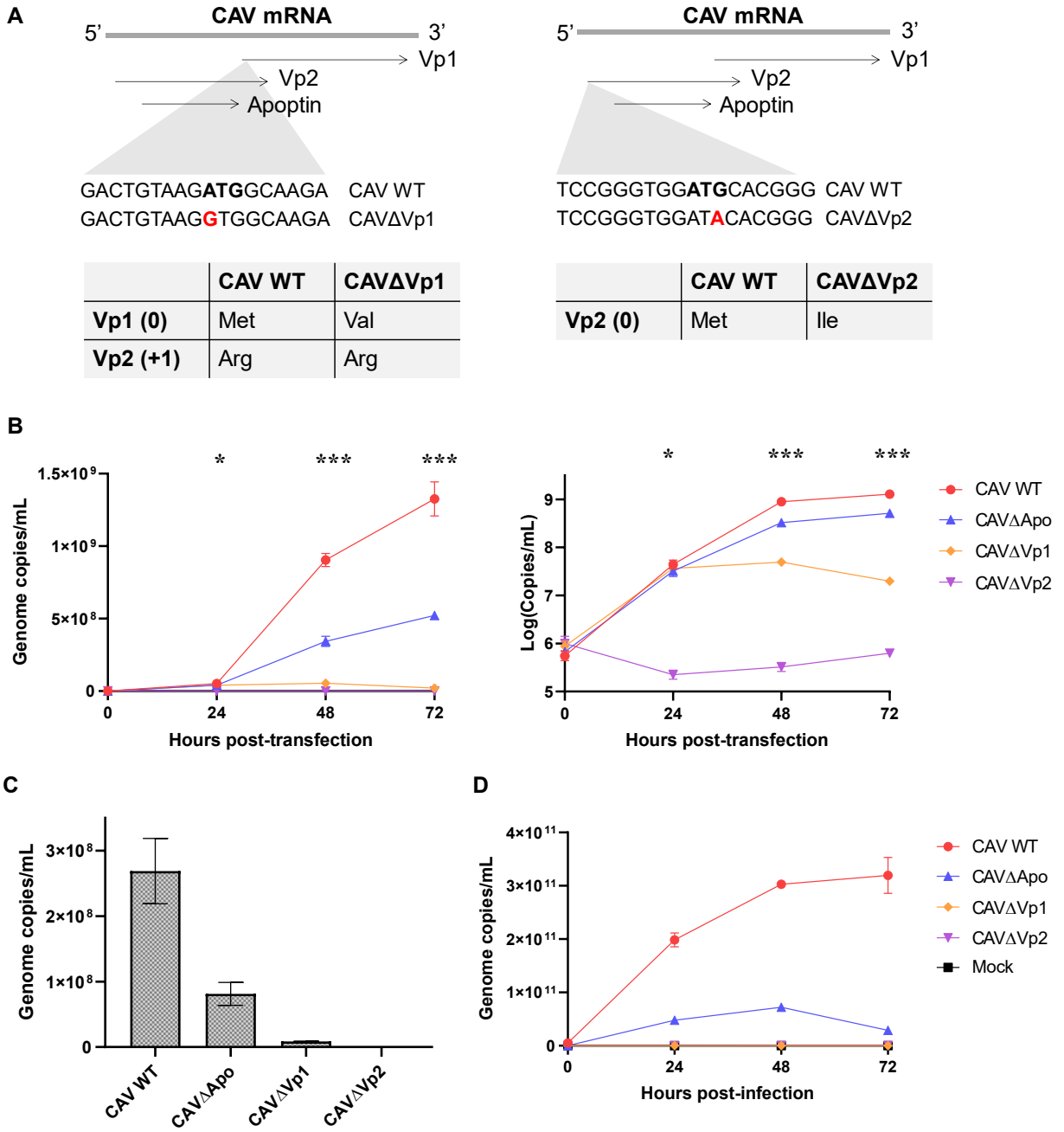
described method for CAVΔApo was used to create replicative form CAV DNA for transfection into MSB-1 cells lacking expression of either Vp1 or Vp2, respectively referred to as CAVΔVp1 and CAVΔVp2.

In the case of CAVΔVp1, the methionine initiation codon is altered to a valine codon while maintaining an arginine codon in the +1 reading frame which codes for Vp2 (Figure 6A). We hypothesized that transfection of CAVΔVp1 DNA would potentially be capable of genomic replication, based on the expectation that the only protein not being expressed was the capsid, predicted to only be required for later phases of infection. Thus, we believed that while no CAVΔVp1 infectious virus would be harvested from the cell-free supernatant, we expected to see a rise in viral genomes over time after RF DNA transfection when quantifying genomes from lysed cells by qPCR. However, the CAVΔVp1 DNA failed to produce high levels of viral genome replication, in stark contrast to both the wild-type CAV RF DNA and the CAVΔApo RF DNA (Figure 6B). Rather, the CAVΔVp1 appeared to increase in genome copy number in the first 24 hours after transfection, in levels similar to those of CAV and CAVΔApo, but did not continue to amplify after 24 hours, reaching a plateau in genomic replication about one order of magnitude below that of the viable viruses. Since CAVΔVp1 lacks the structural protein and should not be able to form a viral particle, we would not expect to find a high quantity of viral genomes in the cell-free supernatant. However, quantification of the cell-free media pooled from CAVΔVp1 transfections after 72 hours did indicate the presence of a very low number of viral genomes, but this was much less than the viral genome titres found in CAV and CAVΔApo transfections (Figure 6C). In addition, since the quantification by qPCR is based on the detection of viral DNA, this presence of viral genomes does not necessarily indicate any presence of viral particles in the supernatant. Nevertheless, to confirm that there were no viral particles from

CAVΔVp1 transfection capable of infection, the collected cell-free media in which the genomes were quantified after CAVΔVp1 transfection were used to test "infect" MSB-1 cells (like a mock infection). Quantification of viral genomes in the lysed cells at each time point after introduction of cell-free media from the CAVΔVp1 transfection resulted in no growth in copy number of viral DNA, supporting that CAVΔVp1 would not form viable infectious viral particles (Figure 6D).

