Characterization of the Poxviral Encoded Ubiquitin Ligase p28

by

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ABSTRACT

Many cellular processes are regulated by the ubiquitin proteasome system (UPS), which utilizes the polypeptide ubiquitin to mark proteins for destruction via the 26S proteasome. Since the UPS plays an important role in cellular homeostasis, many viruses have evolved strategies to regulate the process of ubiquitination to their own advantage. Poxviruses also manipulate the UPS by encoding E3 ubiquitin ligases to target cellular or viral proteins for ubiquitination. This allows poxviruses to exploit the UPS and manipulate a wide range of cellular processes.

p28, a poxvirus encoded ubiquitin ligase, was first described in ectromelia virus (ECTV). p28 contains an N-terminal KilA-N DNA binding domain and a C-terminal RING-domain that is necessary for the ubiquitin ligase activity. Buller and colleagues demonstrated that deletion of p28 from ECTV results in a dramatic reduction in virus virulence, indicating its importance during viral infection. p28 is a highly conserved ubiquitin ligase that is expressed in a wide range of poxviruses, including Avipoxviruses. One distinguishing feature of the Avipoxviruses is their large genome, as exemplified by the prototypical members of fowlpox virus (FWPV) (288 kbp) and canarypox virus (CNPV) (365 kbp), encoding a number of yet uncharacterized proteins. FWPV and CNPV each contain two p28-like ubiquitin ligases. In contrast, non-Avipoxviruses encode only one p28-like ubiquitin ligase. FWPV and CNPV also encode additional KilA-N only genes lacking a RING domain; an observation not seen in other members of the poxvirus family. The data presented in this thesis demonstrate that both p28 homologues in FWPV, FWPV150 and FWPV157 are functional ubiquitin ligases located at the virus factories. Intriguingly, expression of FWPV150 started early in infection,

while FWPV157 was expressed late. We further demonstrated that each of the eight KilA-N domain-containing proteins were expressed during FWPV infection. Since many cellular and viral ubiquitin ligases are regulated through ubiquitination, we investigated whether this was true of p28. Our data confirmed a role for the RING domain for the self-ubiquitination of p28, but we further found that p28 is also regulated by another E3 ubiquitin ligase(s). It remains to be investigated whether ubiquitination changes p28 activity or if the ubiquitination is solely required for the proteasomal degradation of p28 at a certain stage during infection.

Finally, we conducted a mass spectrometry screen for p28 substrates, and identified heat shock protein 70 (HSP70) as an interaction partner of p28 during VACV infection. While we were unable to show that HSP70 is a substrate of p28, we demonstrated HSP70 enrichment at the virus factory in the presence of p28 when compared with VACVCop infected cells alone. This effect of HSP70 enrichment was conserved within the investigated p28 homologues. We further found that p28 residues 184-204 were important for recruiting HSP70 from the cellular nuclear and cytoplasmic pool to the virus factory. The importance of the p28 mediated HSP70 recruitment to the virus factory is to be determined.

In summary, this thesis characterized the expression, cellular localization and ubiquitin ligase activity of p28 homologues in FWPV. In addition, p28 RING and KilA-N mutants were characterized in terms of their ubiquitin ligase activity and interaction with ubiquitin and the UPS; in the end a mass spectrometry screen and follow up validation confirmed HSP70 as an interaction partner of p28.

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LIST OF ABREVATIONS

ANOVA	Analysis of variance
ALCAM	activated leukocyte cell adhesion molecule
APC	anaphase-promoting complex
APC11	anaphase promoting complex subunit 11
AraC	b-D-arabinofuranoside hydrochloride
ATCC	American type culture collection
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
BGMK	baby green monkey kidney cells
BLAST	Basic Local Alignment Search Tool
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
BSL4	biosafety level 4
BTB	Bric-a-brack/tram-track/broad complex protein domain
CAS	cellular apoptosis susceptibility protein
CD4	cluster of differentiation 4
CD95	cluster of differentiation 95
CDC	Centres for Disease Control and Prevention
CMC	carboxyl-methyl cellulose
CNPV	canarypoxvirus
CMV	Cytomegalovirus
CPXV	cowpox virus
CRL	Cullin-RING ubiquitin ligases
Crm	Cytokine response modifier
CV-1	African green monkey kidney cells
CYLD	cylindromatosis
DAPI	4', 6' diamidino-2-phenylindole
DAVID	Database for Annotation, Visualization and Integrated Discovery
DMEM	Dulbecco's minimal essential media
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DUB	deubiquitinating enzyme
DUF	domain of unknown function
dsDNA	double-stranded deoxyribonucleic acid
dsRNA	double-stranded ribonucleic acid
DTT	dithiothreitol
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ubiquitin ligase

E6AP	E6-associated protein
EBNA1	Epstein–Barr nuclear antigen 1
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescence
ECTV	Ectromelia virus
EDTA	ethylenediaminetetraacetic acid
EEV	extracellular enveloped virus
EGFP	Enhanced green fluorescent protein
EV	enveloped virus
Fwd	Forward
FWPV	fowlpox virus
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescence protein
MCV	Molluscum contagiosum virus
HA	Hemaglutinin
HBV	Hepatitis B virus
HC	heavy chain
HCMV	Human cytomegalovirus
HECT	homologues to E6-associated protein carboxy terminus
HI-FBS	heat inactivated foetal bovine serum
HIV	Human immunodeficiency virus
HPV	Human papilloma virus
HRP	Horse radish peroxidase
HSP	Heat shock protein
HSV	Herpes Simplex Virus
IBD	Institute for Biomolecular Design
ICAM-1	Intercellular Adhesion Molecule 1
ICP0	infected-cell polypeptide 0
ICTV	International Committee on the Taxonomy of Viruses
IFI16	gamma-interferon-inducible protein 16
IFN	interferon
IL	interleukin
IMP	inflammation modulatory proteins
IP	immunoprecipitate
IPTG	isopropyl β-D-1-thiogalactopyranoside
IRF3	interferon regulatory factor 3
ITR	inverted terminal repeat
IV	immature virus
JAMM	Jab1/MPN domain-associated metalloisopeptidase
kbp	kilobase pairs
kDa	kilodaltons
LacZ	β-galactosidase
LB	Luria-Bertani broth
LC	light chain
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LMB	Leptomycin B

LMP	low melting point
LMP1	latent membrane protein 1
LMP2A	latent membrane protein 2A
LUBAC	linear ubiquitin chain assembly complex
MARCH	membrane associated RING-CH
MCMV	murine cytomegalovirus
MCP	Molecular and Cellular Proteomics guidelines
Mdm2	Mouse double minute 2 homolog
mg	milligram
MHC	major histocompatibility complex class I
MJD	Machado-Josephin-domain
mL	milliliter
mM	milimolar
MOI	multiplicity of infection
MPXV	monkeypox virus
mRNA	messenger ribonucleic acid
MSH2	DNA mismatch repair protein 2
MSH6	DNA mismatch repair protein 6
MVA	Modified vaccinia Ankara
MYXV	myxoma virus
MV	mature virus
MVA	modified vaccinia Ankara
MyD88	myeloid differentiation primary response 88
N/A	not applicable
NCBI	National Centre for Biotechnology Information
NCS	newborn calf serum
NEM	N-Ethylmaleimide
NES	nuclear export signal
NLS	nuclear localization signal
ns	non significant
NBF	neutral-buffered formalin
NFκB	nuclear factor kappa B
ORF	open reading frame
OUT	ovarian tumor domain proteins
PAGE	polyacrylamide gel electrophoresis
PARG	Poly(ADP-ribose) glycohydrolase
PBS	phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	polymerase chain reaction
PECAM-1	Platelet endothelial cell adhesion molecule 1
pE/L	synthetic poxviral early/late promoter
PKR	protein kinase R
PML	Promyelocytic leukemia protein
PVDF	polyvinylidene fluoride
PFA	paraformaldehyde
PFU	plaque forming unit

Rev	Reverse
RING	Really Interesting New Gene
RIPA buffer	Radioimmunoprecipitation assay buffer
RLU	relative light units
RNA	ribonucleic acid
RNF8	ring finger protein 8
RNF168	ring finger protein 168
ROI	Region of interest
Rpm	rotations per minute
RT-PCR	Real time polymerase chain reaction
SCF	Skp-1/Cullin-1/F-box
SDS	sodium dodecyl sulfate
Serp1	stress-associated endoplasmic reticulum protein 1
SILAC	Stable isotope labeling of amino acids in cell culture
SOC	super optimal broth with catabolite repression
SPI	serpin
SSC	standard saline citrate
SPICE	smallpox inhibitor of complement enzymes
ssDNA	single-stranded DNA
STAT1	Signal Transducers and Activators of Transcription family of transcription factors 1
SUMO	Small Ubiquitin-like Modifier
TAGC	The Applied Genomics Centre
TAE	Tris-acetate buffer
TBK1	TANK-binding kinase 1
TBST	tris-buffered saline plus Tween 20
TCC	Training Consulting Centre
TK	thymidine kinase
TNF	tumor necrosis factor
TMRE	tetramethylrhodamine ethyl ester
TRAF6	tumor necrosis factor receptor-associated factor 6
tRNA	transfer RNA
U	Unit
Ub	ubiquitin
Ubc13	Ubiquitin-conjugating enzyme 13
UCH	ubiquitin C-terminal hydrolase
UPS	ubiquitin-proteasome system
USP	ubiquitin specific protease
v/v	volume per volume
VACV	vaccinia virus
VACVCop	vaccinia virus copenhagen
VARV	variola virus
VCP	valosin-containing protein
vFLIPs	viral Fas-associated death domain-like interleukin-1β converting enzyme inhibitory protein
vCD30	viral cluster of differentiation 30

w/v	weight per volume
WB	Western blotting
WCL	whole cell lysates
WGA	wheat germ agglutinin
WHO	World Health Organization
WR	Western Reserve
WT	wild type
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactosidase

CHAPTER 1: INTRODUCTION

1.1 Poxviruses

Poxviridae are double-stranded DNA (dsDNA) viruses capable of infecting a wide range of hosts (1). Viruses within this family are enveloped and large in size, with dimensions of 200 nm x 300 nm, and appear brick-shaped under an electron microscope. Unlike most other families of DNA viruses, poxviruses replicate exclusively in the cytoplasm in so-called virus factories **(Figure 1.1)** (1). Since poxviruses complete their life cycle in the cytoplasm, their genomes encode the majority of the structural proteins and enzymes needed for gene expression and replication (2). The rest of the poxviral genome is dedicated to host-specific virulence factors, which are crucial for infection *in vivo* and dictate host range, immune evasion strategies and host cell manipulation in general (2, 3). Studying poxviral virulence factors is a powerful way to understand the complexity of virus-host interactions, host immune response and cellular pathways. This work focuses on characterizing a poxviral virulence factor called p28 and the role it plays during infection.

1.2 The *Poxviridae* family

The *Poxviridae* family contains over forty-six different poxviruses, which infect a wide range of hosts, both vertebrate and invertebrate (1). The International Committee on the Taxonomy of Viruses (ICTV) divided poxviruses into two subfamilies, called *Chordopoxvirinae* and *Entomopoxvirinae* (4). The *Entomopoxvirinae* subfamily consists of fewer members and exclusively infects invertebrates. The more intensively studied

A

Outer membrane
Inner membrane
Core
Core wall
Lateral bodies
DNA genome
Viral enzymes

B

Virus factory

Nucleus
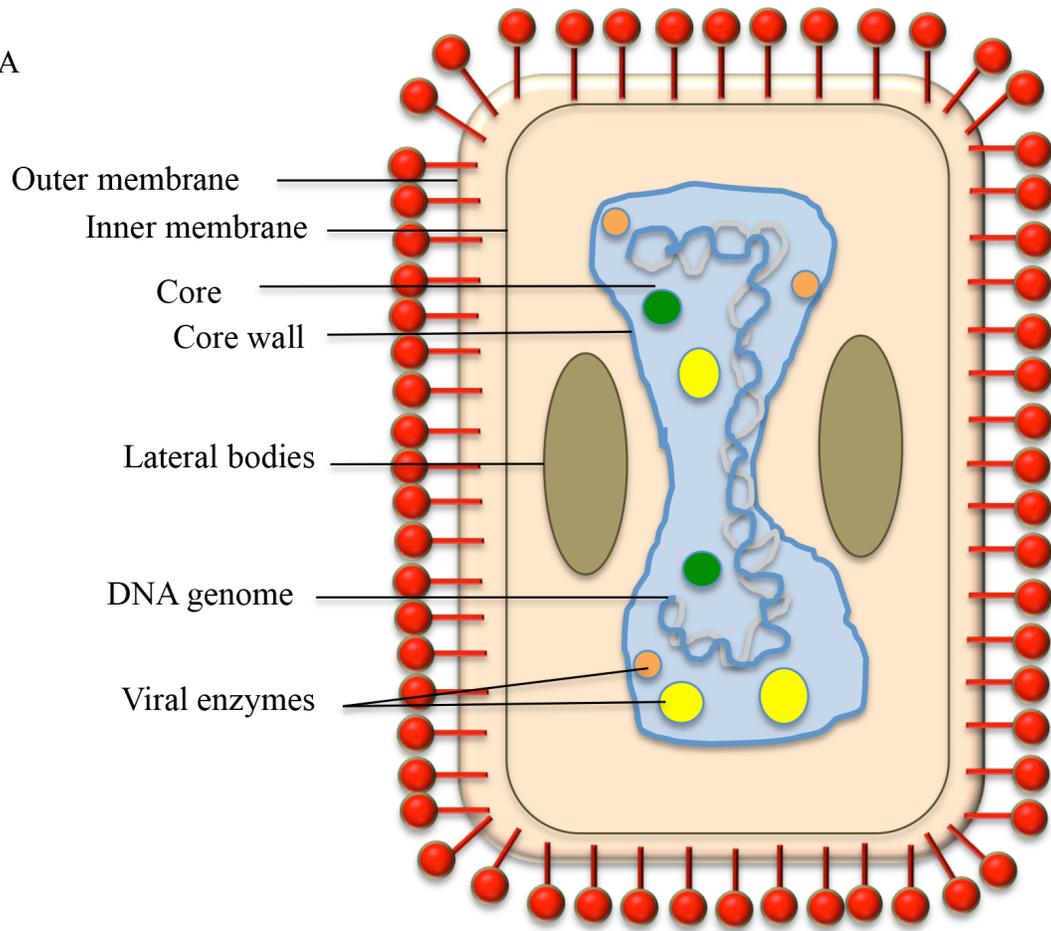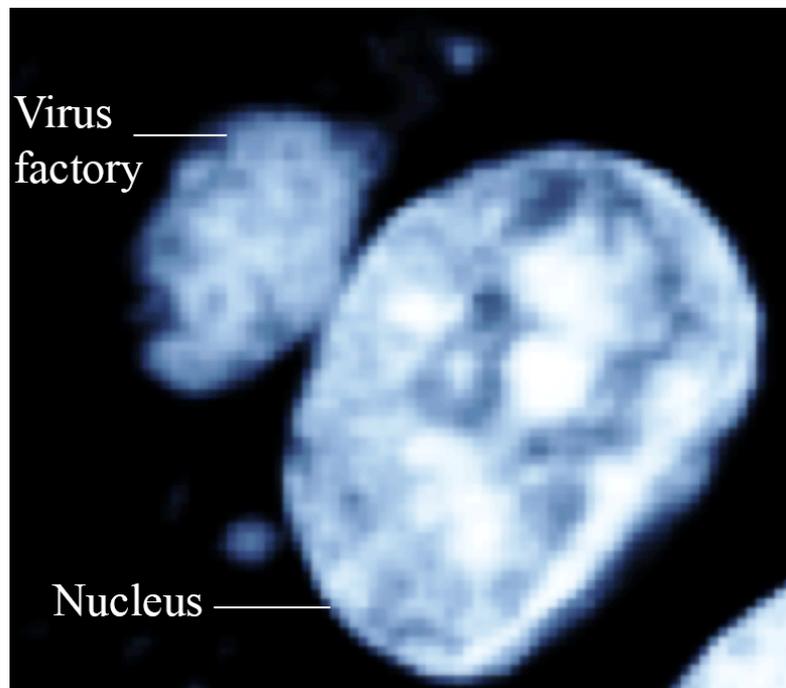
Figure 1.1 Poxvirus structure and virus. (A) Virion structure of poxviruses. The poxviral dsDNA genomes are enclosed in a dumbbell shaped viral capsid. Lateral bodies, which are protein rich structures, containing enzymes necessary for initiating the next round of infection, are found besides both sides of the core. The virion contains an inner and outer membrane. **(B)** Poxviruses replicate in the cytoplasm in so-called virus factories. Here, we infected HeLa cells with VACVCop for 12 hours, and then fixed and stained with DAPI (4',6-diamidino-2-phenylindole), a fluorescent stain that binds strongly to A-T rich regions in DNA, to visualize nuclei and virus factories.

Chordopoxvirinae subfamily infects vertebrates and can be subdivided into the *Avipoxvirus, Capripoxvirus, Cervidpoxvirus, Leporipoxvirus, Molluscipoxvirus, Orthopoxvirus, Parapoxvirus, Suipoxvirus, and Yatapoxvirus* genera. These genera are classified based on host range, serological cross-reactivity between members, disease pathology and genetic similarity **(Table 1.1)** (1, 4). Two genera are of particular interest to our work on characterizing homologues of the virulence factor p28: Orthopoxviruses and Avipoxviruses.

1.2.1 Orthopoxviruses

Orthopoxviruses contain the most medically relevant poxviruses for humans including variola virus (VARV, discussed more in Section 1.2.1.1), Vaccinia virus (VACV) and monkeypox virus (MPXV) (1). In total, there are fourteen species classified as Orthopoxviruses with different host ranges and severity of disease (1, 5). Orthopoxviruses encode many genes that are homologous between members in this genus including those necessary for virus replication and immune modulation. However, there are still differences, which define their host tropism. VARV and ECTV are restricted to a single host, human and mice, respectively, causing severe symptoms with high mortality rates (6). In contrast, VACV, MPXV, and cowpox virus (CPXV) can infect a variety of hosts (1, 3). While VACV and CPXV cause mild diseases in humans, MPXV has been reported to cause outbreaks of smallpox-like symptoms in the Democratic Republic of the Congo (7-11). Today, VACV is the prototypical member of Orthopoxviruses and serves as an excellent model to study virus-host interactions.

Subfamily	Genus	Member
Chordopoxvirinae	*Avipoxvirus*	Canarypox virus Fowlpox virus Penguinpox virus
	Capripoxvirus	Goatpoxvirus Lumpy skin disease virus Sheeppox virus
	Cervidpoxvirus	Deerpoxvirus
	Leporipoxvirus	Myxomavirus Shope fibroma virus
	Molluscipoxvirus	Molluscum contagiosum virus
	Orthopoxvirus	Camelpox virus Cowpox virus Ectromelia virus Monkeypox virus Taterapox virus Vaccinia virus Variola virus
	Parapoxvirus	Bovine papular stomatitis virus Orf virus
	Suipox virus	Swinepox virus
	Yatapoxvirus	Tanapox virus Yaba-like disease virus Yaba monkey tumour virus
Entomapoxvirinae	*Entemopoxvirus alpha*	*Melontha melontha*
	Entemopoxvirus beta	*Amsacta moorei* *Melanoplus sanguinipes*
	Entemopoxvirus gamma	*Chrionimus luridus*

Table 1.1 The poxvirus family. The Poxvirus family infects vertebrates and invertebrates. The *Entomapoxvirinae* subfamily infects invertebrates and includes three genera. The *Chordopoxvirinae* subfamily contains nine genera, which infect a wide range of vertebrates. The genus *Avipoxvirus* infects birds. The genus *Capripoxvirus* infects sheep, cattle and goats. The members of the *Cervidpoxvirus* genus infect deer. *Leporipoxvirus* members infect rabbits. Members of the *Molluscipoxvirus* can infect humans. *Orthopoxvirus* members can infect camels, cows, mice, monkeys, humans, and other small mammals. *Parapoxvirus* infect sheep, cattle, or goats. *Suipox virus* infects swine, and *Yatapoxvirus* infects humans and non-human primates (12).

1.2.1.1 Variola virus

Historically, VARV is the most infamous member of the Orthopoxviruses, which caused a devastating disease known as smallpox (3, 13). Smallpox is an ancient disease, with smallpox lesions reported as early as 5000 years ago on the remains of Egyptian mummies (14). VARV is a strict human pathogen and has not been reported in non-human hosts (15). It is transmitted via inhalation, which leads to infection of epithelia and respiratory mucosa. In the next stage, VARV infects lymphoid organs, which is followed by viraemia and skin lesions (1). There are two strains of VARV: variola major and variola minor. VARV has a mortality rate of up to 30-40%, while the minor strain is the milder form and has a death rate of less than 1% (1).

1.2.1.2 Orthopoxviruses used as a vaccination strains

At the end of the 18th century, Dr. Edward Jenner used a CPXV strain for vaccination against smallpox. This was the first recorded demonstration of vaccination, which made Dr. Jenner the "Father of Vaccination" (16). Thanks to an extensive vaccination program, the World Health Organization (WHO) declared that smallpox was eradicated in 1979 (16). Over the years, the smallpox vaccine components switched from CPXV and HPXV to VACV. However, the origin of VACV remains unknown and controversial. Some argue that VACV arose from vaccine farms during the eradication campaign (1, 13, 17). VACV is closely related genetically to VARV; however, it rarely causes disease in healthy individuals (17). With a wide host range, VACV provides protective immunity against smallpox. All variola virus stocks were collected in 1978 after a laboratory outbreak in the UK and are currently contained in only two BSL4 facilities worldwide

(The Vector Institute in Russia and the Centres for Disease Control and Prevention (CDC) in Atlanta) (18-21). Today, VACV and other members of the *Poxviridae* family are used as a tool to study host-virus interactions.

1.2.2 Avipoxviruses

Avipoxviruses are evolutionally more divergent from Orthopoxviruses and generally infect birds **(Table 1.1)**. Fowlpox virus (FWPV) and canarypox virus (CNPV) are the best-characterized members of the *Avipoxvirus* family (22, 23). FWPV infection in poultry leads to lesions and scabs on the skin, or in severe cases, to lesions in the upper gastrointestinal and respiratory tracts (24). Typically, transmission depends on contact and shedding from lesions due to increased stress (24). FWPV poses a serious agriculture problem in poultry houses, as it leads to decreased flock performance and egg production, resulting in significant economic loss (24). Luckily, vaccines are available in the form of attenuated FWPV (25, 26). Furthermore, Avipoxviruses like FWPV and CNPV are also suitable candidates for safe vaccination vectors in humans since they infect mammalian cells. In mammalian cells they only express early proteins, but are unable to replicate due to restricted host range (27). Even though FWPV is a prototypical member of Avipoxviruses with applications for vaccines, most of their host range and immune evasion genes still remain unexplored.

1.3 Poxviral genome structure

Poxviruses contain some of the largest viral genomes with sizes ranging from 150-300 kbp, encoding 150 to 350 ORFs (1). The poxviral dsDNA is connected by hairpin loops

at the inverted terminal repeats (ITR) that contain AT-rich regions (1). The VACV genome was originally characterized via a *Hind*III restriction enzyme digestion of the VACV genome, which was used to define genomic fragments. The resulting DNA fragments were alphabetically named (A to P) based on the *Hind*III digested fragment length and were assigned a number reading from the left to the right hand side **(Figure 1.2)**. Furthermore, depending on the direction in which the ORF was transcribed, it was named "R" for right or "L" for left (e.g. F1L). The genome consists of a central region with highly conserved genes, including transcription factors, polymerases, factors important for viral replication, and structural proteins (28). Collectively, these gene products allow poxviruses to replicate in cytoplasmic compartments known as viral factories, mostly independent from the host replication machinery (2). Around 100 of these central genes are conserved within the *Orthopoxvirus* genus, while 50 are conserved across all poxviruses (28, 29). Poxvirus genes do not contain introns and the viral mRNA is not spliced, which allows further independence of poxvirus transcription from the host transcription machinery. Two variable regions flank the central region of the poxviral genome. Typically, these variable regions contain less conserved genes. Many are unique and non-essential ORFs to the individual virus and are typically important for host range, virulence, and immune evasion capabilities (20, 30, 31). Deletion of genes within the variable region usually does not affect the ability of the virus to replicate in cell culture *in vitro*. Rather, these genes are usually important *in vivo* for immune evasion strategies and host range (2, 29). Interestingly, there are no genes within the variable region of the genome that are found in all known members of the

Orthopoxviruses (28, 29). This presents a contrast with genes in the central region, which are crucial for virus replication.

1.4 Virus life cycle/replication

Poxviruses can enter their host cell by binding to a number of different cell surface molecules. These include heparan sulfate, glycosaminglycans, laminin, or chondroitin sulfate (32-35). In the case of VACV, the entry-fusion complex involves proteins A21, A28, H2 and L5 on the outer membrane, which allows fusion and cell entry via endocytic uptake of the virus particle (36-41). Following internalization of the poxvirus core into the cytoplasm, cores travel via microtubules to sites near the nucleus, where virus replication is initiated **(Figure 1.3)**. Unlike other DNA viruses, poxviruses replicate exclusively in the cytoplasm in structures known as virus factories (42-44).

Virus factories are perinuclear DNA rich regions, which allow for viral replication, transcription, translation, and viral progeny formation. The number of virus factories is proportional to the <u>m</u>ultiplicity <u>o</u>f <u>i</u>nfection (MOI) and can originate from a single virion (45, 46). The virus factory is structured and surrounded by cellular endoplasmic reticulum early during infection. This membrane disappears during virus assembly (47). Furthermore, there are subdomains within the virus factories, which provide distinct areas for viral gene transcription and translation (45).

A
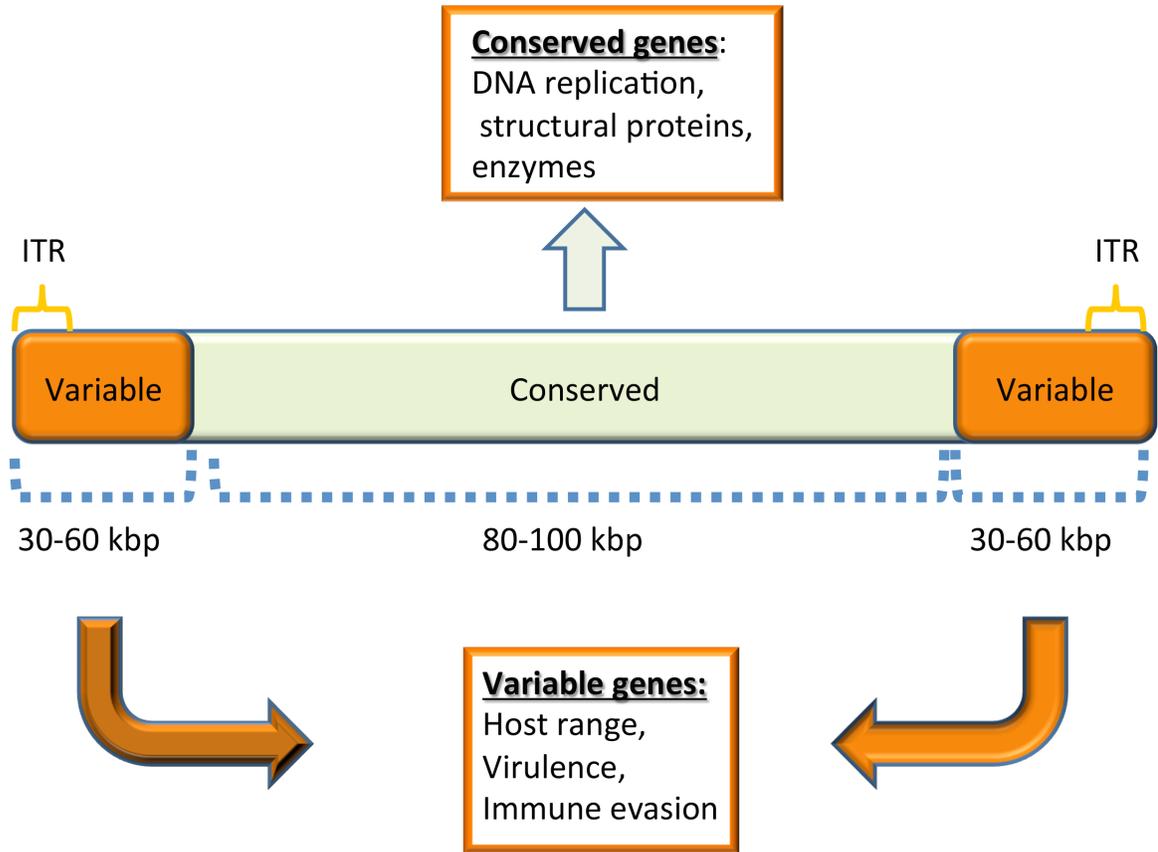
B
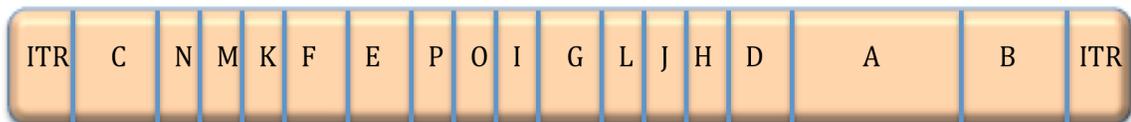
Figure 1.2 The genome of poxviruses (central and variable regions).

(A) The poxvirus genome contains a conserved region in the center of the genome, which is flanked by variable regions. At both the ends of the genome are inverted terminal repeats (ITR), which form hairpin loops to connect the double stranded DNA. **(B)** The genome is organized in letters, which represent the fragments after *Hind*III digestion of the VACVCop genome, A being the biggest and P, being the smallest fragment (48). The genome of poxviruses contains a central conserved region consisting of genes that are important for viral replication as well as structural proteins and enzymes. The terminal variable regions contain numerous genes that are important for tropism, immune evasion, and host range.

Poxvirus replication is organized into temporally distinct steps. These steps are dependent on the type of promoter and comprise early, intermediate, and late gene expression (49, 50). Early genes are transcribed and translated first, even before the virus core reaches the site of establishing a virus factory (2, 51). Early genes comprise roughly half of the genes encoded by the VACV genome and include genes that are important for uncoating, immune evasion, host tropism, expression of intermediate genes, and DNA replication (30, 52, 53). Early transcripts can be detected as early as 20 minutes and up to 4 hours post-infection. After transcription of early genes, intermediate genes are expressed, which encode for proteins that are crucial for DNA replication. DNA replication occurs between early and intermediate stages. Following DNA replication, late genes are expressed, which encode structural proteins required for virion assembly, transcription factors for early genes, RNA polymerase, transcript capping and methylation enzymes and budding from the host cell (2). Some of these late proteins are packed into virions for the next round of infection (2, 29). It is noteworthy that there is a distinct promoter sequence for each class of viral genes (50, 54). However, some genes are driven by promoters, which combine early, intermediate, or late promoters in tandem, upstream of the ORF, so that some genes are expressed during more than one stage of infection (50). A hybrid early/late promoter is used in this study to express transgenes from a vector, called pSC66, which was further used to create recombinant poxviruses that express transgenes from this hybrid promoter (54). After late protein synthesis, poxviruses begin the stage of virion morphogenesis, which is it a maturation process that involves structural changes at the virus factory. It is still controversial whether the outer poxviral membrane of the virion is produced, or if it arises from an intermediate

1. Binding and entry

2. Uncoating,
Early gene expression,
Formation of viral factory

3. Intermediate gene expression
Viral genome replication

4. Late gene expression
And assembly

5. Poxviral morphogenesis
Membrane wrapping

6. Release

Cell surface receptors

Nucleus
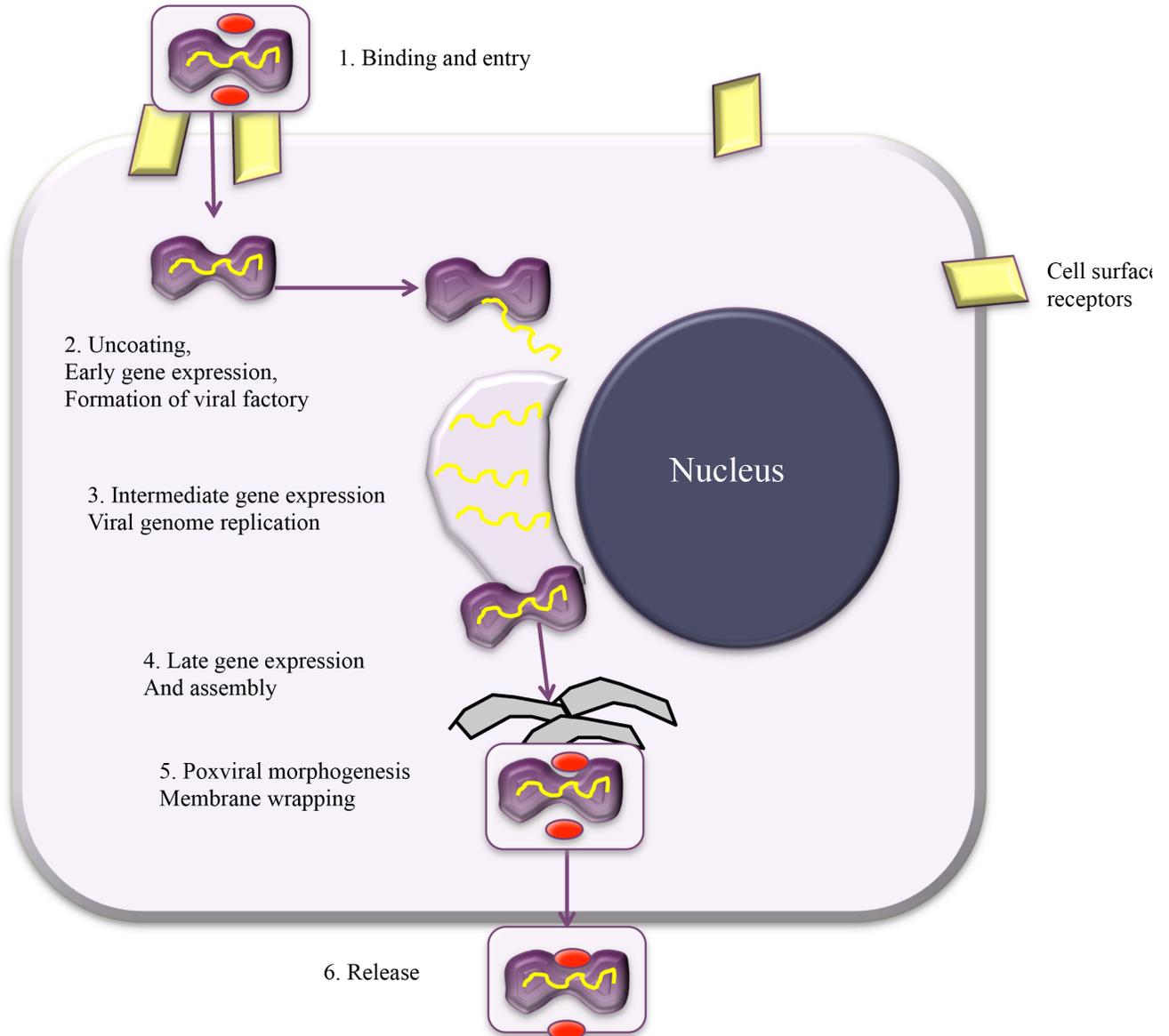
Figure 1.3 Poxvirus life cycle.

1. Poxviruses bind to cell surface receptors, followed by a fusion event or endocytosis that leads to the release of the virion core into the host cell cytoplasm. **2.** The early genes are transcribed, which provide viral factors for immune evasion and uncoating. The virus factory is formed. **3.** Intermediate genes are expressed and the viral genome replicates. **4.** Late poxviral genes are transcribed, which encode structural proteins and enzymes that are packaged into virions, **5.** During poxviral morphogenesis, the genome is packaged into the new cores, which is followed by the formation of crescent shaped structures. **6.** Mature virions (MV) can be released via lysis or budding. MV can also acquire lipid membranes from the endosome or the Golgi-complex to form wrapped virus (WV), which release extracellular enveloped virions (EV) via fusion with the plasma membrane.

compartment of the Golgi apparatus and endoplasmic reticulum (44, 55). Following this first morphogenetic step, the immature virus particles develop into mature virions (MV) with a genome-containing core (2, 33). MVs constitute the majority of infectious poxviruses and are released from the host cell via lysis (33). In addition, some MVs undergo additional wrapping into a double-membrane derived from the endosome or trans-Golgi network to produce wrapped virions (WV), which leave the host cell before cell lysis (56-59). WV are transported to the cell surface through actin filaments. They fuse with the plasma membrane and release extracellular enveloped virions (EV) (33, 60, 61). EV can form actin projectiles, which can push the EV away from the cell to infect neighboring cells. This is crucial *in vivo* for lethal viral infection. In fact, many viral factors are investigated via knockout viruses in an ECTV mouse model to investigate their function during infection in a natural host (7, 62, 63).

1.5 Animal models of poxvirus infection

ECTV infection of mice provides an excellent animal model to study poxvirus infection in a natural host, since it causes severe symptoms and disease in susceptible mouse strains **(Figure 1.4)** (7). Susceptible mouse strains include CBA, A/J, BALB/c and DBA/Z. Resistant mouse strains include C57/BL6 and AKR (3, 64). Resistance seems to correlate with the ability of the mouse strain to evoke a Th1-polarized immune response (65). In support of this, Jackson *et al.* demonstrated that a recombinant ECTV strain expressing interleukin-4, a Th2 cytokine, was lethal in non-susceptible mouse strains (66). This further implies the importance of a Th1 immune response against ECTV infection (66). Natural infection in mice begins following abrasions on the skin; with

virus replication occurring in the skin layers of the epidermis, followed by poxviral spread to the lymph nodes and bloodstream **(Figure 1.4)** (64). ECTV is carried by infected macrophages and replicates and disseminates within the host (67, 68). Macrophages play an important role in controlling virus infection, but can also be used as a tool for virus dissemination. ECTV uses macrophages to gain access to the liver through the bloodstream, where it is taken up by the liver macrophages called Kupffer cells. This leads to an infection of hepatocytes, extensive liver necrosis and death (69-71). Since ECTV belongs to the same *Orthopoxvirus* family as VARV and VACV, it contains a large number of similar immune modulator homologues, which makes it a great tool to study poxvirus virulence factors and pathogenesis (7, 20).

1.6 Poxvirus manipulation of host immune response

Viruses have co-evolved with their hosts for millions of years. Hosts have developed both innate and adaptive immune responses to defeat viral infection and disease (72). In response, viruses have established mechanisms to evade the immune response in numerous ways **(Table 1.2)** (2, 20). Their large genome allows poxviruses to encode a multitude of immune evasion proteins that can inhibit the immune response at both extracellular and intracellular levels. Extracellular mechanisms include poxviral encoded secretory proteins, such as chemokine homologues, cytokine decoy receptors, and poxviral proteins that bind cytokines, chemokines, or complement factors **(Table 1.2)**. Consequently, an inflammatory response in the host is inhibited. Poxviruses also encode proteins that inhibit the induction of intracellular immune signaling and apoptosis, disrupt receptor-ligand interactions and viral antigen presentation (30).

Day

0 — Skin invasion and multiplication

1 — Multiplication in regional lymph node

2 — Dissemination into bloodstream. primary viraemia

3

4 — Multiplication in liver and spleen

5 — Bloodstream. secondary viraemia

6 — Focal infections at distal sites of the skin

7 — Swelling of foot (primary lesion)

8

9 — Early rash (papules)

10

11 — Severe rash (ulceration)
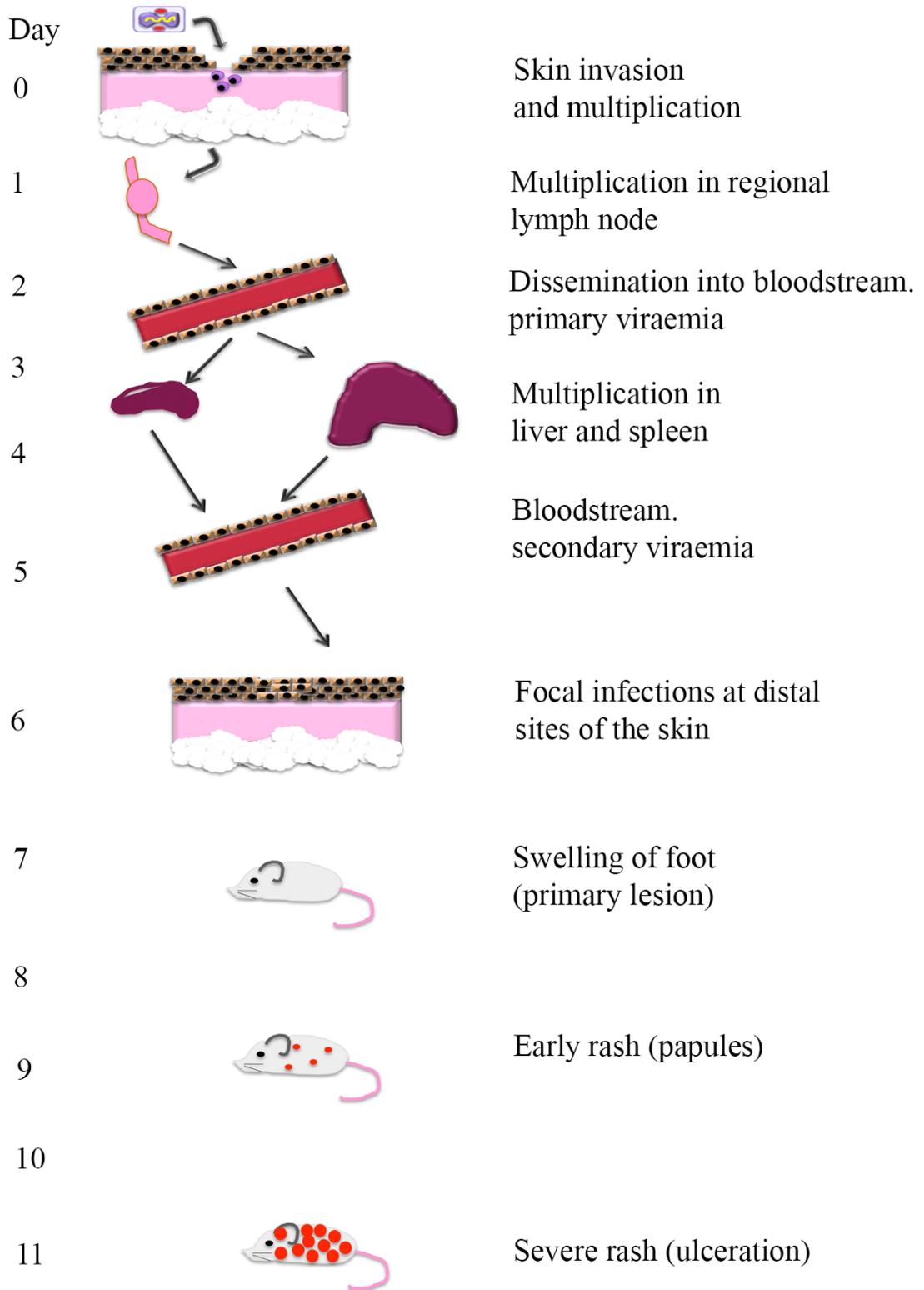
Figure 1.4 ECTV pathogenesis.

Adapted from Esteban et al. (7). ECTV infects the mouse through the skin in the footpad. The virus replicates and disseminates into the lymphatic system. Once ECTV spreads into the bloodstream, primary viraemia begins. This event allows viral infection of liver, spleen and other organs. When the virus is released from the organs, secondary viraemia starts. This results in infection of distal sites of the skin.

Virostealth Genes	Virus	Mode of action	Reference
M153R	Myxoma	Ubiquitin ligase; Down-regulation of MHC class I, CD4, ALCAM, CD95	(73, 74)
Virotransducer			
E3L	Vaccinia	dsRNA binding protein, inhibits PKR-induced IFN response	(75, 76)
H1L	Vaccinia	De-phosphorylates STAT1; prevents IFN response	(77)
K3L	Vaccinia	Prevents PKR-induced IFN response	(78)
K7L	Vaccinia	Prevents TBK1/IKKε activation of IFN	(79)
CrmA, SPI-1, SPI-2	Cowpox	Intracellular serine protease inhibitors	(80-83)
F1L, M11L, FWPV039, DPV022	Vaccinia, Myxoma, Fowlpox Deerpox	Anti-apoptotic proteins	(84-86)
vFLIPs	Molluscum	Signal transduction inhibitors	(87)
A46R	Cowpox	Toll-like receptor inhibitor	(83)

Virokines			
SERP-1, SPI-3	Vaccinia, Myxoma	Extracellular serine proteases	(88, 89)
D7L	Ectomelia	Il-18 binding proteins	(90, 91)
M-T7, M-T1	Myxoma	Chemokine binding proteins	(92-94)
VCP, IMP, SPICE	Vaccinia Cowpox Variola	Complement binding proteins	(95-97)
Viroceptors			
B15R	Vaccinia	IL-1β receptor homologue	(98)
B18R, B8R	Vaccinia	IFN receptor homologues	(18, 99)
M-T2, CrmB, CrmC, CrmD, CrmE, vCD30	Myxoma, Cowpox	TNF receptor homologs	(100-104)

Table 1.2 Poxviridae Immunomodulators

Poxvirus immunomodulatory proteins can be catagorized as virostealth, virotransduction, and viromimicry (30). Virostealth proteins can mask virus infected cells, for example, by reducing cell surface molecules and subsequently preventing recognition by cytotoxic T cells. Virotransduction proteins can modulate cell signaling networks, which aids in minimizing effects of proinflammatory signaling and to create a favourable cellular environment for virus replication. Viromimicry include virokines, which mimic host cytokines or viroreceptors, mimicking cytokine receptors, to block extracellular immune signaling.

Poxviruses also encode a large number of proteins that inhibit NF-κB activation, and subsequent anti-viral mechanisms, including A46, A52, B13, B14, B15, E3, M2, N1, K1, and K7 (79, 98, 105-115). Furthermore, VACV proteins E3 and K3 prevent the host cell sensing of dsRNA (76, 78, 98). To further establish a cellular environment for optimal poxviral replication, poxviruses encode proteins to manipulate the UPS (116, 117).

1.7 The Ubiquitin system

Ubiquitin is a highly conserved 76 amino acid protein with only three amino acid changes between the yeast and human homologue (118, 119). It is a globular protein, which is added post-translationally to lysine residues on the target protein. The C-terminus of ubiquitin contains a di-glycine motif, which forms a covalent bond with the lysine residue of the target protein. This modification is called ubiquitination and is important for a wide array of cellular functions such as cell cycle control, DNA repair, signal transduction, antigen processing and apoptosis (120-122). Ubiquitination can have diverse effects on the target protein depending on the type of labeling. Ubiquitin contains seven distinct lysine residues, K6, K11, K27, K29, K33, K48, and K63 (123). Any of these seven allows for the formation of ubiquitin chains. Furthermore, chemical structure and length of the ubiquitin chain can influence signalling outcomes (122). Monoubiquitination with the addition of a single ubiquitin molecule can lead to histone modification, and regulate endocytosis, protein trafficking, or function of substrates (120). Polyubiquitin chains can lead to different outcomes depending on the type of chain linkage (120). Chains are connected by isopeptide bonds between a specific lysine residue of one ubiquitin and the carboxyl group of the next ubiquitin. Chains linked

through lysine 48 and lysine 63 are well-characterized (122, 124). Lysine 63 chains can modulate protein function in a non-proteolytic manner, including regulation of intracellular trafficking, DNA repair, and signal transduction pathways. In contrast, lysine 48 ubiquitin chains typically mark the proteins for degradation by the 26S proteasome to destroy old proteins or proteins with altered functions so their components can be recycled (120, 122, 125). Tight regulation of ubiquitination, which maintains a fine balance in the amount of proteins in cells, is crucial for avoiding cellular dysfunction that may result in diseases such as neurodegenerative disorders and cancer (120-122).

Regulation occurs through an enzymatic cascade including enzymes E1, E2, and E3 **(Figure 1.5)**. E1 catalyzes formation of a thiol-ester bond in an ATP-dependent manner between itself and the carboxyl terminal glycine of ubiquitin, leading to the activation of ubiquitin (126). The E1 ubiquitin-activating enzyme is highly conserved and there is only one copy in most eukaryotic genomes (126). Activated ubiquitin is then transferred from E1 to the E2 ubiquitin conjugating enzyme (127). The human genome encodes at least 25 E2 conjugating enzymes, which interact with a distinct group of E3 ubiquitin ligases (122). The E2 enzymes contribute to the chain type and length of ubiquitin chain formed. For example, the E2 enzyme Ubc13 is important for K63-linked ubiquitin chains (128). In the final step, the E3 ubiquitin ligase binds both the target protein and E2 thioesterified with ubiquitin, and mediates the transfer of activated ubiquitin onto the target protein (122). Genes encoding hundreds of putative E3 ubiquitin ligases have been identified in the human genome. It is thought that every E3 ligase has its own unique set of target proteins for ubiquitination.

1.7.1 Types of E3 ubiquitin ligases

Two major types of E3 ligases are found in eukaryotes: homologues to E6-associated protein carboxy terminus (HECT) and Really Interesting New Gene (RING) proteins (129). HECT domain E3s were first discovered in Human papilloma virus (HPV) infection, where viral E6 proteins redirect cellular E6-AP to ubiquitinate and degrade p53 (130). HECT domains usually form thiol-ester intermediates with ubiquitin and transfer it onto the substrate, whereas RING domain E3 ligases transfer ubiquitin from E2 directly to the target protein. RING domains are conserved from yeast to humans and contain a highly conserved motif of 7 cysteines and one histidine buried in the 3D structure in the domain's core. This is important for maintaining the overall structure through binding of two zinc atoms (131). Only one quarter of RING domain E3 ligases contain the substrate-binding site in the same protein. The majority of RING E3 ligases act in multi-subunit protein complexes, where substrate recognition is mediated by a separate subunit (131). Examples of multi-subunit E3 ligases include the Skp-1/Cullin-1/F-box (SCF) complex and other Cullin-RING ubiquitin ligases (CRL) with diverse substrate-recognition subunits, like F-box or BTB domains (132). There are further subfamilies of RING ubiquitin ligases (133), including U-box and RING-CH domain-containing proteins, which are structurally similar, but have variations within the RING domain (134, 135). The U-box is structurally similar to the RING domain, but the zinc ions are replaced by hydrogen bonds to hold the structure in shape (136). The RING-CH domain, or membrane associated RING-CH (MARCH) domain, has a C4HC3 configuration in its RING domain (137). MARCH E3 ligases are predominantly membrane bound and are

associated with down-regulation of cell surface proteins (135). In this thesis, I will focus only on RING domain E3 ligases.