Similarly, site-directed mutagenesis was used to create a single point mutation at the site of the Vp2 start codon, knocking out expression of Vp2 by altering the methionine translation initiation codon to one for isoleucine (Figure 6A). In the same set of experiments as described above, we investigated the impact of removing Vp2 from CAV. Due to its putative roles in replication, we predicted that any construct of CAV unable to express Vp2 would not be capable of any form of viral replication (no increase in genomic viral DNA nor an ability to package viral particles). In the transfection of CAVΔVp2 RF DNA into MSB-1 cells, no amplification of viral genomes was detected, aligning with our predictions (Figure 6B). As well, in the cell-free media from the CAVΔVp2 transfected cells, no significant levels of viral genomes were detected by qPCR, suggesting that there was no viral DNA produced and liberated from the transfected cells (Figure 6C). Finally, the CAVΔVp2 harvested media was used in the same manner as a mock infection to confirm no presence of infectious viral particles, and again no increase in viral genome copies was seen over 72 hours (Figure 6D).

Together, these results provide evidence that no form of CAV lacking Vp1 or Vp2 expression would be capable of viral reproduction and propagation, and support that both Vp1 and Vp2 viral proteins are required for the production of infectious viral particles.



	CAV WT	CAVΔVp1
Vp1 (0)	Met	Val
Vp2 (+1)	Arg	Arg

	CAV WT	CAVΔVp2
Vp2 (0)	Met	Ile

Figure 6. Elimination of Vp1 and Vp2 from CAV genome abolishes viral replication. (A) Schematics showing mutagenesis strategy used to generate CAVΔVp1 (left) and CAVΔVp2 (right). For CAVΔVp1, a A→G point mutation eliminates the Vp1 start codon but maintains the Vp2 reading frame codon. A G→A point mutation removes the Vp2 start codon in CAVΔVp2. (B) Comparison of growth in viral genomes over 72 hours in MSB-1 cells after

transfection with RF DNA of CAV (red circle), CAVΔApo (blue triangle), CAVΔVp1 (orange diamond) or CAVΔVp2 (purple triangle). Samples taken every 24 hours and quantified by qPCR are shown in both linear (left) and logarithmic (right) scale for visual clarity. Data are means and SEM. N=3. Student's *t* test; * denotes $p < 0.05$, CAVΔVp2 to other samples. *** denotes $p < 0.05$, for all samples. (C) Quantification by qPCR of viral genomes found in filtered cell-free media of CAV variant-transfected MSB-1 cells after 72 hours. Data display means and SD. N=3. Student's *t* test; $p < 0.05$, for all samples. (D) Comparison of growth in viral genomes over 72 hours after infection with CAV (red circle), CAVΔApo (blue triangle), CAVΔVp1 (orange diamond) or CAVΔVp2 (purple triangle) in MSB-1 cells. Mock infection (black square) performed as control. Error bars are SD. N=1.

3.4 Specific inhibition of the APC/C appears to promote CAVΔApo replication

As CAVΔApo was determined to be the only mutant virus capable of creating functional virus, but at a decreased efficiency, we sought to examine how its replicative efficiency could be rescued. Based on past research in the lab and prior experiments (Figure 5), we believed that apoptin's induction of cell cycle arrest contributes to favouring viral replication. Thus, we explored the effect of chemical compounds as cell cycle inhibitors on the replication efficiency of the mutant CAVΔApo virus. Two chemical inhibitors were tested: Nocodazole, which causes mitotic arrest through depolymerization of the microtubules (preventing cell division in an APC/C-independent manner), and tosyl-arginine methyl ester (TAME), an APC/C-specific small molecule inhibitor (150, 151).

Nocodazole, when added to MSB-1 cells infected with CAVΔApo, did not result in an increase in viral genome copies over time (Figure 7A). This suggests that CAVΔApo maintains

decreased genomic replication even in the presence of a mitotic arrest induced in an APC/C-independent manner. In fact, 25 ng/mL Nocodazole introduced in MSB-1 cells (resulting final concentration 80 nM) appeared to be rather effective in mimicking the cell cycle profiles caused by wild-type CAV virus after infection (Figure 7B). For both wild-type CAV infection and CAVΔApo with Nocodazole in MSB-1 cells, over 50% of cells are arrested in G2/M by 24 hours after infection, and by 48 hours, the vast majority (over 80%) of cells in the population have died. In comparison, CAVΔApo infection without the addition of Nocodazole does not result in a G2/M peak at 24 hours (in line with what we previously observed) and accordingly, the cells do not undergo apoptosis by 48 hours.