1.7.2 Types of ubiquitination

Ubiquitination can occur in the form of mono-, multi-mono-, or poly-ubiquitination. Mono or multi-mono-ubiquitination can initiate the internalization of cell surface receptors and subsequent degradation in lysosomes (138, 139). Polyubiquitination chains can form through any of the seven lysine residues in ubiquitin (K6, K11, K27, K29, K33, K48, K63) (140). Furthermore, linear polyubiquitin chains can be formed by the catalytic activity of the linear ubiquitin chain assembly complex (LUBAC) (141). Since different ubiquitin chains have distinct structures, the type of ubiquitin chain dictates the fate of the target protein (138). To date, most investigations have focused on K48 and K63 (142-144). K63-linked chains are elongated, while K48-linked are compact chains (142, 143). A K48 tetramer chain is the minimal signal for proteasomal degradation (145). While the K48-linked chain is mostly known for proteasomal degradation, K11-linked chains can also lead to the degradation of target proteins. Targets for K11-linked chains include proteins that are involved in the cell cycle, and ER-associated proteins (146, 147). K29-linked chains play a role in lysosomal protein degradation (148). K63-linked chains are involved in NF-κB activation, DNA damage response, and down-regulation of cell
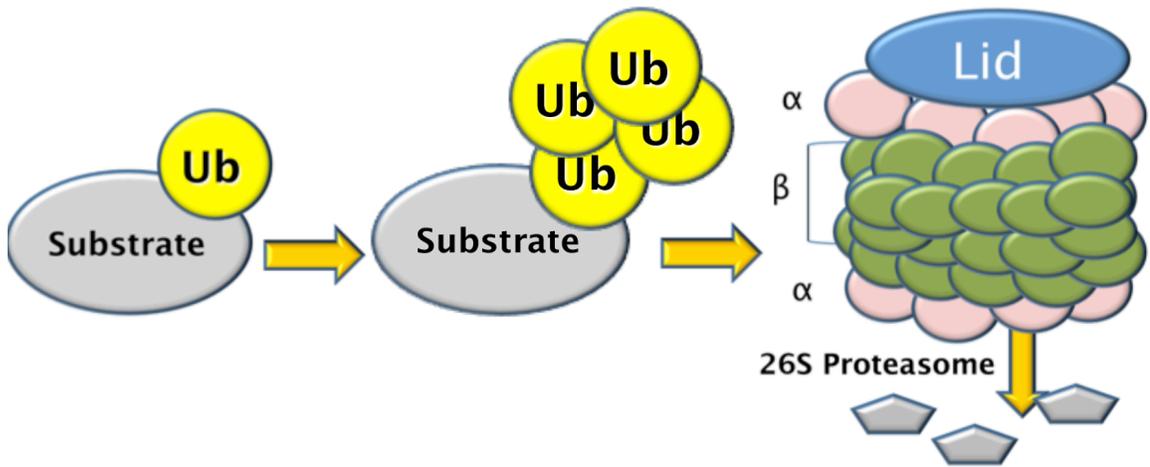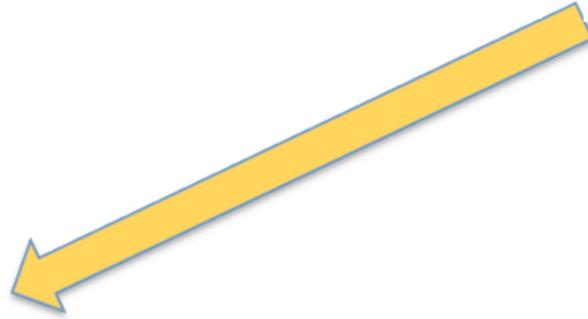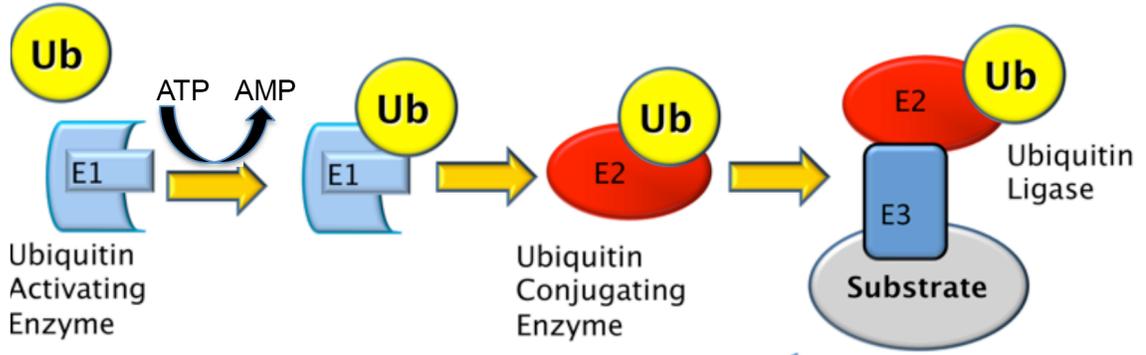
Figure 1.5 Ubiquitination of target proteins.

Free ubiquitin (Ub) is activated by the E1 activating enzyme in an ATP-dependent manner. Ub is transferred to the E2 ubiquitin conjugating enzyme. The E3 ubiquitin ligase interacts with both E2 and the protein substrate and mediates the transfer of activated Ub onto the target protein. In the case of K48-linked polyubiquitination, the labeled protein is targeted to the 26S proteasome for degradation.

surface receptors (128, 149-151). Recently, head to-tail-linked linear polyubiquitin chains formed by LUBAC has been demonstrated to be a critical regulator of the NF- κB pathway (152).

1.7.3 Regulation of E3 ubiquitin ligases

Ubiquitin ligases are the key regulators of target proteins and therefore have to be tightly regulated (139). Post-translational modifications can target a protein for ubiquitination, but can also influence E3 ubiquitin ligase activity For example, when the yeast E3 ubiquitin ligase APC is phosphorylated, there is increased ubiquitin ligase activity compared to the non-modified APC (153). Commonly, ubiquitin ligases are themselves regulated by ubiquitination as a feedback regulatory mechanism. This can occur through autoubiquitination or via another ubiquitin ligase (139). TRAF6 is an example of autoubiquitination, which occurs during stimulation of the NF-κB signal transduction pathway (154). When enough of a substrate is present for the ubiquitin ligase, it tends to be protected from autoubiquitination. In the absence of substrates, autoubiquitination serves as a regulatory mechanism, since their service is not needed (139, 155).

1.7.4 Substrate regulation by ubiquitination

Most intracellular and many extracellular proteins experience regular turnover (156). Old or misfolded proteins are hydrolyzed down to amino acids and new proteins are built to replace them. This process is regulated by ubiquitin ligases and is very important for homeostatic functions. Either excessive breakdown, or accumulation of proteins can lead

to diseases (157). Deregulation of ubiquitination can lead to abnormal activation or shutdown of pathways, which can play a role in oncogenesis (158, 159). Furthermore, incorrect assembly of protein complexes can result in interference with inflammatory responses or impairment of DNA repair (160). Moreover, impaired ubiquitination can lead to accumulation of misfolded proteins, which is thought to play a role in neurodegenerative diseases and mislocalization of proteins (161).

The turnover rate for a given protein varies from minutes for some regulatory enzymes, to days/weeks for actin and myosin in skeletal muscle, to months for hemoglobin in erythrocytes (156). In some instances, ubiquitination is used to rapidly degrade specific proteins to permit adaptation to new physiological conditions. This includes the regulation of many transcription factors in order to modulate gene transcription (162). Ubiquitination of transcription factors can lead to their removal or re-location (163, 164). Monoubiquitination typically does not lead to proteolysis, but can lead to internalization of cell surface proteins (165) or regulation of transcription factors (166).

Different types of post-translational protein modification can stimulate ubiquitination. One of the most common is phosphorylation of the target protein. However, there are other mechanisms, which recruit E3 ubiquitin ligases to substrates, including glycosylation, nitrosylation, and deacetylation (156).

1.7.5 De-ubiquitinating enzymes

De-ubiquitinating enzymes (DUB) play a further regulatory role by removing ubiquitin chains from the target protein and opposing the action of ubiquitin ligases. There are over

90 DUBs found in the human genome, which can be grouped into two families, including cysteine proteases and ubiquitin-specific metalloproteases (167). The former comprise the ubiquitin specific protease (USP), ubiquitin C-terminal hydrolase (UCH), ovarian tumor domain proteins (OTU), Machado-Josephin-domain (MJD), while the metalloproteases contain JAMM domain proteins (167-170).

USP7 is an example of a DUB, which targets ubiquitin ligases Mdm2, ICP0, MARCH7, and RING1B and regulates their stability (171). CYLD and A20 are DUBs that both have an inhibitory effect on the NF-κB pathway, via removing K63 ubiquitin chains from TRAF6 (172-174). Some DUBs have important roles as tumor suppressors and are mutated in a number of cancers (158, 159, 175).

1.7.6 The 26S Proteasome

Many polyubiquitinated proteins are targeted to the 26S proteasome for degradation (176, 177). The 26S proteasome is a multi-protein complex, which consists of a barrel shaped 20S core particle with a 19S regulatory particle at either or both ends **(Figure 1.6)** (178, 179). Substrate entry is catalyzed by the 19S particle. This outer lid can bind the polyubiquitin chains and disassemble the ubiquitin chain to recycle ubiquitin for degradation of other proteins (180). The target proteins are then unfolded and translocated into the 20S core, which contains the catalytic activity of the proteasome (178, 179). The 20S core consists of four stacked rings. The outer two rings consist of non-catalytic α subunits, while the inner two rings consisting of β subunits have the catalytic activity. All proteins that are targeted to the proteasome are subsequently processed and degraded into small peptides (181). An estimated 80-90% of intracellular

Free ubiquitin

Substrate

Lid subcomplex

19S regulatory complex

ATPase

α subunit

β subunit

20S catalytic core complex

α subunit

19S regulatory complex
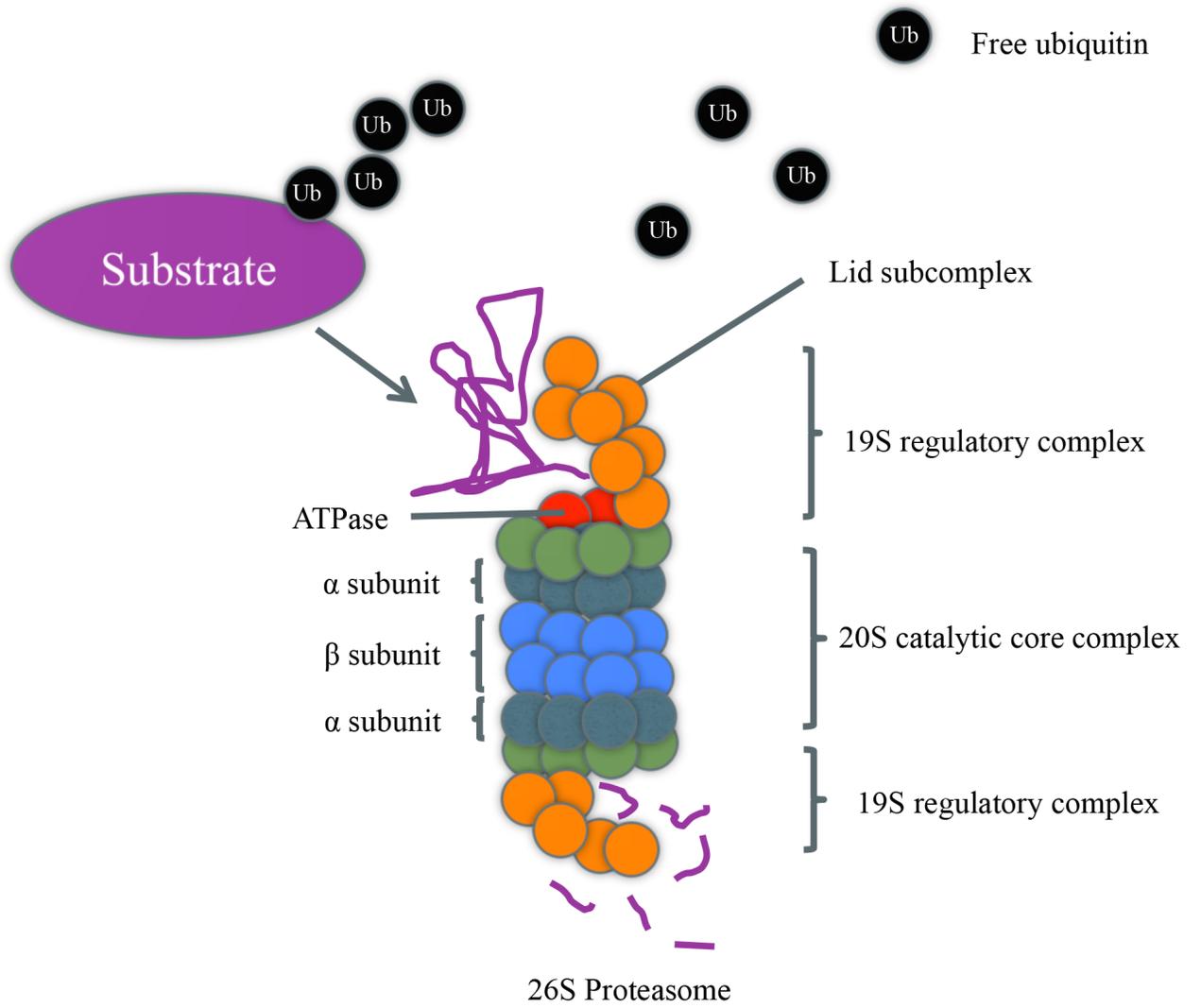
26S Proteasome

Figure 1.6 The 26S Proteasome.

Polyubiquitinated substrate proteins are targeted for proteolysis by the 26S proteasome. The ubiquitinated proteins are recognized by the lid subcomplex of the 19S regulatory complex and are deubiquitinated to reuse free ubiquitin for ubiquitination of other targets. Proteins are then unfolded and translocated into the 20S catalytic core complex. The 20S core consists of non-catalytic α subunits and catalytic β-subunits.

proteins are degraded by the 26S proteasome (182). There are numerous chemical inhibitors of the 26S proteasome (183, 184). One of the most widely used proteasome inhibitors for cell biology experiments is the synthetic peptide aldehyde MG132, which inhibits the proteasome by targeting the chemotrypsin-like β5 subunit (93, 185). Some inhibitors are used in clinical settings, especially in cancer research (186, 187).

1.8 Viruses manipulate the ubiquitin-proteasome system

Since viruses have adapted to interfere with cellular pathways, it is not surprising that many viruses have developed ways to manipulate the ubiquitin-proteasome pathway to their own advantage (116, 117, 188-199). It has been extensively shown that proteasome inhibitors interfere with the replication cycle of numerous human pathogens, such as herpesviruses (188, 189), adenoviruses (191), influenza viruses (192), coronaviruses (196), picornaviruses (198), hepadnaviruses (190), retroviruses (193-195), paramyxoviruses (197), rotaviruses (199), and poxviruses (116, 117). This underlines the importance of a functional ubiquitin-proteasome system for viruses. One of the first examples was discovered in HPV, where the viral E6 protein interacts with a cellular ubiquitin ligase E6AP (UBE3A), which leads to the ubiquitination and subsequent degradation of p53 to prevent apoptosis of the host cell (130). In addition to inhibition of apoptosis, ubiquitination is required for functional gene expression during infections such as with Epstein-Barr virus (EBV) and human immunodeficiency virus-1 (HIV-1) (200-202). EBV proteins LMP2A and LMP1 regulate replication by interacting with ubiquitin ligases or DUBs (203, 204). Viruses also exploit ubiquitination for manipulation of the immune response, downregulation of immune signaling proteins, inhibition of MHC

Class I antigen presentation, inhibition of viral induced apoptosis, modulation of NF-κB activation, interferon regulatory factor 3 (IRF3), and type I interferon (IFN) responses (205-212).

Some large DNA viruses encode their own ubiquitin ligases or adapter proteins. Kaposi's Sarcoma-Associated Herpesvirus encodes viral E3 ligases K3 and K5 that are responsible for immune evasion strategies. These target MHC class I antigen presentation to cytotoxic T cells and modulation of ICAM-1 and PECAM-1 to diminish antiviral cytokine release (213, 214). Another example is Herpes Simplex Virus (HSV) protein ICP0, which contains a RING finger domain and acts directly as an E3 ligase responsible for ubiquitination of cellular proteins PML, SUMO, IFI16, MyD88, NF-κB, IκB, PARG, RNF168, RNF8, and USP7 (215, 216). Furthermore, ICP0 is an important mediator for reactivation of HSV from its latent state to a state of lytic infection.

Furthermore, there are several reports of viral proteins interacting with cellular de-ubiquitinating enzymes (DUBs). HSV protein ICP0 and EBV protein EBNA1 interact with the cellular DUB USP7 (217, 218). This leads to the removal of ubiquitin chains from cellular p53, subsequently preventing its degradation (219, 220). Moreover, ICP0 is a substrate of USP7, which leads to ICP0 stabilization. In general, EBV infection leads to an increase in activity of cellular DUBs, including USP5, USP7, USP9, USP13, USP15, and USP22 (221). Measles infection leads to the upregulation of the cellular DUB, A20, which removes ubiquitin from TRAF6 and interferes with NF-κB activation (222). Some viruses encode their own DUBs including HSV-1, EBV, MCMV, and HCMV (223-226). Overall, viruses extensively use the ubiquitin-proteasome system for their life cycle and immune evasion.

1.9 Importance of ubiquitination and a functioning proteasome during poxvirus infection

Like other virus families, poxviruses encode a variety of proteins to manipulate the ubiquitin-proteasome pathway. The importance of the ubiquitin-proteasome system during poxviral infection was recently highlighted by the observation that poxviruses require a functional ubiquitin-proteasome system for replication and late gene expression (116, 117). Interestingly, some poxviruses, including canarypox and entomopoxvirus, encode a homologue to human ubiquitin (22, 23). However, the role of poxvirus-encoded ubiquitin remains unknown, since poxviruses also incorporate host cell ubiquitin into their virions (180, 227, 228). Additionally, poxviruses contain a putative DUB I7L, although its activity remains to be confirmed (229). Below, I will discuss some of the better characterized mechanisms of manipulation of the ubiquitin-proteasome pathway by poxviruses.

1.10 Poxviral E3 ubiquitin ligases and adapter proteins

Poxviruses encode a variety of proteins to manipulate the UPS **(Figure 1.7)**. One of the first described poxviral E3 ligases was M153R, found in MYXV, a poxviral strain that infects rabbits. M153R is a Membrane-Associated RING-CH (MARCH) E3 ligase (73, 74). MARCH has a modified RING domain at its N-terminus combined with a transmembrane domain, which keeps it localized to the membrane. At the plasma membrane, M153R is involved in down-regulating cell surface immune molecules like MHC class I, MHC class II, and CD4 coreceptor (73, 74, 230-232). This prevents

A MARCH (M153R)
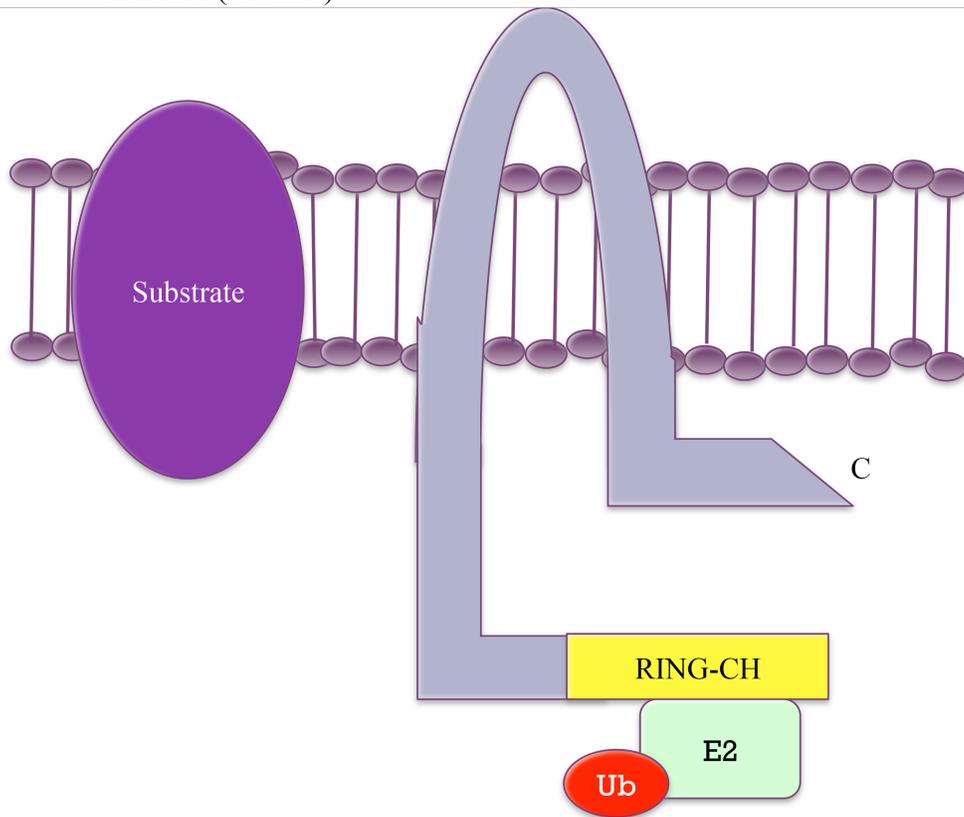
B RING (p28)
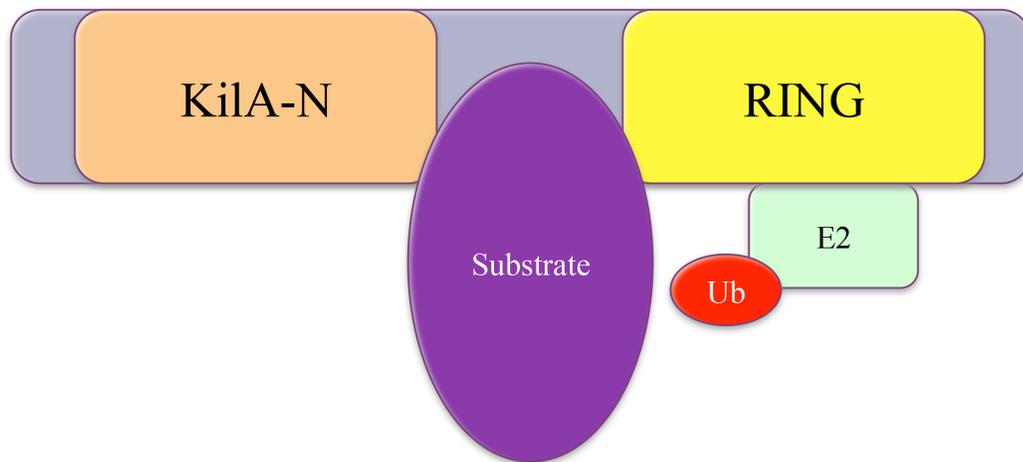
C   BTB/Kelch
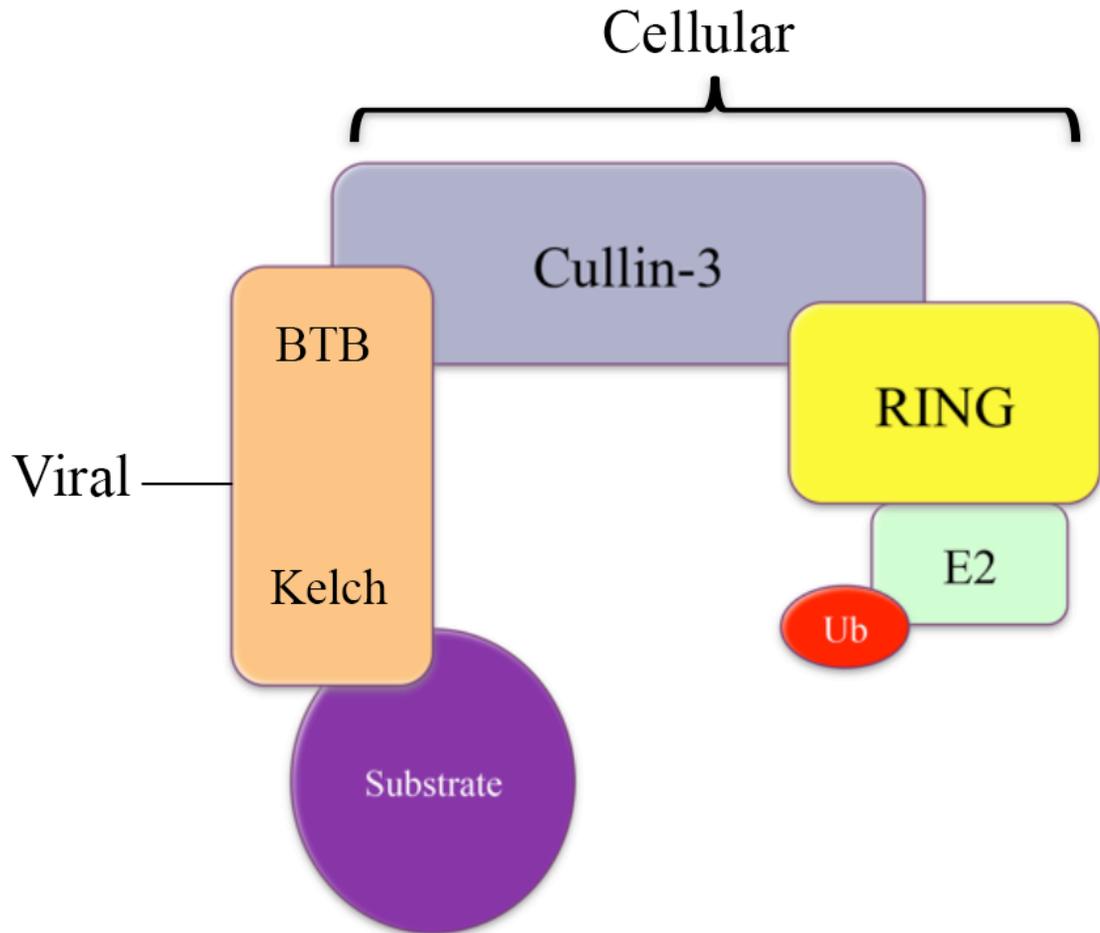
Figure 1.7 Poxviral E3 ubiquitin ligases and adapter proteins.

Adapted from Barry *at el.* (233). **(A)** MARCH (membrane-associated RING-CH) has an N-terminal RING domain and a C-terminal trans-membrane domain. **(B)** p28 is a single subunit ubiquitin ligase with an N-terminal KilA-N DNA binding domain and a C-terminal RING domain. **(C)** Multi-subunit ubiquitin ligase. This is a member of the cullin protein family, which interacts with linker protein and RING finger protein.

the presentation of viral peptides to T cells and subsequently suppresses the adaptive immune response.

Parapoxviruses, *Molluscipoxviruses*, crocodilepox and squirrelpox viruses are also known to express APC11 homologues with similarity to RING-finger proteins (234). However, this thesis will focus on the poxviral E3 ligase p28 and its homologues in FWPV.

In addition to encoding E3 ubiquitin ligases, the family of Orthopoxviruses expresses adapter proteins that facilitate recruitment of substrates to multi-subunit cullin-based ubiquitin ligases. These adapter proteins can contain BTB/Kelch (Broad-complex, Tramtrack and Bric-a-Brac) domains or Ankyrin/F-box proteins (208, 229, 233). The Barry laboratory (University of Alberta, Edmonton, Canada) has demonstrated that ECTV encodes four Ankyrin/F-box proteins (EVM002, EVM005, EVM154 and EVM165), which interact with the cellular SCF ubiquitin ligase to inhibit NF-κB activation (235, 236). Of note, EVM005 can inhibit NF-κB activation through manipulation of the host SCF ubiquitin ligase complex, but also acts as an NF-κB-independent virulence factor (237). Furthermore, ECTV encodes four BTB/kelch adaptor proteins, EVM018, EVM027, EVM150, and EVM167, which are associated with the cellular cullin-3 ubiquitin ligase complex (233, 238-240). It was recently demonstrated that EVM150 inhibited both TNF-α- and IL-1β-induced NF-κB nuclear translocation, without blocking the degradation of IκBα (241). In addition to the role of EVM150 as an adapter for cullin-3-based ubiquitin ligases, the BTB domain of EVM150 was sufficient to suppress NF-κB activation (241).

1.11 p28 and homologues

While poxviruses utilize several strategies for manipulating the ubiquitin pathway, this thesis is mainly focused on characterization of the poxviral encoded p28 E3 ligases. p28 is a 28 kDa protein found in diverse members of the poxvirus family. It was first described in VACV and ECTV (242, 243), but homologues are also found in myxoma virus (MYXV), shope fibroma virus, VARV and birdpoxviruses **(Table 1.3)**. p28 is the first RING domain protein demonstrated to be associated with virulence. Interestingly, p28 is mostly found in highly pathogenic poxviruses, but is absent in the attenuated VACV strains used for vaccination. p28 is not essential for replication in tissue culture (242, 244, 245). The first indication that p28 acts as a virulence factor was demonstrated by the Buller group (244). Mice survived infection with ECTV devoid of p28, demonstrating a dramatic reduction in virus virulence compared to WT ECTV infection. This was due to the fact that the p28 knockout virus could no longer replicate in macrophages, which is important for viral dissemination from skin lesions to lymph nodes and the liver (244, 245).

p28 consists of two highly conserved functional domains **(Figure 1.8)**. The C-terminus has a RING domain, which defines its E3 ligase activity. The N-terminal domain is a DNA binding domain named KilA-N domain, which is largely uncharacterized. KilA-N domains were previously found in large DNA viruses, bacteria and bacteriophages and seem to bind non-specifically to DNA (246). p28 is the only known protein to our knowledge which pairs a KilA-N DNA binding domain with a RING domain. Previous work with N1R, the p28 homologue in shope fibroma virus, revealed that the KilA-N domain seems to be responsible for localizing p28 to the viral

factories where the virus replicates (247). Dissection of diverse parts of the KilA-N domain revealed that this 112 amino acid KilA-N domain contained a highly conserved 7 amino acid area from residue 44-50 (YINITKI), which is crucial for this localization **(Figure 1.8)**.

The VACV-WR strain contains only a truncated version of p28 lacking the RING domain, while VACVCop completely lacks p28. Previous work in the laboratory of Dr. Barry in collaboration with Dr. Klaus Frueh (Oregon Health and Science University, Beaverton, OR) demonstrated the E3 ligase activity of p28 in an *in vitro* ubiquitination assay. It was also shown that the first two highly conserved cysteines C173 and C176 within the RING domain were crucial for E3 ligase activity. When these two cysteines were mutated to serines, the E3 ligase activity of p28 was abolished, since the mutations disrupted the ability of the RING domain to form a complex with zinc (243). Even though p28 was demonstrated to be a virulence factor during viral infection, no substrates for p28 have been identified to date. Previous reports suggest that p28, and the p28 homologue in shope fibroma virus (N1R) might be involved in inhibition of apoptosis, which leads to the speculation that p28 might target pro-apoptotic proteins for ubiquitination and proteasomal degradation (20, 247, 248). However, this awaits confirmation.

Since p28 localizes to viral factories, potential substrates might be located there as well. It is known that p28 expression starts early during infection before the virus factories are formed (245, 249). Therefore, other substrates might be found in the cytoplasm of the host cell as well.

Genus	Virus	Gene/Protein	Length	Accession
Avipoxvirus	CNPV	CNPV197	275	VP0043670
		CNPV205	318	VP0043678
	FWPV	FWPV150	276	VP0037882
		FWPV157	311	VP0037889
Capripoxvirus	GTPV-Pellor	127	240	VP0044947
	LSDV-Nee	140	240	VP0040345
	SPPV-A	136	240	VP0044645
Leporipoxvirus	MYXV-Lau	M143	234	VP0038572
	RFV-Kas	gp143R (N1R)	234	VP0038740
Orthopoxvirus	CMLV-CMS	14R	242	VP0041112
	CPXV-GRI	C7R	242	VP0042678
	ECTV-Mos	12	241	VP0040932
	MPXV-ZAR	D5R	242	VP0040369
	VACV IHD-W	p28	243	N/A
	VARV-BGD75major	D6R	242	VP0038767
	RPXV-Utr	8	242	VP0041370
Suipoxvirus	SWPV-Neb	138	246	VP0040694
Yatapoxvirus	TANV-COD	143R	234	VP0067759
	YMTV-Amano	143R	236	VP0043181

Table 1.3 p28 homologues

KilA-N   RING

C173
C176 C196 C202 C222
      H198 C205 C225

p28

19 44 51   132 167   228 242
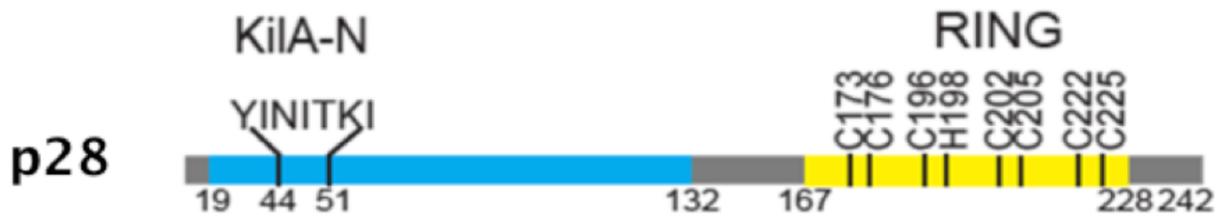
DNA Binding Domain:
Localizes to viral factories
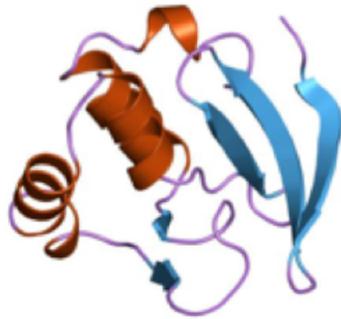
RING Domain:
E3 Ligase activity

X_{4-48}

C1   C5   H1   C6

X_2 Zn^{+2} X_2 X_{2-3} X_{1-3} Zn^{+2} X_2

C2   C4   C3   C7

X_{9-39}
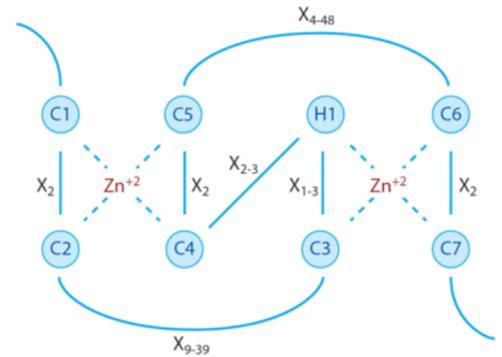
Figure 1.8 p28 E3 ubiquitin ligase.

A schematic representation of p28 illustrates the N-terminal KilA-N DNA binding domain and the C-terminal RING domain; The KilA-N domain is an illustration of the related APSES DNA binding domain, adapted from Iyer *et al*. (246). The p28 KilA-N domain contains a YINITKI motif, which is necessary for localization to the virus factory. The highly conserved cysteine and histidine residues are further highlighted in the RING domain; adapted from Deshaies *et al.* (139).

Interestingly, p28 has been associated with K63 ubiquitin chains, since a yeast-2 hybrid associated p28 with E2 conjugating enzyme Ubc13, an enzyme that is solely utilized for formation of K63-linked chains (242). This suggests that p28 could be responsible for either targeting proteins for proteasomal degradation, or altering their functions.

1.12 Hypothesis and study objectives

Previous work from our group and other labs suggested that p28 acts as an important E3 ligase. We were also interested in investigating homologues of p28 in other members of the poxviruses family and determining their role in host cell infection. Unlike other members of the poxvirus family, both Avipoxviruses FWPV and CNPV contain two homologues of p28. In FWPV p28 homologues were named after their ORF number FWPV150 and FWPV157, and had not been studied at the time I began my thesis. Notably, the critical key residues within RING domains (seven cysteines and one histidine motif) and KilA-N domains (YINITKI motif) are conserved in both FWPV150 and FWPV157, suggesting that these proteins likely function as ubiquitin ligases as well. However, the rest of the sequences do not contain high sequence homology. Protein sequence identity between FWPV150 and FWPV157 is 36%, whereas sequence identity between p28 and FWPV150 is 24% and between p28 and FWPV157 is 43%. FWPV also encodes eight KilA-N domain-containing proteins lacking the RING domain and canarypox virus encodes twenty-three KilA-N only proteins. The function of these KilA-N domains during virus infection were unknown at the time that my thesis work began. The substrates of p28 and its homologues were also unknown. I was interested in

characterizing the mechanism by which these proteins function as virulence factors during virus infection.

Hypotheses:

My working <u>hypotheses</u> are:

1) FWPV150 and FWPV157 act as E3 ligases due to their domain similarity to p28

2) p28 targets cellular and/or viral targets for ubiquitination during viral infection.

I will test these hypotheses by pursuing the following objectives.

Thesis Objectives:

<u>Objective 1</u>: Characterize the ubiquitin ligase activity, expression and cellular localization of FWPV150 and FWPV157 in FWPV.

<u>Objective 2</u>: Characterize p28 RING and KilA-N mutants, their interaction with ubiquitin, and their ability to act as ubiquitin ligases.

<u>Objective 3</u>: Identify interaction partners and potential substrates for p28.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell Culture and Viruses

2.1.1 Cell Lines

The cell lines and viruses used in this study are listed in **Table 2.1**. Human fibroblasts (HeLa), CV-1, J774, Raw 264, baby green monkey kidney (BGMK), and HuTK$^{-/-}$-143B cells were obtained from the American Type Culture Collection (ATCC, Burlington, ON, Canada) and maintained at 37°C and 5% CO_2. HeLa, CV-1, J774, Raw 264 and HuTK$^{-/-}$-143B cells were cultured in Dulbecco's modified Eagle's Medium (DMEM; Invitrogen, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS; Invitrogen), 50 U/mL penicillin, 50 µg/mL streptomycin, and 200 µM $_L$-glutamine (Invitrogen). HuTK$^{-/-}$ -143B cell media additionally contained 25 µg/mL 5-bromo-2'-deoxyuridine (BrdU; Sigma-Aldrich, Oakville, ON, Canada). BGMK cells were cultured in DMEM supplemented with 10% newborn calf serum (NCS; Invitrogen), 50 Units (U) of penicillin/ml, 50 µg/mL streptomycin, and 200 µM $_L$-glutamine. Quail fibrosarcoma (QM5) cells were a generous gift from R. Duncan (Dalhousie University, Halifax, NS) and maintained in Dulbecco's Modified Eagle medium supplemented with 10% heat inactivated fetal bovine serum (HI-FBS) (Invitrogen), 50 U of penicillin/ml (Invitrogen), and 200 µM glutamine (Invitrogen).

2.1.2 Viruses

VACVCop (no endogenous p28), VACVWR (truncated version of endogenous p28(1-184)), and CPXV were provided by G. McFadden (University of Florida, Gainesville,

Cell Line	Cell Type	Characteristics	Source
HeLa	Human Fibroblast		ATCC
BGMK	Baby Green Monkey Kidney		ATCC
CV-1	Monkey Kidney Fibroblast		ATCC
HuTK$^{-/-}$-143B	Human 143B Osterosarcoma	Deficient in Thymidine Kinase	ATCC
QM5	Quail Fibroblast		D. Evans
J774	Mouse Macrophage		ATCC
Raw 264	Mouse Macrophage		ATCC
Viruses	Strain	Characteristics	Source
VACV(Cop)	Copenhagen	No endogenous p28	G. McFadden
VACVWR	Western Reserve	Trucated p28	G. McFadden
VACV-IHDW	IHDW	Endogenous p28	D. Evans
VACV-FLAG-M143R	Copenhagen	Expresses FLAG-M143R instead of TK	K. Früh (243)
VACV-HA-Ubiquitin	Copenhagen	Expresses HA-Ubiquitin in place of TK	K. Früh (243)
VACV-FLAG-p28	Copenhagen	Expresses FLAG-p28 in place of TK	K. Früh (243)

VACV-FLAG-p28(C173S/C176S)	Copenhagen	Expresses FLAG-p28(C173S/C176S) in place of TK	K. Früh (243)
VVT7lacOI	WR		B. Moss (250)
VACVΔF1L-FLAG-FWPV039	Copenhagen	Expresses FLAG-FWPV039 instead of TK; lacks endogenous F1L	L. Banadyga (84)
CPXV	Brighton Red		G. McFadden
ECTV	Moscow		M. Buller

Table 2.1 Cells and viruses used in this study

FL). FWPV and VACV-IHDW were generously provided by D. Evans (University of Alberta, Edmonton, AB). Recombinant VACV-FLAG-M143R, Recombinant VACV-HA-Ubiquitin and VACV-FLAG-p28, and VACV-FLAG-p28(C173S/C176S) were previously described (243). VACV strain Western Reserve expressing T7 polymerase VACVT7lacOI (VACVT7) and ECTV were a generous gift from B. Moss (National Institute of Allergy and Infectious Diseases, Bethesda) (251) Recombinant VACVΔF1L-FLAG-FWPV039 was generated as previously described (84) All viruses were propagated in BGMK cells and purified as previously described, except for FWPV, which was propagated in QM5 cells (252).

2.2 DNA Methodology

2.2.1 Polymerase Chain Reaction

Polymerase chain reactions (PCR) were carried out in volumes of 50 µl containing 60 mM Tris-SO$_4$, 20 mM (NH$_4$)$_2$SO$_4$, 2 mM MgSO$_4$, 3% glycerol, 0.06% NP-40, and 0.05% Tween-20 (pH 9.0 at 25°C). Each reaction contained 1 pmole of each primer, 10 mM deoxyribonucleotide triphosphates (dNTPs; Invitrogen), 2.5 U of LongAmp Taq DNA polymerase (New England Biolabs, Whitby, ON, Canada) and either 10 ng of plasmid DNA or 100 ng of viral DNA template. All primers used in this study are listed in **Table 2.2**. Temperature cycles were performed with a C1000 thermal cycler (BioRad, Mississauga, ON, Canada) using the following parameters: Initial denaturation for 5 minutes at 95°C followed by 30 cycles of denaturation at 95°C for 1 minute, primer annealing at 55°C for 30 seconds, and primer elongation at 65°C for 1 minute/kilobase

Primer Name	Primer Sequence (5' – 3')	Restriction Site	Description	Source
FLAG-FWPV150 *Sal*I Fwd	GTCGACATGGACTAC AAAGACGATGACGAC AAGTCACATCTTCATC TTAAT	*Sal*I	Used to clone pSC66-FLAG-FWPV150 and RT-PCR test for gene expression	This study
FWPV150 *Not*I Rev	GCGGCCGCTTAACAT GATTTTATATATAC	*Not*I	Used to clone pSC66-FLAG-FWPV150 and RT-PCR test for gene expression	This study
FLAG-FWPV157 *Sal*I Fwd	GTCGACATGGACTAC AAAGACGATGACGAC AAGAAGGAGGACGAC TCATCA	*Sal*I	Used to clone pSC66-FLAG-FWPV157 and RT-PCR test for gene expression	This study
FWPV157 *Not*I Rev	GCGGCCGCTTATATTT TTATTGTTCTTAT	*Not*I	Used to clone pSC66-FLAG-FWPV157 and RT-PCR test for gene expression	This study

FWPV150(C196S/C199S) Fwd	CGATAAATCCGGTATT TCTTTGGATGC	N/A	Used as overlapping primer for site directed mutagenesis	This study
FWPV150(C196S/C199S) Rev	GCATCCAAAGAAATA CCGGATTTATCG	N/A	Used as overlapping primer for site directed mutagenesis	This study
FWPV157(C218S/C221S) Fwd	CCAGTAAAAAGTCTG GCATATCCATAGAAG	N/A	Used as overlapping primer for site directed mutagenesis	This study
FWPV157(C218S/C221S) Rev	CTTCTATGGATATGCC AGACTTTTTACTGG	N/A	Used as overlapping primer for site directed mutagenesis	This study
FLAG-p28-*Sal*I-(Fwd)	GTCGACATGGACTAC AAGGACGACGACG	*Sal*I	Used to clone pSC66-FLAG-p28(1-184) and pSC66-FLAG-p28(1-204)	This study
p28(1-184) *Not*I Rev	GCGGCCGCTCAGCGC TTGCTG	*Not*I	Used to clone pSC66-FLAG-p28(1-184)	This study
p28(1-204) *Not*I Rev	GCGGCCGCTCAGGTG ATGCCA	*Not*I	Used to clone pSC66-FLAG-p28(1-204)	This study

FWPV085 Fwd	TGGAAATAGCTAGAG AAACGCTAA	N/A	RT-PCR late gene control	This study
FWPV085 Rev	AAACGAAGTATTCTTC CTGCTG	N/A	RT-PCR late gene control	This study
FWPV037 Fwd	GAATTCATGACGCTAT TCGAATATATA	N/A	RT-PCR early gene control	This study
FWPV037 Rev	GGATCCGATTGTGACT TTTCAGACTTTTTACT	N/A	RT-PCR early gene control	This study
GAPDH Fwd	TGATGACATCAAGAA GGTGGTGAAG	N/A	RT-PCR housekeeping gene	This study
GAPDH Rev	TCCTTGGAGGCCATGT GGGCCAT	N/A	RT-PCR housekeeping gene	This study
FWPV075 Fwd	GTCGACATGGACTAC AAAGACGATGACGAC AAGGAGTTTGTACCTA ACACC	N/A	RT-PCR test for gene expression	This study
FWPV075 Rev	GCGGCCGCTTATTTTA TGGATAAACC	N/A	RT-PCR test for gene expression	This study
FWPV124 Fwd	GTCGACATGGACTAC AAAGACGATGACGAC AAGGACTTCTCGGATC TCGTT	N/A	RT-PCR test for gene expression	This study
FWPV124	GCGGCCGCTTAACCG	N/A	RT-PCR test for	This

Rev	ATTAAAACAGG		gene expression	study
FWPV155 **Fwd**	GTCGACATGGACTAC AAAGACGATGACGAC AAGATGTTTAATAGTA TGATA	N/A	RT-PCR test for gene expression	This study
FWPV155 **Rev**	GCGGCCGCTTAGTTAT CTGAAAATAT	N/A	RT-PCR test for gene expression	This study
FWPV159 **Fwd**	GTCGACATGGACTAC AAAGACGATGACGAC AAGTCTACTATTACCT GTTAT	N/A	RT-PCR test for gene expression	This study
FWPV159 **Rev**	GCGGCCGCTTAGATG AAAAAAATTAGCTT	N/A	RT-PCR test for gene expression	This study
FWPV161 **Fwd**	GTCGACATGGACTAC AAAGACGATGACGAC AAGGATAAAGATAT ATATCA	N/A	RT-PCR test for gene expression	This study
FWPV161 **Rev**	GCGGCCGCCTACATGT TGTATGAATT	N/A	RT-PCR test for gene expression	This study
FWPV163 **Fwd**	GTCGACATGGACTAC AAAGACGATGACGAC AAGAATACCTTACCGT ATATT	N/A	RT-PCR test for gene expression	This study

FWPV163 Rev	GCGGCCGCTTATTTAG ATTTTCTGAATGTTAG	N/A	RT-PCR test for gene expression	This study
FWPV236 Fwd	GTCGACATGGACTAC AAAGACGATGACGAC AAGAAATTTAAGGAA GTTAGA	N/A	RT-PCR test for gene expression	This study
FWPV236 Rev	GCGGCCGCCTATTCGT AATTATTCGCTACCGC TGG	N/A	RT-PCR test for gene expression	This study
FWPV248 Fwd	GTCGACATGGACTAC AAAGACGATGACGAC AAGGAAATCAAAGTA GAATCG	N/A	RT-PCR test for gene expression	This study
FWPV248 Rev	GCGGCCGCCTACATAT TG TTATCATCGTTTAA	N/A	RT-PCR test for gene expression	This study
TK- Fwd	GATCTACTTCCTTACC GTGC	N/A	Sequencing of pSC66 vector, confirmation of purity of recombinant viruses	R. Burton
TK- Rev	GGAACGGGACTATGG ACGC	N/A	Sequencing of pSC66 vector,	R. Burton

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			confirmation of purity of recombinant viruses	

Fwd – Forward, Rev – Reverse, N/A – not applicable

Table 2.2 Oligonucleotides used in this study

(kb). Alternatively, PCR reactions were done in 50µl volumes containing 200 mM Tris-HCl pH 8.4, 500 mM KCl, 50 mM MgCl, 1 pmole each primer, 10 mM dNTPs, 10 ng template DNA, and 2.5 U of Taq DNA polymerase (Invitrogen). Temperature cycles were performed as described above, except that elongations were performed at 72°C.

2.2.2 Agarose Gel Electrophoresis

DNA products of PCR and endonuclease digestion (described in section 2.2.4) were purified and isolated by agarose gel electrophoresis followed by gel extraction. DNA was suspended in a sample loading dye containing 30% glycerol (Fisher Scientific, Ottawa, ON, Canada) and 0.25% Bromophenol blue (BioRad). DNA products were separated by gel electrophoresis in gels made of 1% weight per volume (w/v) UltraPure Agarose (Invitrogen) in 1X TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM ethylenediaminetetraacetic acid (EDTA)). To visualize DNA bands, SYBR Safe DNA gel stain (Invitrogen) was added to the agarose gels at a 1:100 dilution. Electrophoresis was performed in 1X TAE running buffer in a Mini-Sub Cell GT (BioRad) at 100V. DNA bands were visualized using a Gel Doc EZ Imager (BioRad). Bands of interest were excised and subjected to gel extraction as described in Section 2.2.3.

2.2.3 Gel Extraction

Gel extraction was performed using a QIAquick gel extraction kit (Qiagen, Toronto, ON, Canada) according to the manufacturer's protocol. In brief, excised DNA bands were suspended in 3 volumes of solubilization buffer per 1 volume of gel (300 µl per 100 mg) and incubated at 50°C for 10 minutes, or until the gel was completely dissolved. One gel

volume of isopropanol was then added before being run on a QIAquick DNA column followed by an ethanol wash. Bound DNA was eluted in 25 µl of 10 mM Tris-HCl (pH 8.5) or ddH$_2$O. The extracted DNA was assessed by agarose gel electrophoresis.

2.2.4 Restriction Digests

Restriction digests were performed according to the manufacturer's instructions with endonuclease restriction enzymes from Invitrogen or New England Biolabs. In general, restriction digests were performed in a total volume of 50 µl for 1 hour at 37°C. The resulting DNA fragments were isolated by agarose gel electrophoresis.

2.2.5 DNA Ligation

DNA fragments produced by PCR were ligated into the T-A vector pGEM-T (Promega, Madison, WI), PCR was done using LongAmp Taq DNA polymerase to add a single deoxyadenosine to the 3'-end of the fragments. T-A cloning was done in 20 µl reactions containing 2 µl of gel extracted DNA, 50 ng of pGEM-T vector, 3 U of T4 DNA ligase, and 5µl of 2X rapid ligation buffer containing 60mM Tris-HCl (pH 7.8), 20mM MgCl$_2$, 20mM dithiothreitol (DTT), 2mM ATP, and 10% polyethylene glycol. Ligation reactions were incubated at 4°C overnight. For ligation into pSC66, or pEGFP reactions were done in 20µl volumes containing 4 U T4 DNA ligase (New England Biolabs), 50 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 1 mM ATP, and 10 mM DTT. Vector to insert ratios of 1:3, 1:6, or 1:9 were tested for the best ligation efficiency and reactions were incubated at 16°C overnight. Ligations were transformed into competent *E. coli* cells as described in Section 2.2.6.

2.2.6 Bacterial Transformation

Competent *E. coli* DH5α cells (Invitrogen) were transformed with ligation reactions using a heat shock method. Fifty µl of competent DH5α cells were incubated with 5 µl of ligation reactions on ice for 30 minutes followed by heat shock at 42°C for 45 seconds and a 2 minute recovery on ice. Two hundred fifty µl of super optimal broth with catabolite repression (253) media containing 20 mg/mL tryptone, 5 mg/mL yeast extract, 0.5 mg/mL NaCl, and 2.5 mM KCl (pH 7.0) was added and cells were incubated at 37°C for 1 hour. Transformed bacteria were plated on Luria-Bertani (LB) agar plates supplemented with the appropriate antibiotic, either 100 µg/mL ampicillin (Sigma-Aldrich) or 30 µg/mL kanamycin (Sigma-Aldrich). *E. coli* transformed with pGEM-T plasmids were plated on LB agar plates containing 100 µg/mL ampicillin, 80 µg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Rose Scientific, Edmonton, AB, Canada) and 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Rose Scientific) to allow for blue/white screening of recombinants.

2.2.7 Plasmid DNA Isolation

To prepare plasmids used for expression in mammalian cells or sequencing, 200 mL of LB broth containing the appropriate antibiotic (100 mg/mL ampicillin or 60 mg/mL kanamycin) was inoculated with *E.coli* DH5α cells containing the desired plasmid and incubated overnight at 37°C. For low copy plasmids, such as pSC66, cultures were incubated for 8 hours followed by treatment with 170 mg/mL chloramphenicol (Sigma-Aldrich) for another 18 hours. Plasmid DNA was prepared using a Plasmid MaxiPrep kit (Qiagen) according to the manufacturer's protocol. DNA yields and optical densities at

280 nm were determined using a NanoVue Plus spectrophotometer (GE Healthcare, Mississauga, ON, Canada).

2.2.8 DNA Sequencing and Computer Analysis

Sanger sequencing was performed by either The Applied Genomics Centre (TAGC) in the Faculty of Medicine at the University of Alberta, or at the McGill University and Génome Québec Innovation Centre. DNA sequence analyses were performed using the Basic Local Alignment Search Tool (BLAST) provided by the National Centre for Biotechnology Information (NCBI).