In contrast to Nocodazole, TAME inhibits the APC/C specifically as it is a structural mimetic to the isoleucine-arginine (IR) tail found in both APC/C coactivators, Cdh1 and Cdc20 (Figure 7C). proTAME, the prodrug form of TAME, was chosen in our experiment as apoptin also contains a putative IR motif, found in its second- and third-last amino acid positions. In past experiments conducted by former lab member David Sharon, it was demonstrated that proTAME appeared to rescue CAVΔApo genome replication to levels similar to wild-type viral DNA by 72 hours in transfection experiments (147). However, as our past experiments did not include genome quantification across multiple time points (rather, we only quantified viral genomes at the experimental endpoint of 72 hours), we planned to continue performing experiments to better characterize and visualize the likely rescue of replication in CAVΔApo by proTAME. In addition, our follow-up experiments were done with infectious virus rather than RF DNA transfection to better contextualize genome replication in the true viral life cycle.

In order to determine the role of APC/C inhibition on viral replication, proTAME was added to cells infected with CAVΔApo virus and genomic replication was compared to that in

wild-type CAV infection. As expected, CAVΔApo infection without any inhibitor added showed decreased replication efficiency when compared to the wild-type virus; however, in the presence of 10 uM proTAME added, genome amplification of CAVΔApo appeared to increase to levels close to that of the wild-type virus as time post-infection progressed (Figure 7D). This result aligns with previous findings from the lab that proTAME is effective at rescuing the apoptin-mutant virus' ability to replicate its genome at full efficiency, and necessitates further follow-up and replicate trials to confirm. Together these data suggest that APC/C inhibition, whether induced by apoptin or by proTAME, is needed for efficient CAV genomic replication.