2.2.9 Plasmids

All plasmids used in this study are listed in **Table 2.3**. All T-A cloning was done in the pGEM-T vector (Promega), and the pEGFP-C3 vector (Clontech, Mountain View, CA) was used to generate all N-terminal enhanced green fluorescent protein (EGFP)-tagged proteins. pSC66, which contains a synthetic poxviral early/late promoter (pE/L), was provided by Dr. E. Long (National Institute of Allergy and Infectious Disease, Bethesda, MB) (54).

Plasmid	Characteristics	Source
pGEM-T	TA cloning vector; CMV promoter	Promega
pEGFP-C3	Empty EGFP vector with a CMV promoter	Clontech
pSC66	Synthetic poxviral early/late promoter; VACV thymidine kinase sequence flanks LacZ and promoter, and multiple cloning site; Used to generate VACV recombinants	Dr. E. Long (54)
pSC66-FLAG-p28	Full length FLAG-p28	C. Milne
pSC66-FLAG-p28(1-152)	FLAG-p28, only KilA-N domain	C. Milne
pSC66-FLAG-p28(153-242)	FLAG-p28, only RING domain	C. Milne
pSC66-FLAG-p28(Δ44-51)	FLAG-p28, lacking virus factory localization signal	C. Milne
pSC66-FLAG-p28(C173S/C176S)	FLAG-p28, double cysteine mutant	B. Nerenberg (243)
pSC66-FLAG-p28(1-184)	FLAG-p28, truncated	This study
pSC66-FLAG-p28(1-204)	FLAG-p28, truncated	This study
pSC66-FLAG-FWPV150	FLAG-FWPV150	This study
pSC66-FLAG-FWPV157	FLAG-FWPV157	This study

pSC66-FLAG-FWPV150(C196S/C199S)	FLAG-FWPV150(C196S/C199S), double cysteine mutant	This study
pSC66-FLAG-FWPV157(C218S/C221S)	FLAG-FWPV157(C218S/C221S), double cysteine mutant	This study
pSC66-FLAG-FWPV075	FLAG-FWPV075, KilA-N only domain	This study
pSC66-FLAG-FWPV124	FLAG-FWPV124, KilA-N domain with Domain of unknown function	This study
pSC66-FLAG-A50	FLAG-A50	D. Evans
pEGFP-p28	EGFP-p28 for flow cytometry	K. Mottet
pEGFP-p28(C173S/C176S)	EGFP-p28(C173S/C176S) for flow cytometry	K. Mottet
pEGFP-p28(1-184)	EGFP-p28(1-184) for flow cytometry	K. Mottet
pEGFP-p28(1-204)	EGFP-p28(1-204) for flow cytometry	K. Mottet

Table 2.3 Plasmids used in this study

2.2.10 Generation of pSC66-FLAG-FWPV150, pSC66-FLAG-FWPV157, pSC66-FLAG-FWPV075, and pSC66-FLAG-FWPV124

QM5 cells (1 x 10^5) were infected with FWPV, at a multiplicity of infection (MOI) of 5 and genomic FWPV DNA was extracted as described in Section 2.5.8. The FWPV150 gene was amplified from FWPV DNA and FLAG-tagged by PCR using the primers 5`-GTCGACATGGACTACAAAGACGATGACGACAAGTCACATCTTCATCTTAAT-3`(forward) and 5`-GCGGCCGCTTAACATGATTTTATATATAC-3`(reverse). The *Sal*I restriction site was included in the forward primer and the *Not*I restriction site was included in the reverse primer. The FWPV157, FWPV075, and FWPV124 genes were amplified using the primers listed in **Table 2.2**. PCR products were subcloned into pGEM-T (Promega) and the constructs were verified by sequencing at TAGC at the University of Alberta. FLAG-FWPV150, FLAG-FWPV157, FLAG-FWPV075, and FLAG-FWPV124 were subcloned into the pSC66 vector, using *Sal*I and *Not*I, placing the FLAG-tagged genes under the control of a synthetic early/late poxviral promotor provided by Dr. E. Long (National Institute of Infectious Diseases, Bethesda) (54).

2.2.11. Generation of pSC66-FLAG-FWPV150(C196S/C199S) and pSC66-FLAG-FWPV157(C218S/C221S)

The double cysteine mutants pSC66-FLAG-FWPV150(C196S/C199S) and pSC66-FLAG-FWPV157(C218S/C221S) were generated by overlapping PCR using primers listed in **Table 2**, following the instructions from the site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutations were verified by sequencing the resulting plasmids (see Section 2.2.8).

2.2.12 Generation of pSC66-FLAG-p28(1-184) and pSC66-FLAG-p28(1-204)

FLAG-p28(1-184) and FLAG-p28(1-204) were generated using pSC66-FLAG-p28 vector as a template using FLAG-p28(1-184) Fwd along with p28(1-184) Rev, or FLAG-p28(1-204) Fwd along with p28(1-204) Rev, respectively. The PCR products were ligated into pGEM-T and sub-cloned into pSC66 as *Sal*I/*Not*I digested fragments to place them under the control of a synthetic poxviral early/late promoter.

2.3 Transfections

2.3.1 General Transfection Protocol

Transfection of 1 x 10^6 HeLa or QM5 cells was accomplished using Lipofectamine 2000 (Invitrogen) according to the manufacturer's specifications. Five μl of Lipofectamine reagent and 2 μg of plasmid DNA were added to separate 500 μl aliquots of OptiMEM media (Invitrogen) and incubated for 5 minutes. Lipid and DNA suspensions were gently mixed together and allowed to incubate for another 20 minutes. Following incubation, the DNA-lipid mixture was added to cell monolayers along with another 0.5 mL of OptiMEM for a total of 1 mL. Transfections were incubated at 37°C and 5% CO_2 for 2 hours followed by supplementation with recovery DMEM media containing 20% HI-FBS and 2 mM $_L$-glutamine. The cells were incubated at 37°C and 5% CO_2 for an additional 12-16 hours.

2.3.2 General Infection/Transfection Protocol

Twelve hour infection/transfections were performed to express proteins from pSC66 in the context of VACVCop infection. HeLa cells were infected in 500 µl to 5 mL of OptiMEM with the appropriate virus at an MOI of 5, rocking every 10 minutes for 1 hour. During this time, 2-5 µg of plasmid DNA and 5-25 µl of Lipofectamine 2000 were incubated in 500 µl to 5 mL of OptiMEM, as described in 2.3.1. Following the 1 h infection, cells were washed with OptiMEM and the DNA-Lipid mixture was added to the cell monolayer. After 2 hours at 37°C and 5% CO_2, an equal volume of recovery media was added to the existing transfection media, and the cells were maintained at 37°C and 5% CO_2 for another 10 hours.

2.4 Virus Generation and Manipulation

2.4.1 General Virus Infection Protocol

Typically, virus stocks were thawed at 37°C and sonicated using a Sonic Dismembrator (Misonix Inc., Farmingdale, NY) for 20 seconds at 0.5 second pulses (on/off cycle) prior to use. To infect cell monolayers in 6-well plates or 12-well plates with 18mm coverslips, 500 µl of appropriate culture media containing the appropriate virus at the indicated MOI was added. The plates were incubated at 37°C and 5% CO_2 for 1 hour with gentle rocking every 10 minutes to allow for virus attachment to cells. An additional 1 mL of culture media was then added for a total of 2 mL. For cell monolayers in 10 cm dishes, 5 mL of culture media containing virus was added to the dishes, incubated at 37°C and 5% CO_2 for 1 hour. Dishes were gently rocked every 10 minutes, followed by the addition of 5

mL of cell culture media. Infections were allowed to progress for the indicated amounts of time at 37°C and 5% CO_2.

2.4.2 Preparation of Virus Stocks

Viruses were amplified in roller bottles containing 3 x 10^8 BGMK cells by infecting at an MOI of 5 for 12-24 hours. Infected cells were harvested in saline-sodium citrate (SSC), centrifuged at 1000 x g for 5 minutes to pellet the cells, and the pellet was re-suspended in 5 mL of ice cold swelling buffer (10 mM Tris, pH 8.0, 2 mM $MgCl_2$). The infected cells were lysed by 3 freeze-thaw cycles (see Section 2.5.5). Virus was isolated by dounce homogenization on ice using a "B" pestle (Bellco Biotechnology, Gilroy, CA) to lyse the cell membranes but not the virus membranes. After 100 strokes, the homogenate was centrifuged (1000 x g, 10 min) to pellet membranes while freed virus remains in the supernatant. After collecting the supernatant, pellets were re-suspended in 10 mL swelling buffer and dounce homogenized again with 60 strokes. After centrifugation at 1000 x g, 10 minutes, supernatants were collected and centrifuged at 10,000 x g for 1 hour at 4°C to pellet the virus. Virus pellets were re-suspended in DMEM and viral titres were determined as described in Section 2.5.7.

2.4.3 Virus Titre Determination

To determine the number of plaque-forming units per mL (PFU/mL) of virus in a stock, serial dilutions were used to infect BGMK monolayers. In brief, 10 µl of the original stock was added to 990 µl of 1X PBS (phosphate-buffered saline) for a 10^{-2} dilution. One hundred µl of this dilution was serially diluted into 900 µl of 1X PBS 6 times for a series

of dilutions from 10^{-3} to 10^{-8}. Four hundred µl of each dilution was added to 6-well plates of sub-confluent BGMK monolayers in duplicate, and infections were incubated (37°C and 5% CO_2) for 12-24 hours. Infected wells were then fixed in neutral-buffered formalin (NBF) (37% (w/v) formaldehyde (Sigma-Aldrich), pH 7.4, 100 mL 10X PBS) and stained with a solution of 0.1% (w/v) crystal violet (Sigma-Aldrich) and 20% (v/v) ethanol. Single infectious virions, or PFU, are seen as visible regions of cell-clearing that can be counted and, with the known dilution of each well, can be used to calculate the original virus titre. The number of visible PFU multiplied by the reciprocal of the dilution of the well that was counted, divided by the total volume of that dilution added to the well, gives the stock concentration in PFU/mL. The average of the two duplicates was taken as the stock concentration.

To determine what volume was required for a given MOI, the following calculation was performed: the number of cells to be infected was multiplied by the desired MOI to give the total number of PFU required. This value was then divided by the stock concentration to give the volume of virus stock, which contains the desired number of PFU.

Calculation of the concentration of FWPV stocks required a separate protocol, since FWPV does not replicate in non-bird cells and does not form plaques at 24 hours post infection. To titer stocks of FWPV, the virus was serial diluted 10-fold in PBS. A 450 µl volume of the dilutions (10^{-3} to 10^{-8}) was plated onto QM5 cells in 6 well plates and rocked every 10 minutes at 37°C for 1 hour. At 1 hour post infection 2 ml of DMEM supplemented with 50U/ml of penicillin with 50 µg/ml of streptomycin, 1% (w/v) carboxymethylcellulose (CMC) (Sigma-Aldrich), and 5% FBS was added to each well.

Formation of plaques on QM5 cells required incubation for 5-7 days at 37°C. The CMC overlay has a high viscosity and prevents the formation of secondary plaques due to prevention of progeny virus diffusion in the cell culture dish (254). Upon formation of plaques as determined through visualization under a light microscope, QM5 monolayers were stained with 2ml per well of a 0.13% crystal violet, 4.75% ethanol, and 11.1% formaldehyde solution, which was directly added to the CMC containing media. The plates were washed and air-dried. Plaques were counted on wells containing 10-150 plaques. The number of plaques was used to calculate the concentration of the virus stocks.

2.4.4 Preparation of Virus Genomic DNA

To extract and prepare viral genomic DNA for PCR reactions, SDS lysis and phenol:chloroform extraction was performed. QM5 cells (1×10^6) were infected with FWPV at an MOI of 5 for 16 hours. Cell lysis buffer (1.2% SDS, 50 mM Tris-HCl (pH 8.0), 4 mM EDTA, 4 mM $CaCl_2$, 0.2 mg/mL proteinase K) was added to lyse the cells and incubated overnight (37°C and 5% CO_2). The lysate was collected in a microfuge tube and DNA was extracted in half a volume of phenol:chloroform (1:1) by vigorous vortexing. After centrifugation, the aqueous layer was collected and 50 μl of 3 M $NaCH_3CO_2$ and 2.5 volumes of 95% ethanol were added and incubated at -70°C for 10 minutes to precipitate the DNA. The DNA was pelleted by centrifugation (9000 x g, 10 minutes) and re-suspended in 50 μl ddH_2O.

2.5 Protein Methodology

2.5.1 Antibodies

All antibodies used in this study are listed in **Table 2.4**, along with the source and working concentration for each assay. Antibodies used for western blots were diluted in 1% BSA (w/v), or 5% skim milk powder (w/v) in 1% Tris-buffered saline plus Tween-20 (TBST). Alternatively, antibodies were diluted in LI-COR blocking solution (Mandel Scientific Company Inc, Guelph, ON, Canada).

2.5.2 SDS Polyacrylamide Gel Electrophoresis

Protein samples were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples were suspended in SDS sample loading buffer comprised of 6.26 mM Tris, pH 6.8, 2% SDS (Fisher Scientific), 32% glycerol (Anachemia, Norman Lachine, QC, Canada), 0.05M 2-mercaptoethanol (Bioshop, Burlington, ON, Canada), and 0.005% w/v Bromophenol blue. Prior to separation by gel electrophoresis, samples were boiled at 100°C for 15 minutes. In general, 10-30 µl of boiled protein samples were loaded on 8-12% acrylamide gels (1.5 mm) and run in a Mini-PROTEAN Cell system (BioRad) at 150-200V along with 7.5 µl of pre-stained low molecular weight protein markers (Fermentas, Burlington, ON, Canada). Gels were run in 1X SDS-PAGE running buffer (190 nM glycine, 25 mM Tris, and 3.5 mM SDS).

Antibody(#Cat. No)	Dilution/Amount	Source
Immunoprecipitation		
Mouse anti-FLAG M2 (#F3165)	2 µl	Sigma-Aldrich
Mouse anti-HSP70 (#sc-66048)	1:100	Santa Cruz
Microscopy		
Mouse anti-FLAG M2 (#F3165)	1:200	Sigma-Aldrich
Rabbit anti-FLAG (#F7425)	1:200	Sigma-Aldrich
Rabbit anti-MSH2 (#D24B5)	1:200	Cell Signaling
Mouse anti-MSH6 (#610919)	1:200	BD Transduction Laboratories
Mouse anti-HSP70 (#sc-66048)	1:200	Santa Cruz
Rabbit anti- HSP90 (#ADI-SPA-836)	1:200	Enzo
Rabbit anti-ATM (#ab32420)	1:200	Epitomics
Mouse anti-Crm1 (# sc-74454)	1:100	Santa Cruz
Mouse anti-β-tubulin (TM1541)	1:200	ECM Biosciences
Mouse anti-HA (#12CA5)	1:200	Roche
Mouse anti-conjugated ubiquitin (FK2) (#BML- PW8810)	1:200	Enzo Life Sciences
Alexa Fluor 546 Goat anti-Rabbit (#A-11010)	1:400	Life Technologies
Alexa Fluor 488 Goat anti-Mouse (#A-1101)	1:400	Life Technologies
Western Blotting		
Mouse anti-FLAG M2 (#F3165)	1:5,000	Sigma-Aldrich

Rabbit anti-FLAG (#F7425)	1:2000	Sigma-Aldrich
Mouse anti-GFP (#MMS-118P)	1:10,000	Covance
Rabbit anti-MSH2 (#D24B5)	1:1,000	Cell Signaling
Mouse anti-MSH6 (#610919)	1:1,000	BD Transduction Laboratories
Mouse anti-HSP70 (#sc-66048)	1:1,000	Santa Cruz
Rabbit anti- HSP90 (#ADI-SPA-836)	1:1,000	Enzo
Rabbit anti-ATM (#ab32420)	1:1,000	Epitomics
Mouse anti-Crm1 (# sc-74454)	1:1,000	Santa Cruz , Wozniak lab
Mouse anti-HA (#12CA5)	1:2000	Roche
Rabbit anti-VCP (#2648)	1:500	Cell Signaling
Rabbit anti-PCNA (#ab2426)	1:500	Abcam
Mouse anti-β-tubulin (TM1541)	1:1,000	ECM Biosciences
Rabbit anti-A34	1:1000	D. Evans
Rabbit anti-A55	1:5000	C. Upton
Rabbit anti-G1	1:800	SIGA Researchlabs
Mouse anti-I3	1:10,000	D. Evans
Rabbit anti-E9	1:2,000	D. Evans
Donkey anti-mouse-HRP (#715-035-150)	1:25,000	Jackson Immunoresearch
Donkey anti-rabbit-HRP (#711-035-152)	1:25,000	Jackson Immunoresearch
Goat anti-mouse-Alexa Fluor 790 (# A11375)	1:10,000	Life technologies

Goat anti-rabbit-Alexa Fluor 680 (#A21109)	1:10,000	Life technologies

EGFP – enhanced green fluorescent protein, HRP – horseradish peroxidase

Table 2.4 Antibodies used in this study

2.5.3 Semi-Dry Transfer

Proteins resolved by SDS-PAGE were transferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare), or nitrocellulose membranes (Biorad) for 2 hours at 450 mA with a semi-dry transfer apparatus (Tyler Research, Edmonton, AB, Canada). Membranes were blocked in 1% (w/v) BSA, or 5% skim milk powder (w/v) in TBST, or Odyssey blocking buffer at room temperature for at least 1 hour at room temperature.

2.5.4 Western Blotting

To detect proteins of interest, membranes were probed with primary antibodies according to the dilutions listed in **Table 2.4** overnight at 4°C. Three 15 minute washes with TBST removed any excess primary antibodies from membranes. Horseradish peroxidase conjugated secondary antibodies were added (**Table 2.4**) for 1 hour at room temperature, followed by four 15 minutes TBST washes. Proteins were visualized by enhanced chemiluminescence (ECL; GE Healthcare) according to the manufacturer's protocol. Alternatively, proteins were visualized with LI-COR. Membranes already probed with primary antibodies were probed with IR define dye-conjugated secondary antibodies for 1 hour, washed three times for 10 minutes in 1% TBST and once in 1x PBS, before bands were quantified on a LI-COR Odyssey Infrared Imager (LI-COR Biosciences, Lincoln, NE).

2.5.5 Image capture and processing

Western Blots and Commassie gels were scanned on a CanoScan 5600F Scanner (Canon, Calgary, AB, Canada), using the accompanied Image Capture Version 6.2 software

package. Adobe Photoshop CC (Adobe, Ottawa, ON, Canada) was used to align and label images from scans and LI-COR Odyssey Infrared Imager to prepare Figures.

2.5.6 Immunoprecipitation to detect ubiquitination

To detect ubiquitination of FWPV proteins, HeLa or QM5 cells (2×10^6) were infected/transfected with VACVCop or VACVΔF1L-FLAG-FPV039 at an MOI of 5, and 2μg of the following plasmids; pSC66-FLAG-p28(IHDW), pSC66-FLAG-FWPV150 or pSC66-FLAG-FWPV157. Additionally, VACV-FLAG-FWPV039 was used as a positive control for ubiquitination. Cells were lysed in RIPA buffer containing 5mM NEM define (Sigma-Aldrich), 50mM Tris-HCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150mM NaCl and EDTA-free protease inhibitors (Roche Diagnostic, Montreal, QC, Canada). FLAG-tagged proteins were immunoprecipitated and pulled down with protein G beads (GE Health Care) with mouse anti-FLAG M2 (Sigma-Aldrich). Samples were analyzed by western blotting (see Section 2.5.4) with mouse anti-FLAG (Sigma-Aldrich), mouse anti-HA, or mouse anti-ubiquitin to detect ubiquitination (Enzo Life Science, Brockville, ON, Canada). To stabilize ubiquitination, cells were treated with MG132 (10μM) for 6 hrs prior to lysis.

To determine ubiquitination of p28 mutants, HeLa cells (2×10^6) were infected with VACV-HA-Ub define at an MOI of 5 and transfected with 2μg of the following plasmids; pSC66-FLAG-p28, pSC66-FLAG-p28(1-152), pSC66-FLAG-p28(153-242), pSC66-FLAG-p28(Δ44-51), pSC66-FLAG-p28(C173S/C176S), pSC66-FLAG-p28(1-184), or pSC66-FLAG-p28(1-204). Cells were lysed in RIPA buffer and p28 was immunoprecipitated with mouse anti-FLAG M2 and western blotted with rabbit anti-

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FLAG (Sigma-Aldrich), or mouse anti-HA (Enzo Life Science) to detect ubiquitination. To further detect ubiquitination, cells were treated in the absence or presence of MG132 (10 μM) for 6 hours prior to lysis.

To determine, if HSP70 is a substrate of p28, HeLa cells (2 x 10^7) were infected with VACVCop, VACV-FLAG-p28, or VACV-FLAG-p28(C173S/C176S) and co-infected with VACV-HA-Ubiquitin at an MOI of 5. Infected cells were treated with MG132 (20 μM) for the last 4 hours of the 16 hour infection. Cells were lysed in RIPA lysis buffer containing 5mM NEM (Sigma-Aldrich) and EDTA-free protease inhibitors (Roche Diagnostic). Endogenous HSP70 was immunoprecipitated with mouse anti-HSP70. Additionally, VACV-FLAG-p28 was immunoprecipitated with mouse anti-FLAG as a positive control for ubiquitination. We used isotype antibody mouse anti-GFP as a negative control for immunoprecipitation of VACV-FLAG-p28 infected cells. Samples were western blotted with rabbit anti-HSP70, mouse anti-FLAG, or mouse anti-HA to detect ubiquitination.

2.5.7 Acetone Precipitation of Cell Lysates

Ten percent of cell lysates prior to immunoprecipitations were subjected to acetone precipitation to precipitate whole cell lysate proteins. Five volumes of ice-cold acetone (Fisher Scientific) were added to supernatants and protein precipitation was allowed to occur at -20°C for at least 1 hour. Proteins were isolated by centrifugation at 10,000 x g for 10 minutes, air-dried, and re-suspended in 100 μl of SDS sample loading buffer.

2.5.8 *In vitro* ubiquitination assay

QM5 cells (2.5 x 10^6) were infected with FWPV at a MOI of 5 and transfected with pSC66-FLAG-p28, pSC66-FLAG-p28(C173S/C176S), pSC66-FLAG-FWPV150, pSC66-FLAG-FWPV150(C196S/C199S), pSC66-FLAG-FWPV157, or pSC66-FLAG-FWPV157(C218S/C221S). Alternatively, HeLa cells (2.5 x 10^6) were infected with VACVCop at an MOI of 5 and transfected with pSC66-FLAG-p28, pSC66-FLAG-p28(1-152), pSC66-FLAG-p28(153-242), pSC66-FLAG-p28(Δ44-51), pSC66-FLAG-p28(C173S/C176S), pSC66-FLAG-p28(1-184), or pSC66-FLAG-p28(1-204). Sixteen hours post-infection, immunoprecipitations were performed using anti-FLAG M2. The immunoprecipitates were washed with 1% NP-40 lysis buffer (described above) and then additionally washed with wash buffer (50 mM Tris-HCL pH7.5, 50 mM NaCl, 1 mM EDTA pH 8.0, 0.01% NP-40, 10% glycerol) (255). The in vitro ubiquitination protocol followed manufacturer's instructions (BIOMOL International, Kelayres, PA). The ubiquitination reaction was carried out in ubiquitination buffer (0.4 mM Tris-HCL, pH 7.5), 10 U/ml inorganic pyrophosphatase solution (Sigma-Aldrich), 1mM DTT, 5 mM MG-ATP) supplemented with 50 nM E1, 1.25 μM E2 (UbcH3 or 5), and 1.25 μM Biotinylated-Ub. This was directly added to the FLAG-tagged proteins immobilized at the protein G beads. The reaction was carried out at 37°C for 90 minutes and run on a SDS-PAGE gel (Biorad). The western blots were blocked in 1%BSA/TBST overnight and blotted with Streptavidin-HRP (Pierce, Rockford, IL) 1:10,000 dilution to detect ubiquitination.

2.5.9 Infection and immunoprecipitation for Mass Spectrometry

HeLa cells (2×10^6) were infected with VACVCop, VACV-FLAG-p28, or VACV-FLAG-p28(C173S/C176S) at an MOI of 5. To stabilize ubiquitinated target proteins, cells were treated with MG132 (20μM) for the last 4 hours prior to lysis. Sixteen hours post-infection, cells were lysed in NP-40 lysis buffer and immunoprecipitations were performed using the anti-FLAG M2 mAb. Protein G beads were washed with 1% NP-40 lysis buffer. Immunoprecipitated proteins were then eluted from the beads by boiling in SDS-PAGE sample loading buffer.

For mass spectrometry follow up with immunoprecipitations and western blotting, we screened primary antibody staining, including rabbit anti-MSH2, mouse anti-MSH6, mouse anti-HSP70, rabbit anti-HSP90, rabbit anti-ATM, mouse anti-Crm1, rabbit anti-VCP, rabbit anti-PCNA, rabbit anti-A34, rabbit anti-A55, rabbit anti-G1, mouse anti-I3, rabbit anti-E9. Proteins were visualized after secondary antibody stain with donkey anti-mouse-HRP or donkey anti-rabbit-HRP by ECL, according to the manufacturer's protocol. Alternatively, proteins were visualized after IRDye-conjugated secondary antibodies for 1 hour using goat anti-mouse-Alexa Fluor, or goat anti-rabbit-Alexa Fluor 680 (see above).

2.5.10 Coomassie Staining

To assess interacting partners, or substrates of FLAG-p28, anti-FLAG immunoprecipitates were subjected to SDS-PAGE on a Hoefer electrophoresis unit SE 600 series (GE Healthcare). Proteins were visualized using a high sensitivity Coomassie staining protocol (Dr. R. Fahlman, University of Alberta, Canada). The gel was first fixed

in a 50% methanol, 2% phosphoric acid solution for 1 hour. After two 20 minute washes in milli-Q water, a destaining solution comprised of 20% methanol, 10% phosphoric acid, 1.4 mM Coomassie Brilliant Blue G-250, and 0.8 M ammonium sulfate was added to the gel and incubated overnight at 4°C. The gel was washed and stored in milli-Q water.