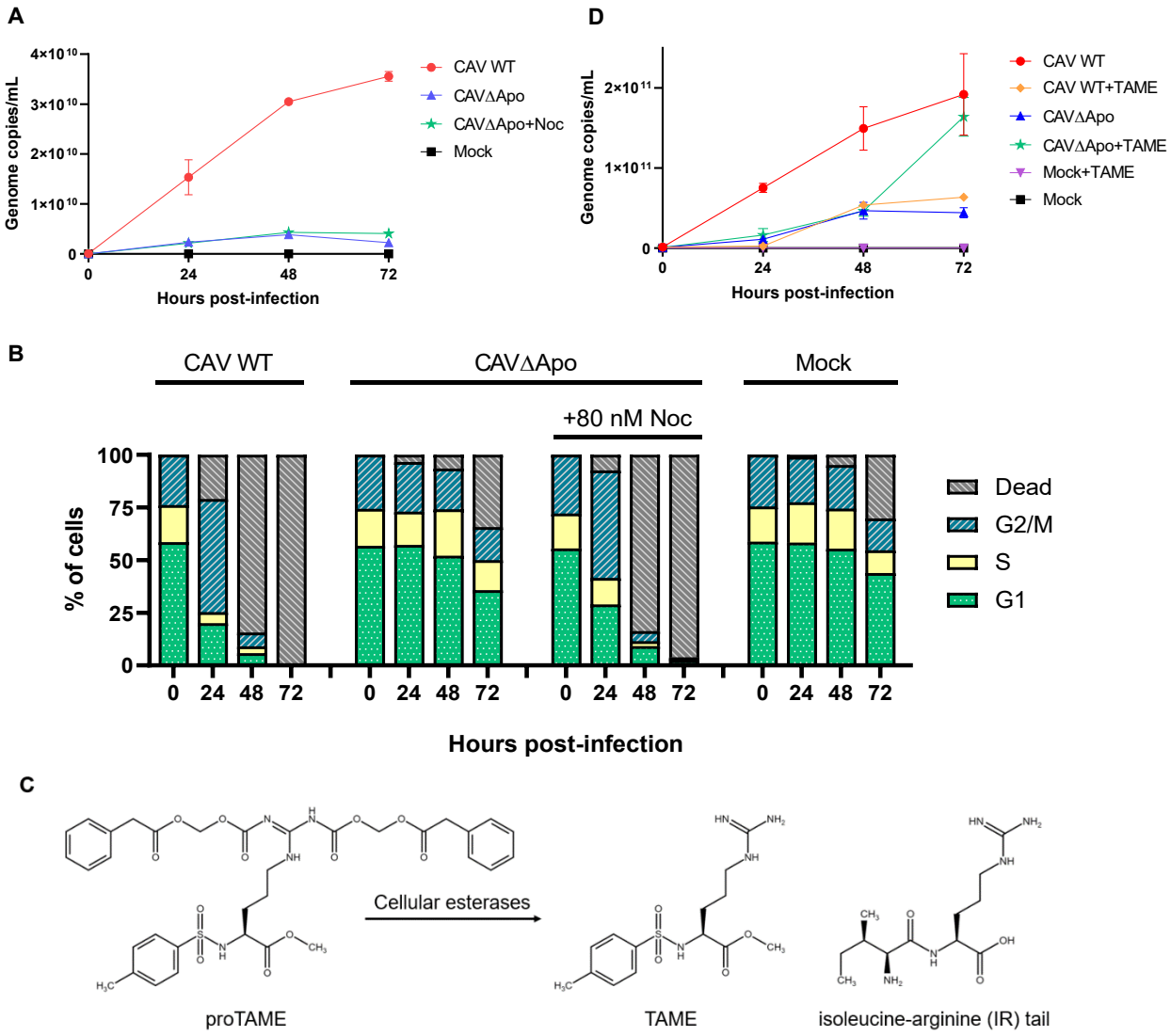


Figure 7. Inhibition of the APC/C rescues CAVΔApo replication efficiency, but cell cycle arrest alone does not. Nocodazole or TAME were added to MSB-1 cells infected with CAVΔApo to assess their effect on viral replication efficiency. (A) Quantification of viral genomes by qPCR over 72 hours after infection with CAV (red circle), CAVΔApo (blue triangle), CAVΔApo with Nocodazole treatment (green star) or no virus (black square). Error bars show SD. N=1. (B) Quantification of cell cycle stages in MSB-1 cells for Nocodazole-treated CAVΔApo infection experiment. G1, S, and G2/M phase distributions determined by flow cytometry analysis of propidium-iodide staining and dead cell fraction determined by trypan blue exclusion. (C) Chemical structures of the prodrug proTAME, its active form TAME, and their similarities to the isoleucine-arginine motif found in APC/C coactivator proteins Cdc20 and Cdh1. (D) Quantification of viral genomes by qPCR over 72 hours with proTAME or vehicle (DMSO) addition during infection with CAV (+DMSO: red circle, +TAME: orange diamond), CAVΔApo (+DMSO: blue triangle, +TAME: green star) or no virus (+DMSO: black square, +TAME: purple triangle). Error bars show SEM. N=2. (Note: WT CAV+TAME and Mock+TAME infection treatments performed only once; N=1).

3.5 Evaluation of CAV mRNA for IRES using bicistronic luciferase reporters

The small genome of CAV and the simplicity of its mRNA suggest that the virus must be functionally efficient. However, initiation of translation along the CAV mRNA is presumed to be regulated by a leaky scanning mechanism, which could suggest difficulty for translation initiation of start codons further downstream on the mRNA, such as that of Vp1 (3). We previously showed delayed expression of Vp1 upon CAV infection, first appearing after expression of apoptin and increasingly expressed following cell cycle arrest in G2/M (Figure 4).

The difference in temporal expression of Vp1 and apoptin suggests that these proteins' translation is differentially regulated to be favoured at different points in the viral life cycle. One hypothetical mechanism of regulation would involve canonical cap-dependent translation for proteins encoded closer to the 5' capped end of the CAV mRNA, such as Vp2 and apoptin, while translation of the downstream Vp1 reading frame could be cap-independent to remain actively expressed during mitotic translational shutdown. As previously noted by former lab members, *in silico* analysis of the region (bases 491-896 in the CAV mRNA) upstream and including the Vp1 start codon predicts a high degree of secondary structure with several stem loops (Figure 8A). Therefore, in order to evaluate for the presence of a cap-independent translational regulatory mechanism, this region of the CAV genome upstream of viral protein Vp1 was cloned into a dual luciferase reporter construct, a common technique for assessing potential IRES activity.

The suspected IRES region, from bases 491-852 of the CAV genome, was cloned into a dual luciferase reporter construct (Figure 8B) to assess the region's ability to promote cap-independent translation – this construct was termed pKS/FF/CAV852/Ren. A similar but slightly longer region from the CAV genome, corresponding to bases 491-896, was also cloned into the bicistronic luciferase plasmid and termed pKS/FF/CAV896/Ren. The choice to include this latter construct was done because some IRESs, such as that of HCV, are highly dependent on the downstream context of the AUG codon for internal initiation (113). The "empty" vector, containing only the multiple cloning site between the two luciferase genes (from Firefly and *Renilla*), was used as a negative control. The HCV IRES was subcloned from a different dual luciferase construct, pSP/$(CAG)_{33}$/FF/HCV/Ren (described previously) into the same vector and termed pKS/FF/HCV/Ren to serve as a comparable positive control which should account for any vector-specific differences (152).

Each reporter construct was transcribed and translated *in vitro* and activity of each luciferase was measured to assess the relative translation induced by the IRES. In the positive control HCV IRES mRNA, higher expression of the downstream luciferase (*Renilla*) was measured compared to the cap-dependently translated Firefly luciferase upstream of the IRES (Figure 8C-8D). However, no increase in the *Renilla*-to-Firefly luciferase translation was seen in either of the suspected CAV IRES-containing mRNAs, resembling the activity of the empty vector control. Together, these results suggest that the region of the CAV mRNA upstream of Vp1 does not initiate translation in a cap-independent manner, raising doubt as to whether an IRES or some other translational mechanism may exist to regulate Vp1 expression.