2.5.11 Mass Spectrometry

Whole lanes of gels from Section 2.5.9 were cut into smaller pieces and sent for identification by mass spectrometry in collaboration with Dr. Richard Fahlman and Dr. Jack Moore at the Institute for Biomolecular Design (IBD), University of Alberta. Proteins in p28 immunoprecipitates were identified with a liquid chromatography-tandem mass spectrometry (LC-MS/MS) using an LCQ Deca XP ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA). We obtained a list of identified proteins via Thermo Proteome Discoverer software (Thermo Scientific) **(Appendix Table A1 and A2)** and considered mass spectrometry hits significant when the protein had at least two peptides identified at a medium confidence according to the common Molecular and Cellular Proteomics (MCP) guidelines. We excluded mass spectrometry hits, found in the negative control of immunoprecipitates from lysates of HeLa cells infected with VACVCop.

2.6 Bioinformatics

2.6.1 Analysis of Functional Annotation Clustering

To extract biological features and meaning associated with the identified mass spectrometry hits, we used DAVID Bioinformatics Resources 6.7 (DAVID; National Institute of Allergy and Infectious Diseases [NIAID, NIH], http://david.abcc.ncifcrf.gov) for the analysis of functional annotation clustering (256).

2.6.2 Protein sequence alignments

Protein alignments for p28(IHDW), M143R, ECTV virus strain Moscow p28(ECTV), canarypox virus (CNPV197 and CNPV205), and fowlpox virus p28(FWPV150, FWPV157, FWPV075, FWPV124, FWPV155, FWPV159, FWPV161, FWPV163, FWPV236, and FWPV248) were performed via ClustralW (1.82) (http://www.ebi.ac.uk/clustalW/#) (257).

2.7 Assays

2.7.1 Reverse Transcription PCR to Detect Virus Gene Expression

RNA transcripts of FWPV150 and FWPV157 were analyzed by reverse transcription polymerase chain reaction (RT-PCR). QM5 cells (1×10^6) were infected with FWPV at an MOI 5 in the absence or presence of 80μg/ml of cytosine b-D-arabinofuranoside hydrochloride (AraC) (Sigma-Aldrich), a DNA replication inhibitor, which subsequently inhibits the expression of late genes. At 4, 12, and 24 hours post infection cells were collected, and RNA was extracted with TRIZOL according to the manufacturer's

protocol (Invitrogen). To prevent genomic DNA contamination, isolated RNA was treated with 1 U/ml DNase (Invitrogen) for 20 min at room temperature. The DNase treatment was inactivated by addition of 1µl 25mM EDTA for 10 min at 65 ºC. To exclude DNA contamination an aliquot was set aside and used as a template for control PCR. Reverse transcription was performed using 1µg of DNase treated RNA, 200U of SuperScriptII reverse transcriptase (Invitrogen), 1µl 500 mg/ml Oligo-dT (Invitrogen), 0.2mM dNTPs (Invitrogen), and 2mM DTT (Invitrogen) and incubated at 42 ºC for 50 min, followed by incubation at 70 ºC for 15 minutes. This first strand RT reaction cDNA was used as a template for PCR using the following primers. FWPV primers for FWPV150, FWPV157, FWPV075, FWPV124, FWPV155, FWPV159, FWPV161, FWPV163, FWPV236, and FWPV248 are listed in **Table 2.2**. To show the inhibition of late gene expression in the presence of AraC, primers specific to FWPV085, I5L homologue, were used (**Table 2.2**). As a control for early gene expression, we used primers specific to the early gene FWPV037. As a positive control, GAPDH was amplified using GAPDH specific primers.

2.7.2 Confocal Microscopy

HeLa or QM5 cells (5×10^5) were seeded on 18mm cover slips and infected/transfected as described in Section 2.3.2. Cells were fixed with 4% paraformalydehyde and permeabilized with 1% NP-40 (Sigma-Aldrich). Cells were stained with antibodies listed in **Table 2.4**. Coverslips were washed with PBS containing 1% FBS and mounted in 4mg/ml of N-propyl gallate (Sigma-Aldrich) in 50% glycerol containing 250 µg/ml 4', 6-diamidino-2-phenylindole (DAPI) (Invitrogen) to visualize nuclei and cytoplasmic viral

factories. Cells were examined using a Leica SP5 confocal microscope (Leica, Concord, ON, Canada) at 405 nm to detect DAPI.

In Chapter 3 and 4, we performed three independent experiments per Figure. We observed on average over 100 cells per sample, which were evaluated across the entire cover slip of the samples. We showed one representative single cells per sample in the Figures.

In Chapter 5, we performed three independent experiments and evaluated 60 cells per sample. Fiji software was used to measure the Region of interest (216) at the virus factories and whole cells. The obtained Raw Integrated Densities values were used to calculate the percentage of antibody-fluorescence labeled proteins at the virus factory in presence or absence of functional p28. The Department of Mathematical and Statistical Sciences, Training Consulting Centre (TCC) was consulted for the statistical analysis. Statistical analysis of data was performed using GraphPad Prism version 7. One-way Analysis of variance (ANOVA) tests with post-Tukey column comparison were used to compare calculate statistical significance. (GraphPad Software, La Jolla, CA) $P<0.05$ was considered significant and statistically significant results were reported as: * $p<0.05$, **$p<0.01$, and *** $p<0.001$. See also Section 2.7.5 Statistical analysis.

2.7.3 Flow cytometry

HeLa cells were transfected with 0.5μg of pEGFP-C3 or 2μg of pEGFP-p28(IHDW), pEGFP-p28(ECTV), pEGFP-p28(1-152), pEGFP-p28(153-242), pEGFP-p28(Δ44-51), pEGFP-p28(1-184), pEGFP-p28(1-204), pEGFP-p28(C173S/C176S) with Lipofectamine according to the manufacturers instructions (Invitrogen).   Sixteen hours post-transfection

cells were treated with or without the proteasome inhibitor MG132 (10μM) (Sigma-Aldrich) for 6 hours. Cells were labelled with 0.2μM tetramethylrhodamine ethyl ester (TMRE) (Invitrogen) to label healthy cells, washed and resuspended in PBS containing 1% FBS and analyzed by two-color flow cytometry (Becton Dickinson, Franklin Lakes, NJ) (36). TMRE fluorescence was measured through the FL-2 channel equipped with a 585-nm filter (42nm band pass) and EGFP fluorescence was measured through the FL-1 channel equipped with a 489nm filter (42nm band pass). The fluorescence signals were acquired for 20,000 cells per sample at logarithmic gain and analysis was performed using CellQuest software (Bector Dickinson).

2.7.4 Proteasome assay

HeLa cells (1×10^4) were mock-infected or infected for 14 hours with VACVCop, CPXV, or ECTV at an MOI of 10. QM5 cells (1×10^4) were mock-infected or infected for 14 hours with FWPV at an MOI of 10. Cells were left untreated or treated with 10μM MG132 at 12 hours post infection 6 hours prior to lysis (Sigma-Aldrich). The chymotrypsin-like activity of the proteasome was assessed using the cell-based Proteasome-Glo assay, according to the manufacturer's directions (Promega). Luminescence was recorded 10 min after the addition of the Proteasome-Glo reagent using an EnVision 2104 multilabel reader (Perkin-Elmer, Waltham, MA). The assay was performed in triplicate, and average relative light units (RLU) were presented. The measurements of relative light units from the proteasome assay were subjected to analysis using two-way ANOVA to explore statistical relationships between MG132 treatment

and between mock and virus infection. The level of statistical significance was set at p<0.001. See also Section 2.7.5 Statistical analysis.

2.7.5 Statistical analysis

Statistical analysis for confocal samples was performed as described in Section 2.7.2. In brief, a total of 60 cells per sample were collected from three independent experiments and the percentage of antibody-fluorescence labeled proteins at the virus factory in presence or absence of functional p28 was calculated. The Department of Mathematical and Statistical Sciences, Training Consulting Centre (TCC) was consulted for the statistical analysis. Statistical analysis of data was performed using GraphPad Prism version 6. One-way Analysis of variance (ANOVA) tests with post-Tukey column comparison were used, to test one dependent variable across different groups to calculate statistical significance. $p<0.05$ was considered significant and statistically significant results were reported as: * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

Statistical analysis for the Proteasome assay was performed as described in Section 2.7.4. Statistical analysis of the results was computed using Prism-graph. The measurements of relative light units from the proteasome assay were subjected to analysis using two-way ANOVA with post-Tukey column comparison since statistical relationships were tested between two factors, MG132 treatment and between mock and virus infection. The level of statistical significance was reported as $p<0.001$.

CHAPTER 3: FOWLPOX VIRUS ENCODES TWO P28-LIKE UBIQUITIN

LIGASES THAT ARE EXPRESSED EARLY AND LATE

A version of the data presented in this chapter has been published:

Bareiss B. and Barry M. (2014) Fowlpox virus encodes two p28-like ubiquitin ligases that are expressed early and late during infection. *Virology*.462-463:60-70.

All of the experiments presented in this chapter were performed by myself. The original manuscript was written by myself with editorial contributions from Robyn-Lee Burton, Dr. Thibault, Dr. Noyce, and Dr. Barry.

3.1 Introduction

p28 is a poxvirus encoded ubiquitin ligase, which acts as a virulence factor during ECTV infection (37, 242, 243, 245). p28 is found in VACV-IHDW and ECTV, while p28-homologues are expressed in a wide range of poxviruses, including MYXV, VARV, shope fibroma virus, and CPXV (**Table 1.3**). Interestingly, Avipoxviruses, such as CNPV and FWPV, contain two p28-like ubiquitin ligases, an observation not seen in other members of the poxvirus family (22, 23). Both p28 homologues in FWPV, FWPV150 and FWPV157 contain a putative KilA-N DNA binding domain, important for localization to the virus factory, and a putative RING domain, crucial for the ubiquitin ligase activity (22). Additionally, FWPV encodes eight KilA-N-only proteins, which are classified as p28-like genes, but are lacking the RING domain (22).

The work in this chapter focuses mainly on characterizing the two p28-like ubiquitin ligases, FWPV150 and FWPV157, encoded by FWPV. The data presented here shows that both FWPV150 and FWPV157 are functional ubiquitin ligases. Furthermore, both co-localize with conjugated ubiquitin at the virus factory and are ubiquitinated during poxvirus infection. Our data indicate that FWPV150 and FWPV157 are actively transcribed during FWPV infection. Intriguingly, FWPV150 was transcribed early on during infection, while FWPV157 was expressed late. Furthermore, all eight KilA-N only genes were actively transcribed early during infection. The presence of numerous KilA-N only proteins and FWPV-encoded ubiquitin ligases indicates their importance during FWPV infection.

3.2 Results

3.2.1 FWPV and CNPV encode p28-like ubiquitin ligases

Poxviruses promote infection by expressing a variety of effector proteins (20, 30). For example, ECTV encodes the virulence factor p28 that functions as a ubiquitin ligase (37, 245). Homologues of p28 are encoded by a wide range of poxviruses including ECTV, IHDW and CPXV, each encoding a single homologue. In contrast, members of the *Avipoxvirus* family, such as FWPV and CNPV, encode two homologues of p28-like ubiquitin ligases that have not been thoroughly investigated (22, 23). Compared to p28(IHDW) and p28(ECTV), the sequence identity/similarity of FWPV150, FWPV157, CNPV197, and CNPV205 demonstrate 23%, 43%, 43%, and 42% sequence identity, and 40%, 62%, 54%, and 56% sequence similarity, respectively. The sequence identity between FWPV150 and FWPV157 is 35%, whereas the sequence identity between CNPV197 and CNPV205 is 36%. Significantly, the cysteine and histidine residues within the RING domain, which were previously shown to be critical for ubiquitin ligase activity (242), are highly conserved among the four *Avipoxvirus* proteins and are similar to IHDW and ECTV **(Figure 3.1)**. Additionally, residues 44 to 50 within the KilA-N domain, which are crucial for DNA binding, were similar among the proteins. Therefore we sought to investigate if both ubiquitin ligases, FWPV150 and FWPV157, functioned during infection.

```
p28(IHDW)  : -MEFDPAK NTSS-----------IDH TILQYIDEPN ERI T CIIRNINNITY FIN TK NTHLANC RA KKRI :  65
p28(ECTV)  : -MEFDPAK NTSS-----------IDH TILQYIDEPN IRI P CIIRNINNITY FIN TK NPDLANC RA KKRI :  65
FWPV150    : -------- SHLH----------- NNGDTEYRVIEDNGFS IL LKHTE------ YIN VTK CKIHNKE FY WKRLI :  52
CNPV197    : -------- SCII-----------PPH DTKYKVLVDG CH MFMIRNG------ YIN VTKMCKINNKE FY WKRLV :  52
FWPV157    : MKEDDSSN NNIHGKYSVSDLSQDDYV EC DGSFDSIKYR IKV IL MKNNG------ YVN SKI CKMRNKY SS RA LRLS :  74
CNPV205    : -MEHEYNGNGTLV----------- KS NEFFNYVEYN KN T MRVNG------ YVN CSKI CKSVNKY S SR WKRLS :  59
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```
p28(IHDW)  : AGRDY TNLSRDTGIQQSKLTETIRNCQKN N- Y G YIHY VINV IDW TDVIVQSI RGLVN W IAN--------- : 135
p28(ECTV)  : AGRDY TNLSRDTGIQQSKLTETIRNCQKN N- Y G YIHY VINV IDW TDVIVQSI RGLVN W IAN--------- : 135
FWPV150    : SAGRI ETVSRDISNQGFESPLVYNRKGN E-FY G YAHPQ ALY AKW SE-DIFNK KHL S TISDKTVVIKDF : 129
CNPV197    : NSSKI EDIEKKYALPES-SSLIYIERKNN D- Y G YAHP IAIE AKW SQ-DVVNS VKK VSI N---EGVNMNLV : 125
FWPV157    : TSKAL DI----YNNKSVDNAIVKVYGKGK LI TG YLKQ MIRY IEW GD-DFTND IYKMI FY NALFGNDELKIV : 148
CNPV205    : RAKAI EI----KKVQNGDDVIIRVHGKGTMNS I G YLRQ IVQY VSEW GDNNFVEEVT II N NSFHSGKKFKPL : 134
```

KilA-N domain

```
p28(IHDW)  : ------- TYT ------------NTPNNTTTISE DI K NKYEDVYR SKEKE------------------- : 172
p28(ECTV)  : ------- TYN ------------NTPNSTTTISE DI K DKYEDVYR SKEKE------------------- : 172
FWPV150    : SYC---- ELC DAIIGKCCKTKSSCEYVHGDICD CGFEA HPTDIDKR THE KVCMQ------------LLCKEDIKY : 193
CNPV197    : CVE---- EEL YDVLGECEKGE-YCEYVHGDICD CG RA HPNDLEQ RNHE ICIKFTLRTSGLFTRNNILDKKIDN : 200
FWPV157    : SCE---- TLC FIELGRCYYGK-KCKYIHGDQCD CG YI HPTDINQR VSHR KTCLV---------DRDSLIVFKRST : 214
CNPV205    : VIERPQD VLC FLKLGRCYYGK-KCKYIHGNKCD CD YI HPTDYIQR NHR NCTE--------TRKLLPPFIRSI : 204
```

```
                          *  *              *   *  *   *              *  *
p28(IHDW)  : ------ CG CY V YS R E DRYFGLLDS THIFC TC NI HK RRE GASD C IC RTRFRN TM KFY KLVN---- : 242
p28(ECTV)  : ------ CG CY V YS R E DRYFGLLDS THIFC TC NI HR RRE GASD C IC RTRFRN TM KFY KLVN---- : 242
FWPV150    : DK---- CG C DA KG----KKP YGILS DQNHMFC NC KTWMT --INSKKQ CE CR VPSKY IQS PI TVDKVSKN : 262
CNPV197    : DKRIHI CS CL DTRRK P -NDRV YGILS KQNHIFC KC NP MK ---STARDRC E CR QVSKN IIPS YIRIDEKKDN- : 275
FWPV157    : SKK--- CG C B NK K -SEQYFGILP SQNHIFC SC HRP AD TRN DTENTC E CR IVFP IPS RY IDNKYDKK : 290
CNPV205    : NKT--- CS VC L R YEKE I -NKQ FGIL PNQKHVFC FYC QRWMSIIKG NTEGTC E VCRTVSVF VPNRY IDDKYEKR : 280
```

RING domain

```
p28(IHDW)  : ---------------------------------------- :  -
p28(ECTV)  : ---------------------------------------- :  -
FWPV150    : QLSVSYKT--------------------VYIKSC : 276
CNPV197    : ---------------------------------------- :  -
FWPV157    : ILYNRYKK--------------MIFTKIPIRTIKI-- : 311
CNPV205    : LIINKYKKDRKTYKAFKHYIGRYVLFYTVNNSLFVTND : 318
```

Figure 3.1 Alignment of p28 homologues in FWPV and CNPV demonstrates conservation of the KilA-N and RING domains.

Protein alignments for p28(IHDW), p28(ECTV), FWPV150, FWPV157, CNPV197 and CNPV205 were performed via Clustral W (1.82) (http://www.ebi.ac.uk/clustalW/#) (8). The KilA-N domain is underlined in black and the RING domain is underlined in grey. Asterisks highlight the conserved cysteines and histidines within the RING domain. The thick black line marks the motif in p28, which is important for DNA binding (44-50). Conserved amino-acid residues were shaded using GeneDoc (258, 259). The Genedoc shading mode was set to conservation, with shading level 4 and the conserved percent was primary 100% (black), secondary 80% (dark grey), and tertiary 60% (light grey). The shade style was set to amino acid identity.

3.2.2 FWPV150 and FWPV157 localize to viral factories

Previous studies have demonstrated that p28(IHDW) localizes to viral factories (243). Given the conservation of key residues among p28(IHDW), FWPV150 and FWPV157, we sought to investigate whether FWPV150 and FWPV157 localize to virus factories as well. We used infection accompanied by transfection to investigate the cellular localization of proteins during infection. HeLa cells were mock-infected **(Figure 3.2; panel a-d)**, infected with VACVCop **(Figure 3.2; panel e-h)**, or infected with VACVCop and transfected with pSC66- FLAG-FWPV150 **(Figure 3.2; panel i-l)** or pSC66-FLAG-FWPV157 **(Figure 3.2; panel m-p)**. DAPI was used to visualize both the nucleus and cytoplasmic virus factories and I3L, a ssDNA binding protein, which localizes to the virus factory, was used as a marker for virus factories. Staining for FLAG visualized FWPV150 and FWPV157. Using this approach, we observed that both FWPV150 and FWPV157 localized to virus factories during VACVCop infection. Given that p28(IHDW) co-localizes with conjugated ubiquitin (243), we determined if the same was true for FWPV150 and FWPV157. We included mutant versions of both FWPV150 and FWPV157 containing mutations of two cysteine residues in the RING domain, FWPV150(C196S/C199S) and FWPV157(C218S/C221S) **(Figure 3.3; panel q-t and panel y-bb)**. Cells were stained with DAPI and co-stained with anti-FLAG to visualize FLAG-FWPV150, FLAG-FWPV157, FLAG-FWPV150(C196S/C199S), FLAG-FWPV157(C218S/C221S) and co-stained with an antibody specific for conjugated ubiquitin (122). As expected, infection with VACVCop resulted in a lack of conjugated ubiquitin accumulating at the virus factory **(Figure 3.3; panel f)**. In contrast, conjugated ubiquitin was concentrated at the viral factory and co-localized in p28(IHDW),
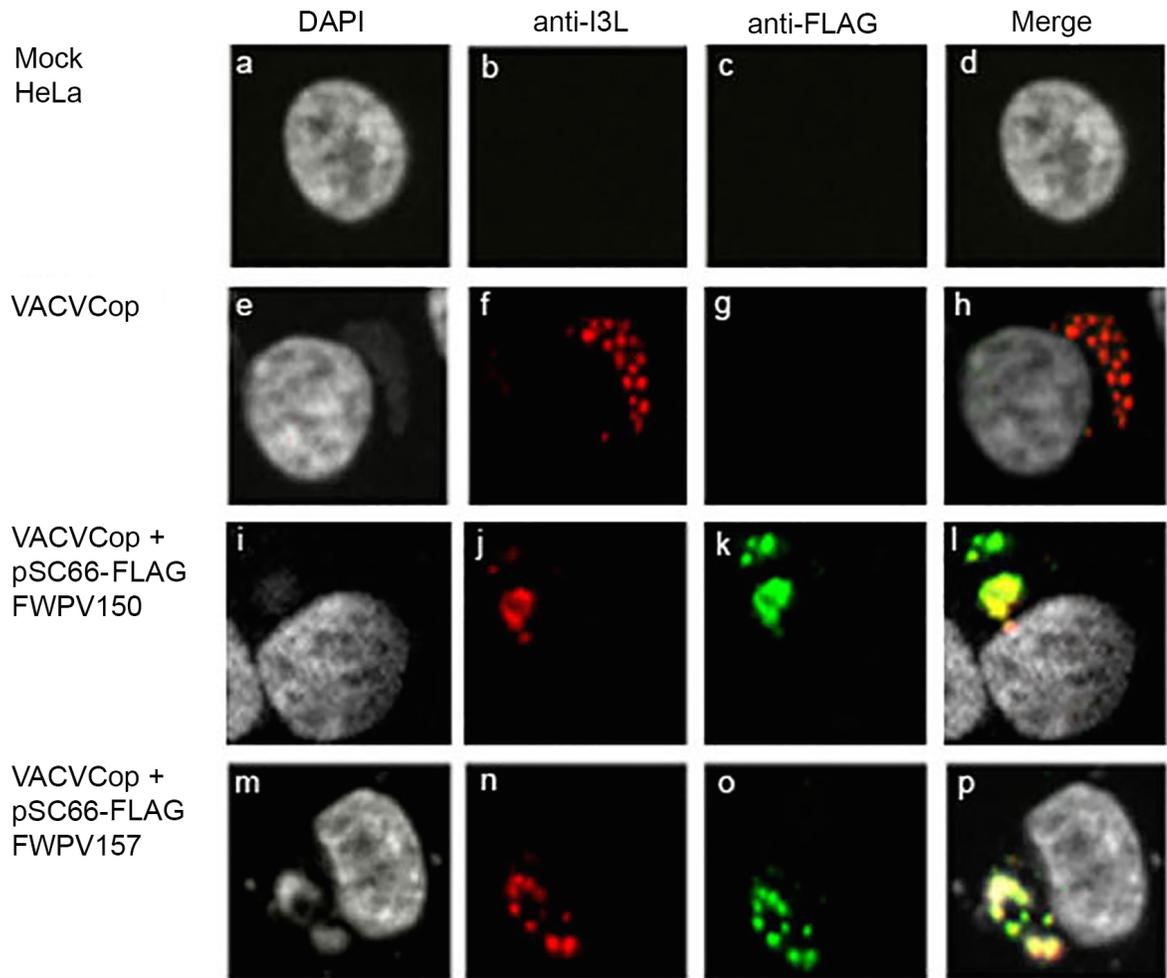
Figure 3.2 FWPV150 and FWPV157 localize to viral factories.

(A) HeLa cells were mock-infected or infected with VACVCop at an MOI of 5 and transfected with pSC66-FLAG-FWPV150 or pSC66-FLAG-FWPV157 for fourteen hours. Cells were fixed and stained for DAPI to visualize nuclei and virus factories. Anti-FLAG was used to visualize FWPV150 and FWPV157, and anti-I3L to visualized the virus factories. Co-localization of both FLAG-tagged proteins with I3L was visualized by confocal microscopy. This experiment was performed three independent times and we observed on average over 100 cells per sample, which were evaluated across the entire cover slips of the samples. We showed one representative cells per sample in this Figure.

	DAPI	anti-FK2	anti-FLAG	Merge

Mock
HeLa

VACVCop

VACVCop +
pSC66-FLAG
p28

VACVCop +
pSC66-FLAG
FWPV150

VACVCop +
pSC66-FLAG
FWPV150
(C196S/C199S)

VACVCop +
pSC66-FLAG
FWPV157

VACVCop +
pSC66-FLAG
FWPV
(C218S/C221S)
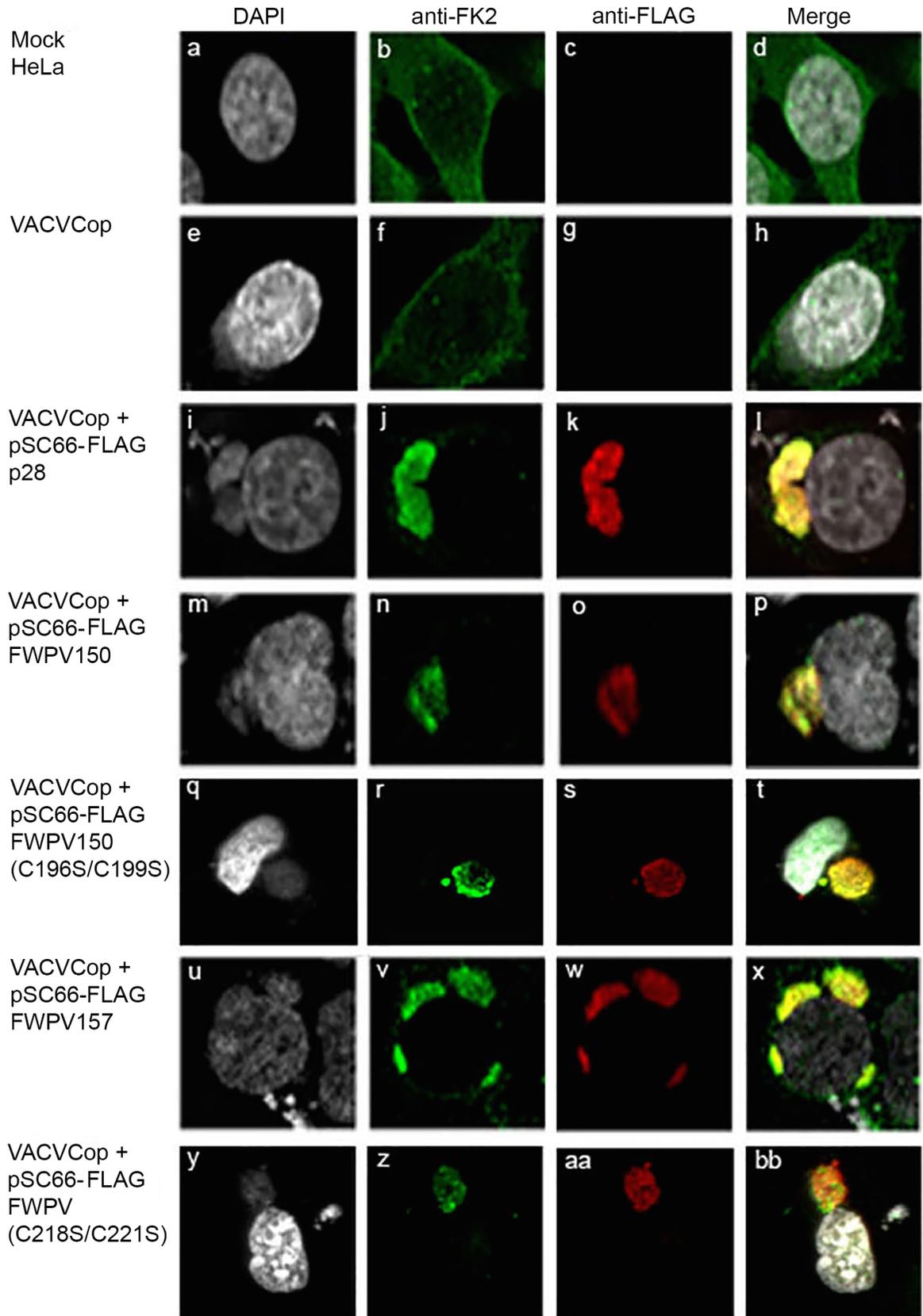
Figure 3.3 FWPV150 and FWPV157 co-localize with conjugated ubiquitin to viral factories.

HeLa cells were mock-infected or infected with VACVCop at an MOI 5 and transfected with pSC66-FLAG-p28, pSC66-FLAG-FWPV150, pSC66-FLAG-FWPV150(C196S/C199S), pSC66-FLAG-FWPV157, or pSC66-FLAG-FWPV157(C218S/C221S). Cells were fixed and treated with DAPI, to visualize the nucleus and virus factories, and stained with anti-FLAG to visualize FLAG-tagged proteins and anti-FK2 to recognize conjugated ubiquitin. Co-localization was visualized by confocal microscopy. This experiment was performed three independent times and we observed on average over 100 cells per sample, which were evaluated across the entire cover slips of the samples. We showed one representative cells per sample in this Figure.

FWPV150 and FWPV157-expressing cells **(Figure 3.3; panel i-l)**. In a similar manner, FWPV150(C196S/C199S), and FWPV157(C218S/C221S) also co-localized with conjugated ubiquitin at the virus factories upon disruption of the RING domain function **(Figure 3.3; panel t and bb)**. Taken together, these data indicate that the accumulation of conjugated ubiquitin at the virus factory occurs in cells expressing 28(IHDW), FWPV150 and FWPV157 and that disruption of the RING domains of FWPV150 and FWPV157 does not prevent conjugated ubiquitin from accumulating at the factory.

Our previous experiments were conducted in HeLa cells during VACVCop infection, we next wanted to determine if conjugated ubiquitin was also found at the virus factory during FWPV infection. We used QM5 quail cells as a source of a bird cell line, since FWPV cannot fully replicate in mammalian cells and failed to produce progeny viruses; FWPV only replicated in bird cells due to host tropism (260). QM5 cells were mock-infected **(Figure 3.4; panel a-d)**, infected with FWPV **(Figure 3.4; panel e-h)**, or infected with FWPV and transfected with pSC66-FLAG-FWPV150 **(Figure 3.4; panel i-l)**, pSC66-FLAG-FWPV150(C196S/C199S) **(Figure 3.4; panel m-p)**, pSC66-FLAG-FWPVFLAG157 **(Figure 3.4; panel q-t)** or pSC66-FWPV-FLAG-157(C218S/C221S) **(Figure 3.4; panel u-x)**. Cells were co-stained with anti-FLAG to visualize FWPV150 **(Figure 3.4; panel k)**, FWPV157 **(Figure 3.4; panel s)**, FWPV150(C196S/C199S) **(Figure 3.4; panel o)** or FWPV(C218S/C221S) **(Figure 3.4; panel w)** and with FK2 to detect conjugated ubiquitin **(Figure 3.4; panel b, f, j, n, r, v)**. Infection with wildtype FWPV resulted in a modest accumulation of conjugated ubiquitin at viral factories **(Figure 3.4; panel f)**. FWPV150 and FWPV157 co-localized with conjugated ubiquitin

	DAPI	anti-FK2	anti-FLAG	Merge
Mock QM5	a	b	c	d
FWPV	e	f	g	h
FWPV + pSC66-FLAG FWPV150	i	j	k	l
FWPV + pSC66-FLAG FWPV150 (C196S/C199S)	m	n	o	p
FWPV + pSC66-FLAG FWPV157	q	r	s	t
FWPV + pSC66-FLAG FWPV157 (C218S/C221S)	u	v	w	x
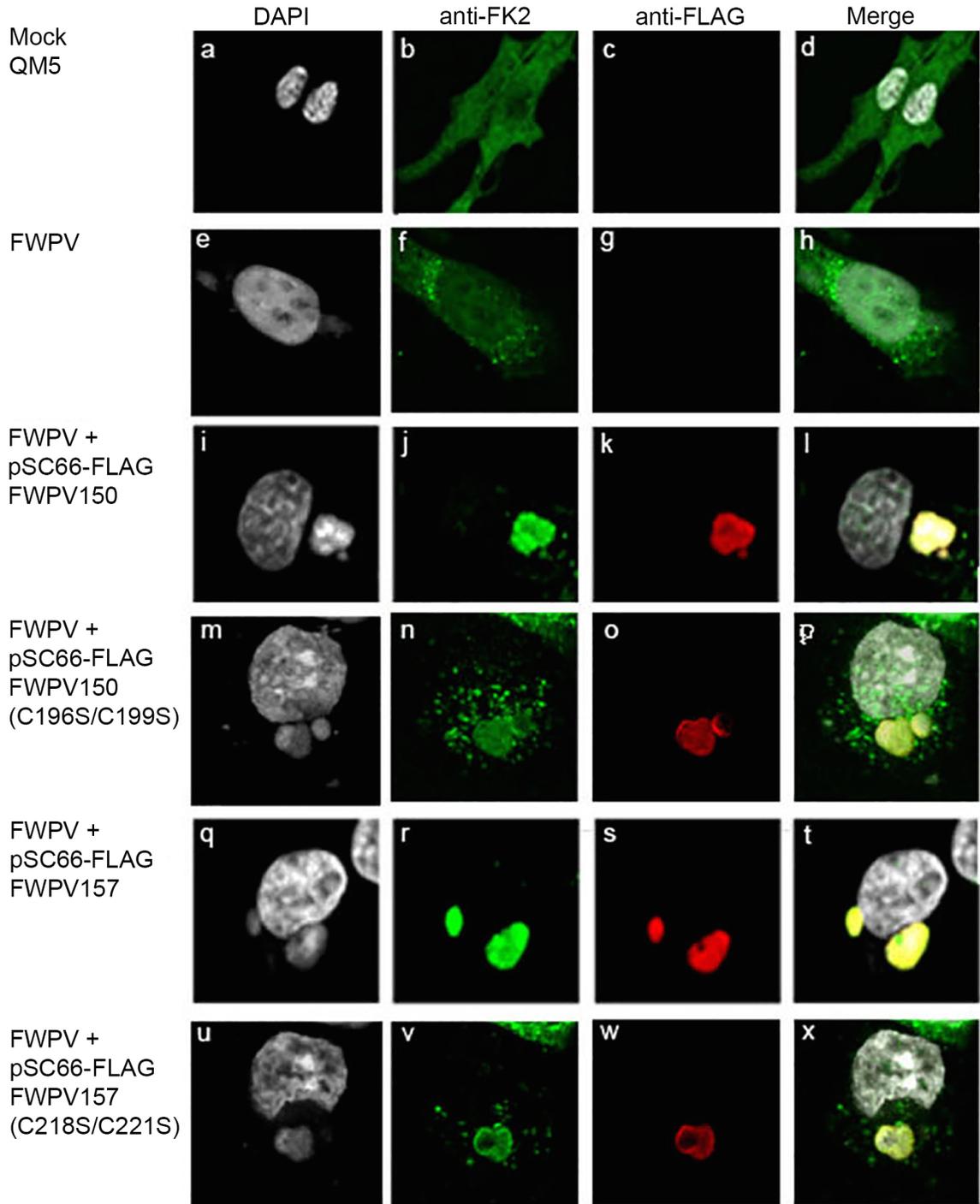
Figure 3.4 FWPV150 and FWPV157 localize with conjugated ubiquitin at virus factories in quail cells.

QM5 cells were mock-infected or infected with FWPV at an MOI of 5 and transfected with pSC66-FLAG-FWPV150, pSC66-FLAG150(C196S/C199S), pSC66-FLAG-FWPV157, or pSC66-FLAG-FWPV157(C218S/C221S). Cells were fixed and treated with DAPI, to visualize the nucleus and virus factories, and stained anti-FLAG to visualize FLAG-tagged proteins and anti-FK2 to recognize conjugated ubiquitin. Co-localization was visualized by confocal microscopy. This experiment was performed three independent times and we observed on average over 100 cells per sample, which were evaluated across the entire cover slips of the samples. We showed one representative cells per sample in this Figure.

at the viral factories **(Figure 3.4; panel l and t)**. Despite the lack of key residues within the RING domain, both FWPV150(C196S/C199S) and FWPV157(C218S/C221S) still accumulated conjugated ubiquitin at the virus factory; however not to the same degree that FWPV150 or FWPV157 were able to **(Figure 3.4; panel l, p, t, and x)**. Both RING domain mutants demonstrated some punctate staining of conjugated ubiquitin in the cytoplasm, which did not co-localize at the virus factories **(Figure 3.4; panel p and x)**. We next investigated, whether FWPV150 or FWPV157 are regulated by ubiquitination, and if they indeed act as ubiquitin ligases.

3.2.3 FWPV150 and FWPV157 are ubiquitinated during infection and act as ubiquitin ligases

Many cellular ubiquitin ligases are regulated by ubiquitination and subsequent proteasomal degradation (139). We next investigated whether FWPV150 and FWP157 were regulated by the ubiquitin-proteasome pathway. HeLa cells were infected with VACV-HA-Ubiquitin at a MOI of 5 and transfected with pSC66-FLAG-p28, pSC66-FLAG-FWPV150 or pSC66-FLAG-FWPV157. As a further control for a ubiquitinated protein, cells were also infected with VACV-FLAG-FWPV039, an anti-apoptotic protein that is regulated by ubiquitination (84) **(Figure 3.5A)**. Cells were lysed in the presence of NEM, thus preventing the removal of ubiquitin chains. FLAG-tagged proteins were immunoprecipitated with mouse anti-FLAG M2 and ubiquitination of the proteins was detected by western blot. Western blots showed high molecular bands for FLAG-p28, FLAG-FWPV150, FLAG-FWPV157 and FLAG-FWPV039, when stained with anti-
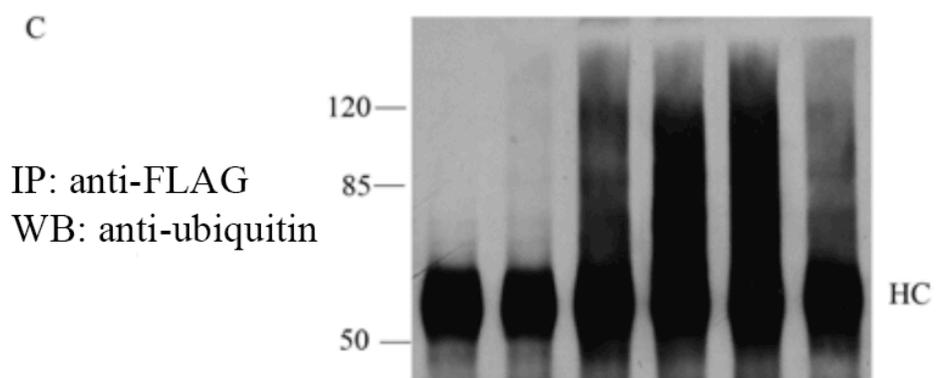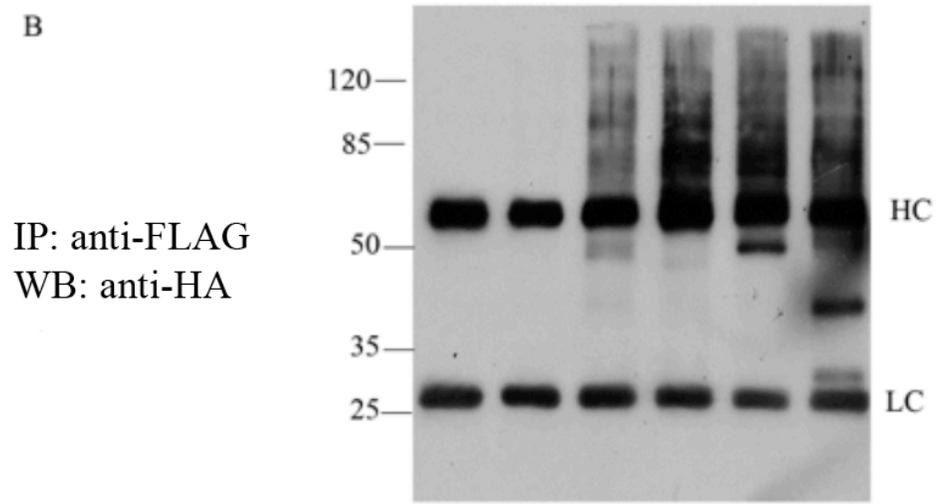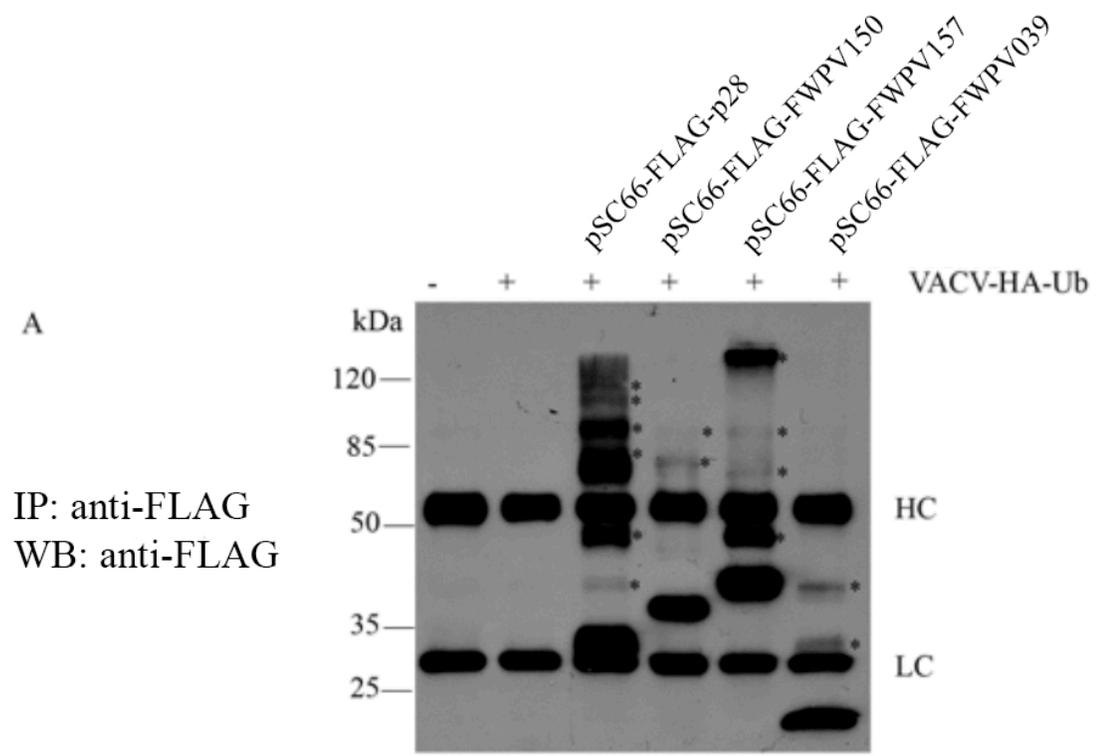
Figure 3.5 FWPV150 and FWPV157 are regulated by ubiquitination.

(A) To detect ubiquitination HeLa cells (2 x 10^6) were mock-infected or infected with recombinant virus VACV-HA-Ub, expressing HA-tagged ubiquitin, at an MOI of 5 and transfected with pSC66-FLAG-p28 (FLAG-p28 predicted molecular weight: ~ 30 kDa), pSC66-FLAG-FWPV150 (FLAG-FWPV150 predicted molecular weight: ~ 35 kDa), pSC66-FLAG-FWPV157 (FLAG-FWPV157 predicted molecular weight: ~ 39 kDa), or infected with VV-FLAG-FWPV039 (FLAG-FWPV039 predicted molecular weight: ~ 22 kDa), an anti-apoptotic protein that serves as a control ubiquinated protein (195). To stabilize ubiquitination, cells were treated with MG132 (10µM) for 6 hours. Cells were lysed in RIPA buffer, containing 5mM NEM, and FLAG-tagged proteins were immunoprecipitated with mouse anti-FLAG M2 and western blotting with mouse anti-FLAG to detect ubiquitination. To further visualize ubiquitination, the FLAG-tagged proteins were immunoprecipitated with mouse anti-FLAG M2 and **(B)** western blotted with mouse anti-HA, or **(C)** were western blotted with mouse anti-ubiquitin. * indicates higher molecular weight proteins in **(A).** HC stands for heavy chain and LC for light chain.
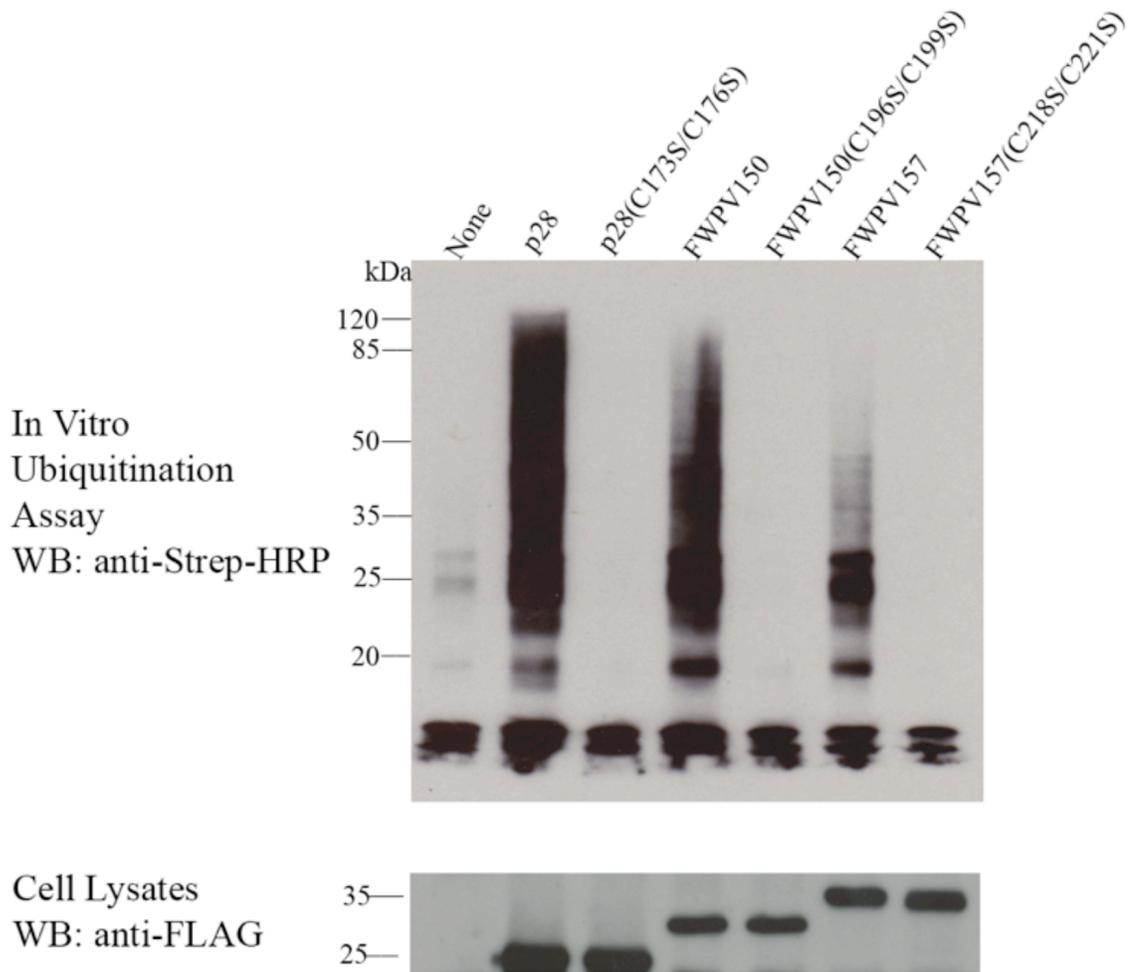
Figure 3.6 Ubiquitin ligase activity of p28, FWPV150, and FWPV157.

Protein G beads containing FLAG tagged proteins were combined in an *in vitro* ubiquitination reaction with E1, E2, ATP, and biotin-labeled ubiquitin. Reactions were separated by SDS-PAGE and were probed with streptavidin-tagged HRP. Western blotting shows the appearance of high-molecular mass ubiquitin adducts in reactions containing either p28, FWPV150, or FWPV157. Reactions with no input, or containing the double mutants of p28(C173S/C176S), FWPV150(C196S/C199S), or FWPV157(C218S/C221S) demonstrated no ubiquitination smear.

FLAG **(Figure 3.5A)**, anti-HA **(Figure 3.5B)**, or anti-ubiquitin **(Figure 3.5C)**. Overall, these data indicate that FWPV150 and FWPV157 are ubiquitinated. We performed an *in vitro* ubiquitination assay to show that FWPV150 and FWPV157 act as functional ubiquitin ligases and to further demonstrate the catalytic loss of ubiquitin ligase activity in the double cysteine mutants FWPV150(C196S/C199S) and FWPV157(C218S/C221S) **(Figure 3.6)**. Cells were infected with FWPV, transfected with pSC66-FLAG-p28, pSC66-FLAG-p28(C173S/C176S), pSC66-FLAG-FWPV150, pSC66-FLAG-FWPV150(C196S/C199S), pSC66-FLAG-FWPV157, or pSC66-FLAG-FWPV157(C218S/C221S), and were subjected to immunoprecipitation using an anti-FLAG antibody. The resulting immune complexes were immobilized on Protein G beads, and used as the source of ubiquitin ligase for an *in vitro* ubiquitination assay. The formation of ubiquitin conjugates was detected by western blotting for biotinylated ubiquitin adducts. Cell lysates stained with rabbit anti-FLAG showed the presence of all FLAG immunoprecipitated proteins **(Figure 3.6)**. We previously demonstrated the ubiquitin ligase activity of p28 in an *in vitro* ubiquitination assay; at the same time we showed that the double cysteine mutant p28(C173S/C176S) lost its ubiquitin ligase activity (243). Therefore, we used p28 as a positive control and p28(C173S/C176S) as a negative control to investigate the ubiquitin ligase activity of FWPV150, FWPV150(C196S/C199S), FWPV157 and FWPV157(C218S/C221S) in the *in vitro* ubiquitination assay. Immune complexes precipitated from infected/transfected cells expressing FLAG-p28, FLAG-FWPV150 and FLAG-FWPV157 showed the presence of both free ubiquitin, as well as high molecular weight ubiquitin conjugates **(Figure 3.6)**. These high molecular weight ubiquitin conjugates were absent in the double cysteine

mutants p28(C173S/C176S), FWPV150(C196S/C199S) and FWPV157(C218S/C221S). This confirms that FWPV150 and FWPV157 act as ubiquitin ligases and that the double cysteine mutation abolished their ubiquitin ligase activity.

3.2.4 K48-linked ubiquitin localizes to viral factories in association with FWPV150 and FWPV157

Lysine-48 (K48) polyubiquitin chains are associated with targeting proteins for proteasomal degradation (261). Therefore, to determine if K48 ubiquitin linkages were present at the viral factories we used an HA-Ub-K48 construct under the control of a T7 promoter, lacking all six lysine residues except for lysine 48, allowing only K48-linked ubiquitin chains to be formed (121, 138). HeLa cells were infected with VACV-T7, which expresses the T7 polymerase under the control of an IPTG inducible promoter, and co-transfected with HA-Ub or HA-K48-Ub along with pSC66-FLAG-FWPV150, pSC66-FLAG-FWPV150(C196S/C199S), pSC66-FLAG-FWPV157, or pSC66-FLAG-FWPV157(C218S/C221S) **(Figure 3.7)**. DAPI was used to stain the cells in order to visualize the viral factories, and anti-HA was used to detect ubiquitin. Upon infection and transfection, both HA-Ub and HA-K48-Ub were found diffuse throughout the cytoplasm in cells not expressing FWPV proteins **(Figure 3.7; panels b and f)**. However, in the presence of the FWPV proteins, both HA-Ub and HA-K48-Ub co-localized at the virus factory **(Figure 3.7; panels l, p, t, x, bb, ff, jj, and nn)**. These data indicate that FWPV150, FWPV157, and the double-mutants promote the enrichment of K48-ubiquitin at the virus factories.

	DAPI	anti-HA	anti-FLAG	Merge
VACV-T7 + HA-Ub	a	b	c	d
VACV-T7 + HA-K48-Ub	e	f	g	h
VACV-T7 + HA-Ub + pSC66-FLAG-FWPV150	i	j	k	l
VACV-T7 + HA-K48-Ub + pSC66-FLAG-FWPV150	m	n	o	p
VACV-T7 + HA-Ub + pSC66-FLAG-FWPV150 (C196S/C199S)	q	r	s	t
VACV-T7 + HA-K48-Ub + pSC66-FLAG-FWPV150 (C196S/C199S)	u	v	w	x
VACV-T7 + HA-Ub + pSC66-FLAG-FWPV157	y	z	aa	bb
VACV-T7 + HA-K48-Ub + pSC66-FLAG-FWPV157	cc	dd	ee	ff
VACV-T7 + HA-Ub + pSC66-FLAG-FWPV157 (C218S/C221S)	gg	hh	ii	jj
VACV-T7 + HA-K48-Ub + pSC66-FLAG-FWPV157 (C218S/C221S)	kk	ll	mm	nn

101

Figure 3.7 FWPV150, FWPV157 and double-cysteine mutants co-localize with HA-Ub and HA-K48-Ub.