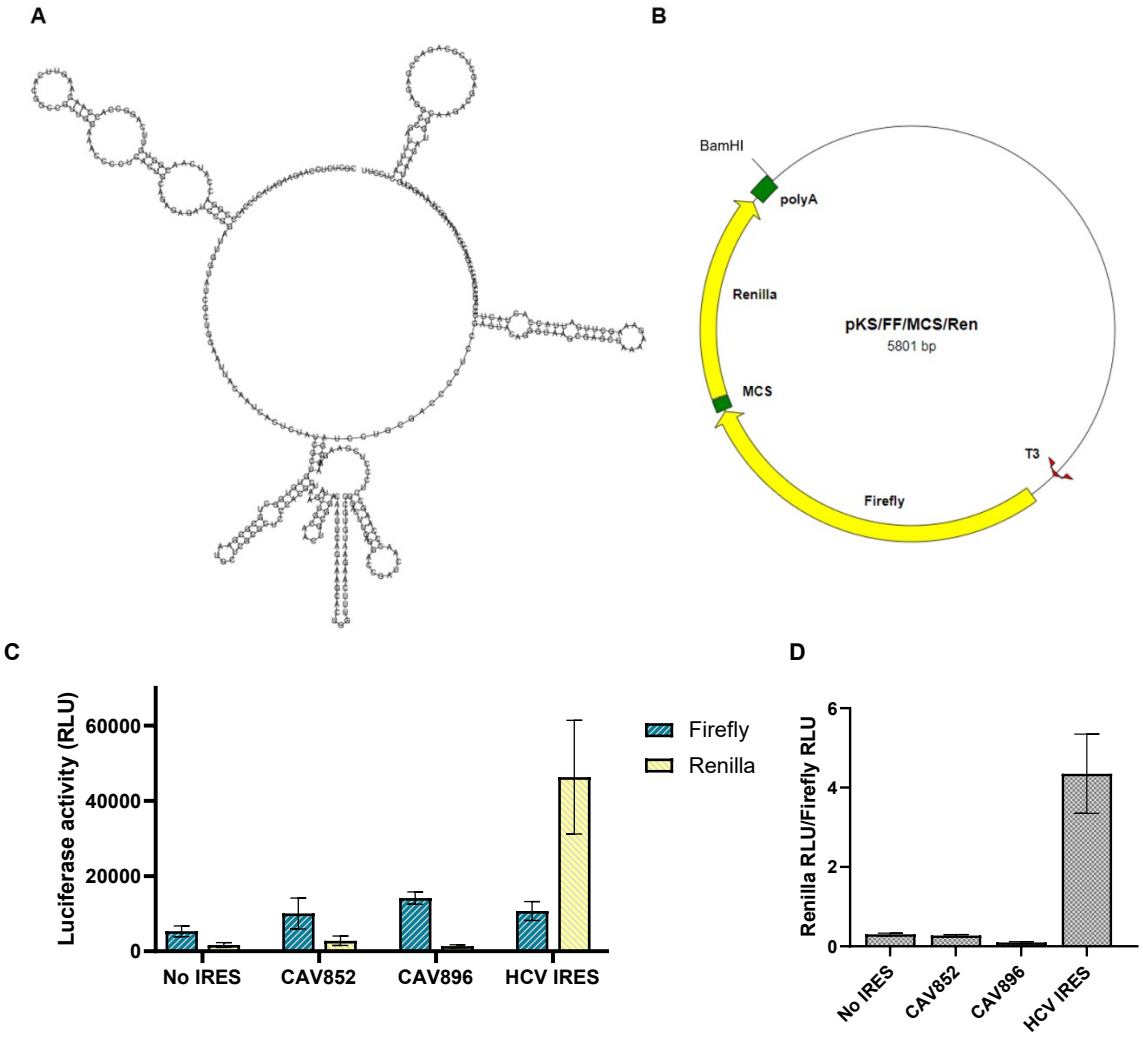


Figure 8. Suspected CAV IRES does not internally initiate translation in bicistronic luciferase constructs *in vitro*. (A) Predicted centroid secondary structure of CAV mRNA from bases 491-896, as computed by the RNAfold WebServer (Institute for Theoretical Chemistry, University of Vienna). (B) Plasmid map of the pKS/FF/MCS/Ren bicistronic luciferase construct containing Firefly and *Renilla* luciferase (yellow arrows), a T3 RNA transcription promotor (red), a multiple cloning site containing restriction enzymes into which suspected CAV IRES regions and the HCV IRES were inserted, a polyA tail, and a unique BamHI restriction site for template linearization. (C) Firefly and *Renilla* luciferase activity in relative light units (RLU) and (D) ratio of *Renilla*/Firefly for pKS reporter constructs containing a multiple cloning site (no IRES), CAV 491-852 (CAV852), CAV 491-896 (CAV896) or HCV 14-381 (HCV) in the intercistronic region. Data show means and SD. N=3. Student's *t* test; $p < 0.05$, HCV to no IRES. n.s., CAV852 or CAV896 to no IRES.

3.6 Exploring translation through generation of CAV mRNA in vitro

To characterize CAV translation specifically without host cell background or the context of the infectious viral life cycle, we decided to conduct *in vitro* translation experiments using the full-length CAV mRNA. We expect to see expression of all three CAV proteins and labelling with ^{35}S-methionine or visualization by Western blot should indicate relational differences in quantity of the viral proteins produced. Their specific translation could then be further investigated through the addition of various inhibitors to translation initiation. A plasmid was constructed to express the CAV mRNA from a T3 RNA polymerase promotor (Figure 9A) and was capable of creating stable full-length CAV mRNA through *in vitro* transcription (Figure 9B). The RNA transcript will subsequently be translated in rabbit reticulocyte lysate and the protein

products assessed. Creation of a similar plasmid, with the same vector backbone but to express the CAVΔApo mRNA instead, is currently in progress and it will also be transcribed and translated *in vitro* to compare how lack of apoptin may affect the expression of other viral proteins.

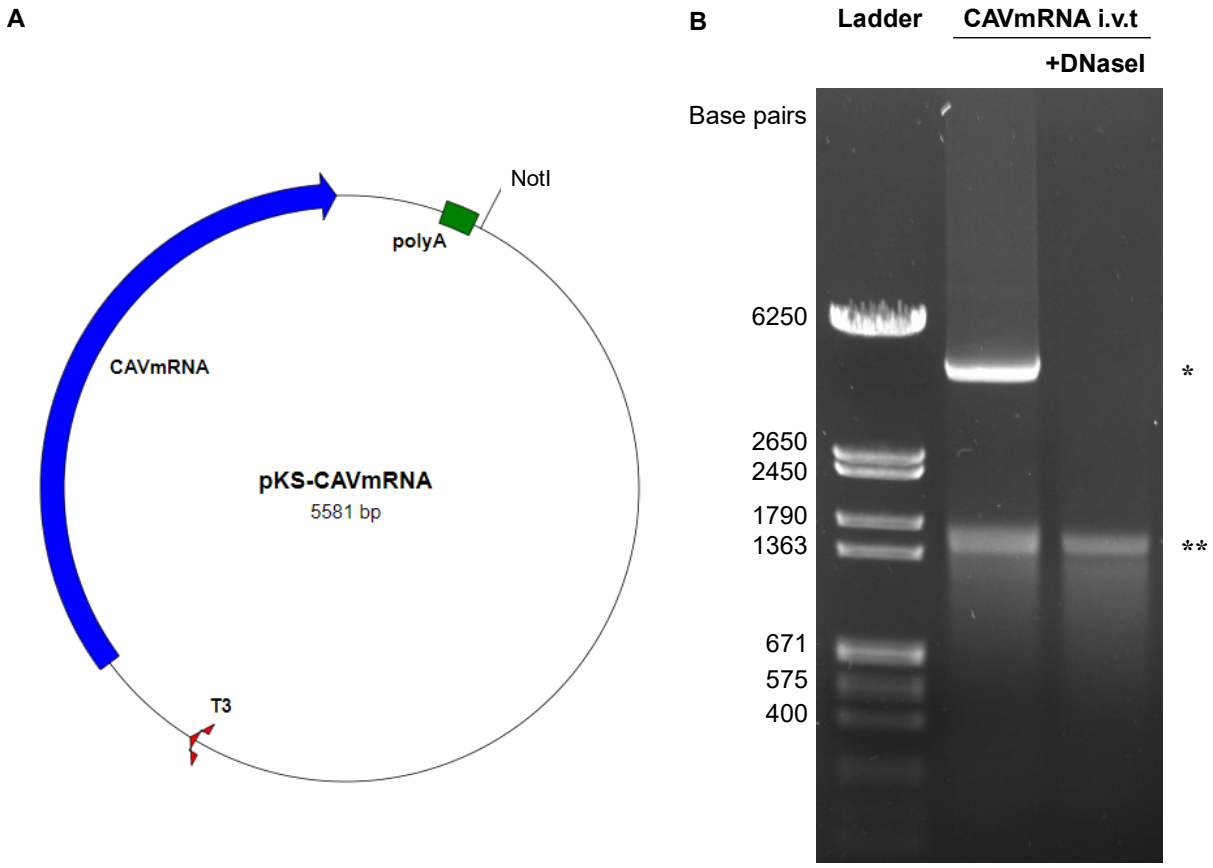


Figure 9. Stable full-length CAV mRNA can be transcribed *in vitro*. (A) A transcription vector was cloned to express the CAV mRNA (blue arrow) under a T3 RNA promotor (red). (B) Visualization of the transcription products before and after addition of DNaseI (to remove DNA template) is shown on a 1% agarose gel with a DNA ladder with sizes labelled on the left. * indicates linearized DNA template and ** indicates RNA transcription product.

4.0 DISCUSSION

In the work presented in this thesis, we sought to better understand the life cycle of CAV through studying its protein functions, gene expression, and viral replication. We showed that the Vp1 capsid protein is expressed later in CAV infection than apoptin, suggesting a difference in gene expression between early and late proteins. We confirmed that apoptin is not required for CAV replication, but contributes to its genomic replication efficiency, with CAVΔApo mutant virus genome amplification at about 40% efficiency compared to that of the wild-type CAV. Moreover, we observed that CAVΔApo virus does not result in G2/M cell cycle arrest or the induction of cell death, validating the role of apoptin in CAV infection. In addition, we demonstrate that both Vp1 and Vp2 are essential to CAV replication and its ability to produce infectious viral particles. We further show that specific inhibition of the APC/C with small molecule inhibitor TAME appears to rescue the viral replication efficiency in a lack of apoptin expression, such as in CAVΔApo infection. Though we were unable to determine an internal region of the CAV mRNA that could potentially initiate translation independently of the 5' mRNA cap, we propose a model in which inhibition of the APC/C by apoptin serves as a novel form of host translational repression and through an alternative translational regulatory mechanism, late protein coding regions on the CAV mRNA are favourably expressed.

Previous work has established that apoptin inhibits the APC/C by associating with the APC1 subunit, causing cell cycle arrest in G2/M by preventing cells from exiting mitosis (24). Past work on this project in our lab has demonstrated that this G2/M arrest is accompanied by translational repression, as ^{35}S pulse-labelled translation assays visualized on autoradiography blot showed the appearance of a putative Vp1 band 24 hours after infection with wild-type CAV (147). Our findings that Vp1 expression occurs later in infection, first detected at 24 hours post-

infection, upon cell cycle arrest during apoptin peak expression, and increasing in late phases of infection, align with these past observations. In addition, these results support some early work by Douglas et al. (1995) containing the only other evidence known to us that Vp1 is expressed later in infection than Vp2 and Vp3, both of which were detected earlier, at 12 hours after infection in MSB-1 cells by immunofluorescence detection using antisera (153). Our use of Western blot and specific antibodies for the viral proteins of interest in this study appear to confirm these observations. Interestingly, former lab member David Sharon's ^{35}S-methionine labelled autoradiography film also included infections using the apoptin-mutant form of CAV, for which translation of the putative Vp1 protein band was not seen at 24 hours after infection (147). While this was interpreted to mean that translation of the CAV mRNA may benefit from host translational repression induced by apoptin, such that CAVΔApo may not sufficiently promote CAV protein expression without inducing G2/M arrest, this result may alternatively suggest that apoptin directly plays a role in facilitating the translation of other protein coding sequences along the mRNA. This is further suggested by the Western blot in our work validating a lack of apoptin expression in the CAVΔApo infection (Figure 5D), as it can be seen that the expression of Vp1 at the same time point in the same imaged blot is greatly decreased in the CAVΔApo infection compared to the wild-type CAV. This alternate interpretation of apoptin's role in Vp1 expression may provide a rationale for the inability of this thesis to confirm an IRES as a novel translational mechanism in the CAV mRNA.

However, a rationale may still exist for the suspicion of an IRES or similar cap-independent mechanism in the region under investigation. Given that CAV encodes a single polycistronic mRNA and the Vp1 start codon is the last from the 5' cap, if the Vp1 ORF is assumed to be translated by a leaky scanning mechanism, one would anticipate low translational

efficiency of the major viral capsid protein (3). Thus, it seems reasonable that some form of virus-mediated alternative to canonical cap-dependent translation regulates the expression of Vp1, especially given our findings that Vp1 is differentially temporally regulated. Unfortunately, our attempt to validate the region upstream of Vp1 as capable of initiating internal translation was unsuccessful in the *in vitro* bicistronic luciferase reporter assay. It remains possible that the RNA structures in this region may require additional help from cellular factors or other viral proteins (such as apoptin) to recruit the ribosome. Given that other viral proteins or host factors may play a role in facilitating Vp1 translation, future experiments to continue investigating the translation of the viral mRNA could involve transfection of a suspected IRES-containing luciferase reporter expressible in cells along with a co-infection of CAV or another method of co-expressing viral proteins. Although the data presented here in an attempt to confirm a CAV IRES is not definitively conclusive, our findings certainly raise doubt of our initial hypothesis concerning the mechanism of Vp1 translation, and support consideration of an alternative by which CAV may regulate the translational switch from early to late protein expression.

Alternatively, other potential RNA stem loops and high secondary structure are predicted in the CAV mRNA in several regions, including at the 3' end of the mRNA. It is possible that these other structures in the CAV mRNA are responsible for coordinating differential translational regulation of early and late proteins, or that folding allows for interaction of these various regions in an infection context to work synergistically. As parasites of cellular translation machinery, viruses have a huge diversity in mechanisms of translation – many may still be 5' cap-dependent but modulate conventional mechanisms to favour viral translation over that of the host cell (154). Other mechanisms may include ribosome shunting, inducing a frameshift, or changing the phosphorylation and activity of various translation factors (154). One possible

assay to assess the role of cap-dependent translation for CAV protein expression involves the use of inhibitors of cap-dependent initiation, such as m^7GTP cap analog, or factor-specific inhibitors such as hippuristanol, which inhibits eIF4A (154, 155). As we have currently generated the wild-type CAV transcript *in vitro*, we intend to use it in such experiments as the full-length mRNA would include any potential regions involved in regulation outside of solely the suspected IRES. If the CAV mRNA does rely on cap-dependent mechanisms for translation initiation, expression of all viral proteins should decrease in response to the addition of inhibitors. If translation of some viral proteins continues or increases in the presence of such inhibitors, it may suggest a cap-independent mechanism and could indicate that further IRES investigation may be needed.

In order to confirm the suspicion that, under G2/M arrest and host translational repression, the CAV mRNA may be preferentially translated, we can perform ribosome profiling (Ribo-Seq) to determine the mRNAs that are actively translated during CAV infection (156). By providing a global snapshot of all the active ribosomes in a cell, Ribo-Seq allows for analysis of the entire translatome, and can provide information such as the distribution of ribosomes on an mRNA, translation start sites, and the speed of translating ribosomes (157). Thus, such a technique could indicate whether CAV mRNA translation is upregulated while other host proteins are translationally repressed in cell cycle arrest and could also suggest cellular mRNAs that remain active during CAV infection as potential host factors used to favour viral translation.

In our work on CAV, we have used apoptin as an example for an early protein of interest and Vp1 as our target for a late protein of interest. However, given that CAV only encodes 3 viral proteins, we are currently in the process of purifying and optimizing the use of a Vp2 antibody from recently custom-ordered rabbit serum (EZ Biolab). Once purified, we hope to confirm Vp2 as an early protein in viral infection through time-course infection Western blots,

possibly with similar kinetics to apoptin. We expect Vp2 to be expressed earlier in infection, as its roles appear to be critical to replication of the viral genome, activation of the DNA damage response, and regulation of apoptin localization to the nucleus (17, 19). The anti-Vp2 antibody could also be used for *in vitro* translation experiments outlined above, to compare translation of the viral protein reading frames under different conditions and using antibodies for detection rather than radioactive labelling. Cloning for a transcription vector expressing the CAVΔApo mRNA is also underway, and repeated experiments with the CAVΔApo transcript would give interesting information on how apoptin may impact the expression of other viral proteins.

To our knowledge, our lab would be the first to show that apoptin is not necessarily required for CAV DNA replication and virus particle production. Previously, Prasetyo et al. (2009) published findings that genomic replication was completely abolished upon transfection of apoptin-knockout replicative form of CAV into cells, but as first shown by former lab student David Sharon and confirmed in this thesis, apoptin-knockout CAV is still able to replicate its genome and package into infectious viral particles, though at significantly decreased efficiency (136, 147). Interestingly, the exact same mutagenesis strategy including the changed base (T→C) was used in both studies to knock out apoptin with the Vp2 reading frame maintained (136, 147). We also confirmed that apoptin was indeed not expressed by CAVΔApo (as seen in Figure 5D of this thesis). Though Prasetyo et al. found CAVΔApo to be unable to replicate, they established that supplementation of apoptin was able to fully complement their CAVΔApo transfection in cells (136). Previously, Sharon included in his thesis a similar experiment to show the rescue of viral genome replication in CAVΔApo transfection when co-transfected with a plasmid expressing apoptin, confirming that exogenous apoptin could rescue DNA replication, cell cycle arrest and cytotoxicity (147). In our continuation of this work, we have begun to replicate these

co-transfection experiments while examining growth in genome quantification over a time-course of 72 hours after transfection (Figure 5F). In addition, varying quantities of co-transfected 3xFLAG-Apoptin vector can be used in follow-up experiments to establish whether an increased presence of apoptin could lead to a complete rescue of viral replication efficiency. Furthermore, we would like to introduce an IR-mutant form of apoptin that has already been cloned in the lab. This plasmid would express a mutant form of apoptin with the isoleucine-arginine motif in the last few amino acids mutated to alanines. If our suspicion is correct that the IR motif of apoptin, similarly to the IR dipeptide tails of the APC/C coactivators Cdc20 and Cdh1, is responsible for interaction with APC1 and inhibition of the APC/C, we would not expect the IR-mutated apoptin expression plasmid to be capable of rescuing CAVΔApo in a co-transfection experiment.

Finally, we plan to repeat and continue the experiments using proTAME inhibition of the APC/C to rescue CAVΔApo genomic replication to achieve more robust data and solidify our results. The experiment included in this thesis (Figure 7D) represent two separate trials, with one missing a proper set of vehicle controls – using DMSO in lieu of proTAME in each of the infection conditions (CAV, CAVΔApo and the uninfected mock). While the observed rescue of viral replication in the CAVΔApo infection with proTAME is promising, the addition of the same concentration of proTAME to the wild-type CAV appeared to hinder viral genome replication. However, this may be due to inhibited cell growth in the proTAME-treated CAV infection (data not shown), which may be expected in APC/C-inhibited cells. This reasoning is supported by the similar kinetics in the genomic replication curve of the proTAME-treated CAV infection compared to its vehicle control, but to a lower absolute quantity of genome copies. In addition, the concentration of proTAME used in this experiment, 10 uM, did not induce a G2/M cell cycle arrest as would have been expected given that the compound inhibits binding of both

Cdc20 and Cdh1 to the APC/C (data not shown). Although this observation requires further validation, this may suggest that an increased concentration of proTAME would be appropriate, especially given that the prodrug must be cleaved within cells to its active form, TAME (150). In fact, when first described by Zeng et al. (2010), proTAME was seen to exhibit inhibitory effects on APC/C binding of both coactivators starting at a concentration of around 10 uM but with increasing dose-response up to 200 uM (150). Moreover, proTAME appeared to more efficiently inhibit Cdc20 binding than Cdh1 binding to the APC/C, meaning that some APC/C^{Cdh1} residual activity even in the presence of some APC/C inhibition by proTAME may remain, preventing a visible cell cycle arrest in G2/M, as active APC/C^{Cdh1} is important for maintaining G1 phase (150). Therefore, in future experiments, we plan to synchronize cells using thymidine at least 12 hours prior to infection, which will induce a reversible cell cycle arrest at the G1/S boundary, allowing the cell population to be more aligned upon initial time of infection and subsequent treatment with the inhibitor proTAME (158). As well, in other experiments (not shown) where we added proTAME at 12 hours post-infection to mimic the onset of apoptin expression in a wild-type CAV infection, rescue of CAVΔApo genomic replication was not observed by 72 hours. Compared with the results in this thesis, where proTAME was added immediately post-infection (at 0 hours), this may suggest that a longer observation period than 72 hours could be needed for proTAME inhibition of the APC/C to have an effect on CAVΔApo genome levels. Future replicates of the proTAME rescue experiment could include later post-infection sampling points than 72 hours to observe whether genome quantities continue to increase beyond the current observation window.

Overall, this thesis supports previous work from our lab in line with the model of the viral life cycle of chicken anemia virus (and other gyroviruses) presented in the introduction

(Figure 3) and in our recently published review (25). We show a time-course of infection that suggests that viral translation of apoptin predominates in "early" infection to induce mitotic arrest and a switch to translation of "late" phase proteins, namely Vp1. We confirm that Vp1 and Vp2, but not apoptin, are absolutely essential to viral replication and reproduction. However, in line with previous findings, apoptin does contribute to the efficiency of genomic replication and greatly hinders the viral infection's primary characteristics, as it is required for the induction of cell cycle arrest and apoptosis. We observed that specific inhibition of the APC/C, but not mitotic arrest in general, appears to rescue the genomic replication levels of CAVΔApo in the absence of any apoptin. Efforts to confirm the presence of an IRES or establish a model of cap-independent translation in the CAV mRNA were unsuccessful, but remain a possibility to be investigated by further means. Future directions thus include assessing translation *in vitro* with the full-length CAV mRNA, to understand how the viral proteins' expressions are related to one another and whether they are regulated by canonical translation factors. Our continued work on the molecular biology of chicken anemia virus aims to contribute to new understandings of Gyrovirus biology for potential applications in both the agriculture industry and human health.

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