HeLa cells were infected with VACV-T7 at an MOI of 5 and transfected with either pBluescript-HA-Ub, pBluescript-HA-K48-Ub, and either with pSC66-FLAG-FWPV150, pSC66-FLAG-FWPV150(C196S/C199S), pSC66-FLAG-FWPV157, or pSC66-FLAG-FWPV157(C218S/C221S). Cells were subsequently treated with 10 mM IPTG for 16 hours, fixed and stained with DAPI to visualize the nucleus and virus factories, anti-HA to visualize ubiquitin and K48-ubiquitin, and anti-FLAG to visualize FLAG-tagged proteins. This experiment was performed three independent times and we observed on average over 100 cells per sample, which were evaluated across the entire cover slips of the samples. We showed one representative cells per sample in this Figure.

K48-ubiquitin chains result in degradation of target proteins, suggesting that potential substrates for FWPV150 and FWPV157 could be located at the virus factories. However, the co-localization of conjugated ubiquitin with the double cysteine mutants of FWPV150 and FWPV157 rather indicate a possibility that the FWPV proteins were ubiquitinated by another ubiquitin ligase. These possibilities will be further argued in the discussion below.

2.3.5 FWPV150 and FWPV157 are expressed at different times during infection

The genome of FWPV encodes two ubiquitin ligases, FWPV150 and FWPV157, therefore we sought to determine if both genes are actively transcribed during infection by examining mRNA levels using RT-PCR **(Figure 3.8)**. QM5 cells were infected with FWPV at an MOI of 5, and RNA was extracted at 4, 12 and 24 hours post-infection and subjected to RT-PCR using primers specific for each FWPV gene. As a control, we used primers specific for an early gene, FWPV037, which encodes a protein that resides at the endoplasmic reticulum and nuclear membrane (Sing-Chi Lam and M. Barry, personal communication), and a late gene, FWPV085, the homologue of VACV I5L (235). FWPV037 transcripts were detected at 4, 12 and 24 hours post-infection. In the presence of AraC transcripts were still detected for early gene FWPV037 **(Figure 3.8; panel a)**. Transcripts of FWPV085 were only detected at 12 and 24 hours post-infection, and treatment with AraC blocked transcription. FWPV150 transcripts were detected early
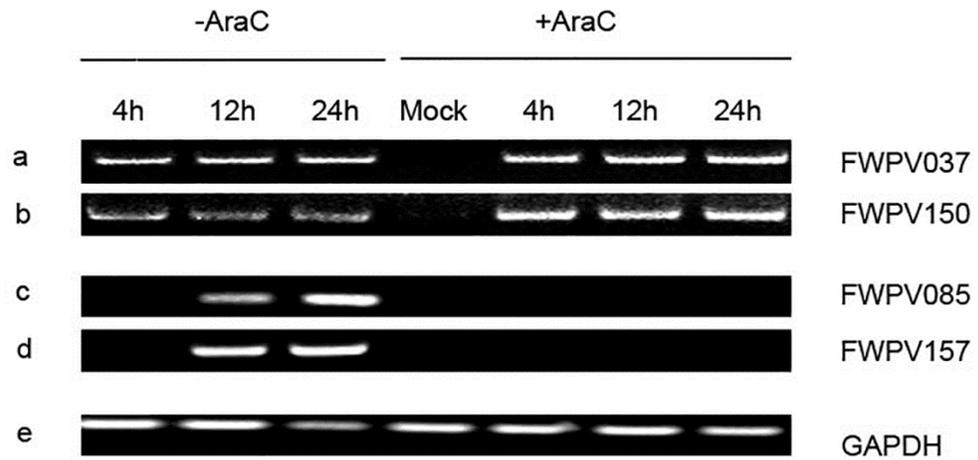
Figure 3.8 FWPV150 is expressed early and FWPV157 is expressed late during FWPV infection.

QM5 were infected with FWPV at an MOI of 5 in the absence or presence of 80μg/ml AraC. At 4, 12, and 24 hrs post infection, total RNA was isolated. Following DNAse treatment, reverse transcription was performed to obtain cDNA, which was subsequently used as a template for PCR with specific primers for FWPV037, FWPV085, FWPV150, FWPV157, or GAPDH. PCR reactions were visualized using 1% agarose gel stained with ethidium bromide.

during infection and also in the presence of AraC **(Figure 3.8; panel b)**, while FWPV157 transcripts were detected only at 12 and 24 hours post-infection and were blocked in the presence of AraC, identifying FWPV150 as an early gene and FWPV157 as a late gene **(Figure 3.8; panel d)**. Levels of GAPDH transcripts were similar for all time points and treatments **(Figure 3.8; panel e)**. Overall, the two p28-like ubiquitin ligases are expressed at different times during FWPV infection, FWPV150 was transcribed starting early during infection, whereas FWPV157 was transcribed only late. We next investigated, whether the eight KilA-N genes, which are also classified as p28-like genes, were actively transcribed during FWPV infection.

3.2.6 Avipoxviruses encode multiple KilA-N domains that are expressed early during FWPV infection:

p28-like proteins are the only known ubiquitin ligases that combine a KilA-N domain with a RING domain (233). Avipoxviruses and Entomopoxvirinae are the only poxviruses that encode KilA-N-only ORFs lacking the RING domain. FWPV encodes six KilA-N-only ORFs, including FWPV075, FWPV159, FWPV161, FWPV163, FWPV236, and FWPV248 (22). FWPV also encodes two KilA-N ORFs, FWPV124 and FWPV155, which contain different C-terminal domain of unknown function (DUF) (22). Bioinformatics indicated that KilA-N only and KilA-N-DUF proteins lacked a RING domain that is crucial for ubiquitin ligase activity (**Figure 3.9**). Although, the KilA-N domains contain low sequence identity, a number of residues were conserved throughout the proteins. Residues 44-50, which were previously shown to be important for

```
                  *            20           *              40            *
p28     : ------------------MEFDPAKINTSSIDHVTILQYIDEPNDIRLPVCIIRNI :  38
FWPV075 : ------------------MEFVPNTVKHID-ENFCFINYAN------IEVIMLKY- :  30
FWPV124 : ------------------MDFSDLVTREID-DRFCYIKYDK------FDLIMMKE- :  30
FWPV150 : --------------------MSHLHLNNGD-TEYRVIEDNG------FSIILLKH- :  28
FWPV155 : ------------------MMFNSMITGYID-EEFCYIQYSG------FHLVMMIS- :  30
FWPV157 : MKEDDSSNINNIHGKYSVSDLSQDDYVIECID-GSFDSIKYRD------IKVIIMKN- :  50
FWPV159 : ---------------------MSTITCYGN-DKFSYIIYDK------IKIIIMKS- :  27
FWPV161 : ------------------MDKRYISTRKLN-DGFLILYYDS------IEIIVMSC- :  30
FWPV163 : ------------------MNTLPYIIQDID-SHFCYIKYDG------ITLTMMKD- :  30
FWPV236 : -------MKFKEVRNTIKKMNITDIKICGIN-EYFMSMKLLD------VEVVIMRS- :  42
FWPV248 : -----------------MEIKVESIN-NNFCKISYED------IEIIMMKE- :  27

          60             *            80            *             100             *
p28     : NNITYFINITKINPDLANQFRAWKKRIAGRDYMTNISRDT--------------GIQQ :  82
FWPV075 : ---NGYINATKICDLGNKNFRQWCRIESSKKLIKTINYKN---------GIY--NKAV :  74
FWPV124 : ---NRFINATKLCKLGGKDFHRWKRIDGSKEIMIKVNEMNEMW--KSAPPPP--DLGG :  81
FWPV150 : ---TEYINVTKLCKIHNKEFYRWKRIISAGRIIETVSRDI--------------SNQG :  69
FWPV155 : ---NCYINASKLCDT--KDFKKWLRIDSSLSLILQEIENTNFPS--EKKFSIK--NSKS :  79
FWPV157 : ---NGYVNCSKLCKMRNKYFSRWLRISTSKALIDIYNNKSV------------DNAI :  92
FWPV159 : ---NNYVNATRLCELRGRKFTNWKKLSESKILVDNVKKIN-----DKTNQLK--TDMI :  75
FWPV161 : ---NHFINISALLAKKNKDFNEWLKIESFREIIDTIDKINYDLGQRYCEEPYGASHSS :  85
FWPV163 : ---NGYINATQLCMLGNKDFKEWIKIDHSIEIIKEIEKNI-----NKETTKY--VKAV :  78
FWPV236 : ---NGFVNITRICNLEGKDFNDWKQIESSRRIINTIKDNN-------------KLHD :  83
FWPV248 : ---NEYINATRLCSSRGRDILDWMSKESSVELINEIDRIN-----RSCNDYY--DYRG :  75

          120            *            140            *             160             *
p28     : SKITETIRNCQKNRNIYGLYIHYNLVINVVIDWIIDVIVQSILRGIVNWY---IAN-- : 135
FWPV075 : LEIGLA-SNSAYKYELVGTYVHIDLVPH-IICWVFPSIALNFSK-ILNSY---ISNSY : 126
FWPV124 : IIIEVNGSNQYTEYDIAGSYVHQDLIPH-IASWISPLFALKVSK-IISCY---VSGKY : 134
FWPV150 : FESPLVYVNRKGNKEFYGFYAHPQIALY-IAKWISEDIFNKIKH-LINSY---TISDK : 122
FWPV155 : VIII----LEKYYHEEVEGYYIHPDIIPH-IVGWLSPTFAISMSK-FINGY---ISNSF : 128
FWPV157 : VKV----YGKGKKLIITGFYLKQNMIRY-VIBWIGDDFTNDIYK-MINFY---NALFG : 141
FWPV159 : IYVK---DIDHKGRDTCGYYVHQDLVSS-ISNWISPLFAVKVNK-IINYY---ICNEY : 125
FWPV161 : VIIEVK-ASNLIDDRTAGFYVHKDLIPY-ILTCISIPFSLKVVR-VLDTY---IGEKL : 137
FWPV163 : ISVRSDYYNSETSNDIKGFYIHGNIMPH-ICAWISSKFAIKVSN-IVHNY---INDRY : 131
FWPV236 : PII----NIRHTRIKINGEYVSQLLIDY-VIPWISPYVATRVSI-IMRYYRRCVALNI : 135
FWPV248 : IVI-----NVVSDSETSELYVHRDLILH-ISHWISPLFSLKVVK-FINSY---IQDSY : 123

          180            *            200            *             220             *
p28     : --------------------------NTYNPNTPNSTTTISELDIIKILDKYEDVY : 165
FWPV075 : CTRLKKG------------SDNEDQIRSGFFSEGISKILYDIHDNEILKLKKNTKKLE : 172
FWPV124 : EFKLKE------------KENKIEELLDLLHKFNNKYDKDTLELKELYREQRKEAKS : 179
FWPV150 : TVVIKDFSYCDELCPD---AIIGKCCKTKSSCEYVHGDICDICGFEALHPTDIDKRLT : 177
FWPV155 : TITEKDDKKYNTLPPSSSYKQGDRNCFIDMLNEMTNKHLNDITELKTHYREQKRELKY : 186
FWPV157 : NDELKIVSCENTLCP----FIELGRCYYGKKCKYIHGDQCDICGLYILHPTDINQRVS : 195
FWPV159 : DIRLSE------------MESDMTEVIDVVDKLVGGYNDEIAEIIYLFNKFIEKYIA : 170
FWPV161 : ENRIK----------------------------LSQSMDLETNNSYNM----- : 157
FWPV163 : VQNDKE--------------EIHQEPDKDIKYIKKQCKLMREIRILFKKNYTRELD : 173
FWPV236 : ETEKDIDHSQE-------LQNQISKIDEVYDRSIKDISNRFKEIETSYYSKLSTYLL : 185
FWPV248 : QL----------------------------------EYELIHKKSL----- : 135
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                    240          *          260          *          280          *
p28       : RV-----------------SKEKECGICYEVVYSKRLENDRYFGL------------ : 193
FWPV075   : EK----------------------------------------------------------- : 174
FWPV124   : LR-------KINERIEEKYDKDTRELKQGLKELKDENKELKFELKKIEERLRDKVINP : 230
FWPV150   : HE---KVCM----QLLCKEDIKYDKCGICLDAIKG----NKKPYGI----------- : 212
FWPV155   : QNTVLSSKITELKNVNDEFRYRIKHFDDSIKEIKDENNTLKSNIKITEKHNKELQRDN : 244
FWPV157   : HK---KTCLVDRDSLIVFKRSTSKKCGICIEEINKKHI-SEQYFGI----------- : 237
FWPV159   : NI-----------------SLSTELSSILNNFINFNKKYNND----------- : 195
FWPV161   : ------------------------------------------------------------- : -
FWPV163   : EL----------KKVRELYYEKNKGLEEYIDKLEYSYTQRMKELTLSIDE-------- : 213
FWPV236   : TK--------------AERVLEKDYSMEQDIDNNEDIRTDEMIAAIEAE-------- : 220
FWPV248   : -------------------------MDQLKEIIL------------------ : 144


                    300          *          320          *          340
p28       : --------------------------------------------------------- : -
FWPV075   : --------------------------------------------------------- : -
FWPV124   : --------------------------------------------------------- : -
FWPV150   : --------------------------------------------------------- : -
FWPV155   : NRLKTLLRELYEKNTSLQNNITELRETIARETKELHNQVIELSKDKGIEPIEEYRVDR : 302
FWPV157   : --------------------------------------------------------- : -
FWPV159   : --------------------------------------------------------- : -
FWPV161   : --------------------------------------------------------- : -
FWPV163   : --------------------------------------------------------- : -
FWPV236   : --------------------------------------------------------- : -
FWPV248   : --------------------------------------------------------- : -


             *          360          *          380          *          400
p28       : -------LDSCNHIFCITCINIWHRTRRETGASDNCPICRTRFRNITMSKFYKLVN-- : 242
FWPV075   : -------YEKTNSLI---------------------NQKISNLEVAVKGLSIK--- : 199
FWPV124   : -------FSPNKHHRLVILQKKIDNNSFKTLRLQAERLNQEMNKYKTNILYFLMHTNL : 281
FWPV150   : -------LSDCNHMFCINCIKTWMTT---INSKKQCPECRVPSKYIIQSPIWTVDKVS : 260
FWPV155   : CFVRNRLHRSNKNNYIIIFQHKKDLFTFKYFKLHIRKVCIELFNYRESHNLFLIIYEP : 360
FWPV157   : -------LPSCKHIFCLSCIRRWADTTRNTDTENTCPECRIVFPFIIPSRYWIDNKYD : 288
FWPV159   : -------IKDIKSLI-LDLKNTSIKLDKKLFDKDNNESNDEKLETEVDKLIFFI---- : 241
FWPV161   : --------------------------------------------------------- : -
FWPV163   : -------LKNSNKQL----KNKLENIEKRIKCINPPTESSKNVIYDRFKKLYHILTFR : 260
FWPV236   : -------IEENNRRYLSIISGIRKQHAEDRINISKIMLSGDSFNEIIVKIRDYIETTA : 271
FWPV248   : -------LNDDNNM------------------------------------------- : 151


             *          420          *          440          *
p28       : ------------------------------------------- : -
FWPV075   : ------------------------------------------- : -
FWPV124   : TQYPVLIG----------------------------------- : 289
FWPV150   : KNQLSVSYKTVYIKSC--------------------------- : 276
FWPV155   : TNKSIIRFKNMLENNEHIELKDNNFKITDTRYTVINILKDINKIFSDN : 408
FWPV157   : KKILYNRYKKMIFTKIPIRTIKI-------------------- : 311
FWPV159   : ------------------------------------------- : -
FWPV161   : ------------------------------------------- : -
FWPV163   : KSK---------------------------------------- : 263
FWPV236   : KPAVANNYE---------------------------------- : 280
FWPV248   : ------------------------------------------- : -
```

Figure 3.9 *Avipoxviruses* encode multiple KilA-N domains.

Protein alignments for FWPV150, FWPV KilA-N only proteins FWPV075, FWPV159, FWPV161, FWPV163, FWPV236, and FWPV248, and KilA-N-DUF proteins FWPV124 and FWPV155 were performed via Clustral W (1.82) (http://www.ebi.ac.uk/clustalW/#). Black bar indicates KilA-N domain and grey bar indicates highly conserved residues within the KilA-N domains, which are important in p28 for localization to the virus factories. Conserved amino-acid residues were shaded using GeneDoc (258, 259). The Genedoc shading mode was set to conservation, with shading level 4 and the conserved percent was primary 100% (black), secondary 80% (dark grey), and tertiary 60% (light grey). The shade style was set to amino acid identity.

localization to the viral factories (247), were conserved throughout the KilA-N containing proteins **(Figure 3.9)**. Therefore, we utilized RT-PCR with specific primers for KilA-N only proteins FWPV075, FWPV159, FWPV161, FWPV163, FWPV236, and FWPV248, and two KilA-N-DUF designated as FWPV124 and FWPV155 to investigate, if there was active transcription of KilA-N and KilA-N-DUF genes during FWPV infection. We used the same cDNA as a template as above in **Figure 3.8**; and the PCRs in 3.8 and 3.10 were done at the same time under the same conditions. Separate controls for GAPDH and early gene FWPV037 were done at the same time and shown in Figure 3.10. The AraC late gene control can be found in **Figure 3.8; panel c**. We again used primers for FWPV037 as a control for an early gene **(Figure 3.10A; panel a)**, and GAPDH as a house-keeping gene **(Figure 3.10A; panel j)**. Interestingly, all of the KilA-N only genes **(Figure 3.10A; panel d-i)** and the two KilA-N-DUF genes **(Figure 3.10A; panel b and c)** were transcribed at 4, 12, and 24 hours post infection, and genes were also transcribed in the presence of the late gene inhibitor AraC. Taken together, all of the KilA-N only and the two KilA-N-DUF genes were actively transcribed starting at early times during FWPV infection. We subsequently chose one representative from each group, FWPV075, containing only a KilA-N domain, and FWPV124, containing a KilA-N-DUF domain, to investigate the subcellular localization during FWPV infection and determine the effect on the localization of conjugated ubiquitin. QM5 cells were mock infected **(Figure 3.10B; panel a-d)**, infected with FWPV **(Figure 3.10B; panel e-h)**, and transfected with pSC66-FLAG-FWPV075 **(Figure 3.10B; panel i-l)**, or pSC66-FLAG-FWPV124 **(Figure 3.10B; panel m-p)**. Cells were co-stained with anti-FLAG to visualize FWPV075 **(Figure 3.10B; panel k)**, FWPV124 **(Figure 3.10B; panel o)**, and with FK2

(Figure 3.10B; panel b, f, j, and n). FWPV075 and FWPV124 localized at the viral factories without an effect on FK2 distribution within the cell **(Figure 3.10B; panel l and p)**. To demonstrate the expression of FLAG-FWPV075 and FLAG-FWPV124, QM5 cells were infected with FWPV at an MOI of 5 and transfected with pSC66-FLAG-FWPV075 or pSC66-FLAG-FWPV124. Whole cell lysates were subjected to western blot analysis with anti-FLAG, or anti β-tubulin as a loading control **(Figure 3.10C)**. Altogether, the KilA-N only protein FWPV075 and the KilA-N-DUF protein FWPV124 localized to the viral factories in QM5 cells during FWPV infection without any effect on accumulation of conjugated ubiquitin **(Figure 3.10B; panel l and p)**. This underlines that a KilA-N domain alone is not sufficient to recruit conjugated ubiquitin and a RING domain is needed.

3.3 Summary and Brief Discussion

Members of Avipoxviruses contain two p28-like ubiquitin ligases (22, 23). Here we demonstrate that FWPV150 is starting to be expressed early during infection, while FWPV157 is expressed late. Bioinformatics analysis also revealed that CNPV encodes two p28-like ubiquitin ligases, CNPV197 and CNPV205 (23). Investigation of the upstream sequences revealed an early promoter for FWPV150 and CNPV197 (early promoter: AAAAATGAAAAAAAA), and a late promoter upstream of FWPV157 and CNPV205 (late promoter: TAAATG). These data suggest that members of the Avipoxviruses contain two distinct p28-like ubiquitin ligases that are expressed early and late during virus infection. In contrast, non-Avipoxviruses encode only one p28-like

A

		-AraC			+AraC			
		4h	12h	24h	Mock	4h	12h	24h

KilA-N-DUF
a — FWPV037
b — FWPV124
c — FWPV155

KilA-N only
d — FWPV075
e — FWPV159
f — FWPV161
g — FWPV163
h — FWPV239
i — FWPV248

j — GAPDH

B

	DAPI	anti-FK2	anti-FLAG	Merge
Mock QM5	a	b	c	d
FWPV	e	f	g	h
FWPV + pSC66-FLAG-FWPV075	i	j	k	l
FWPV + pSC66-FLAG-FWPV124	m	n	o	p

111
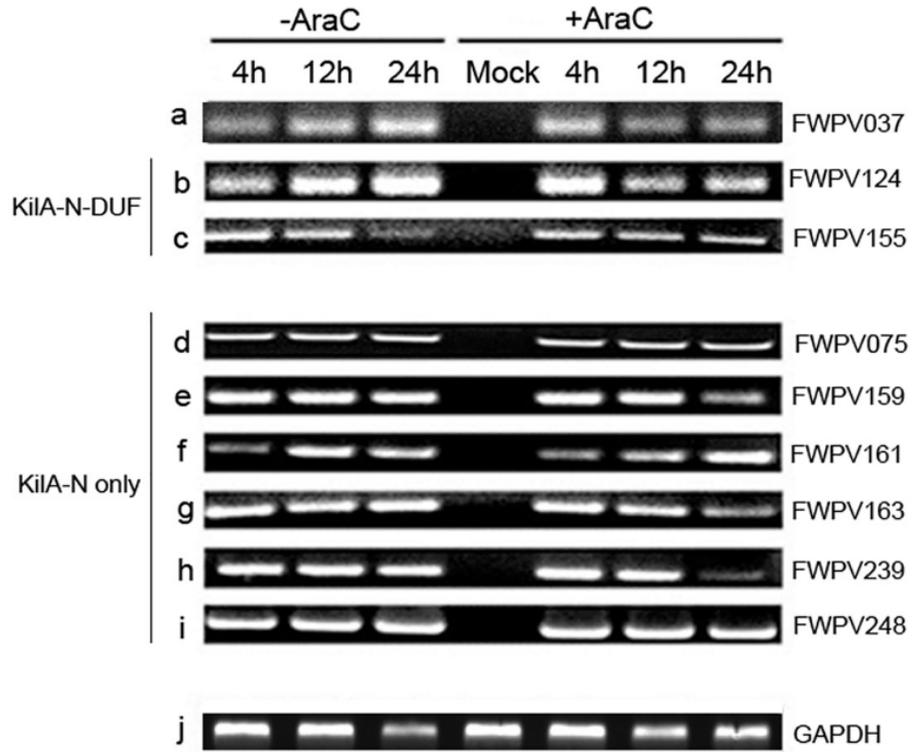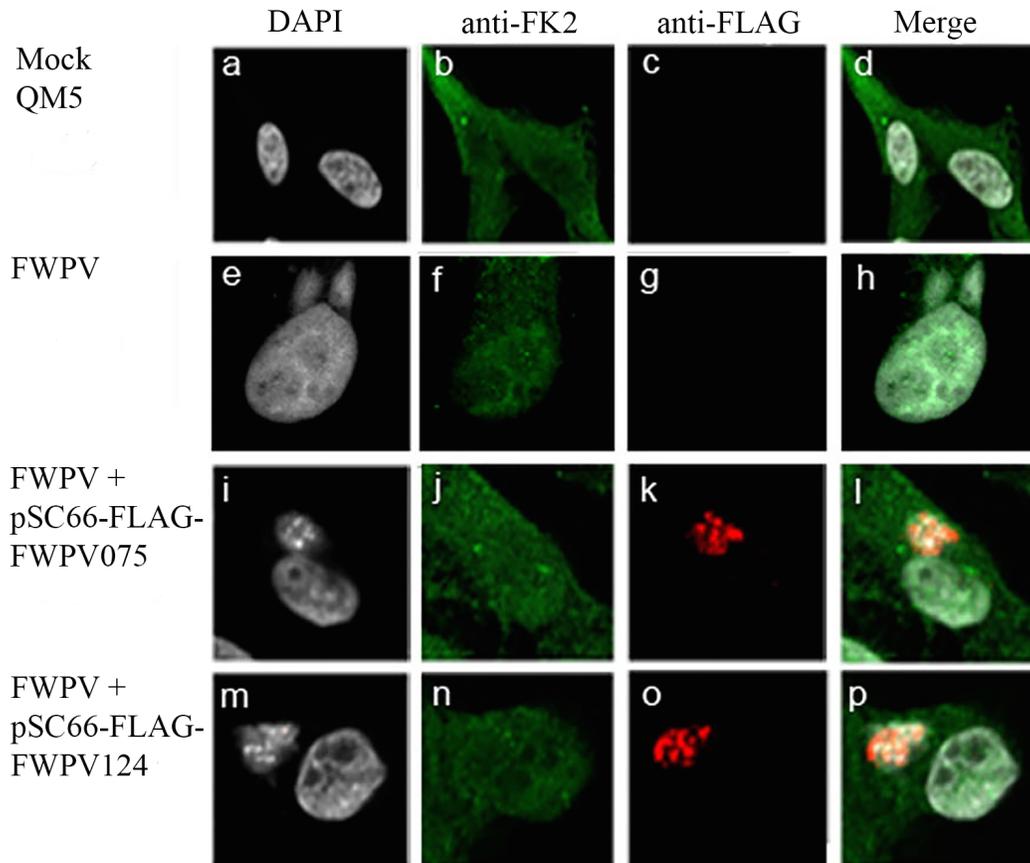
C
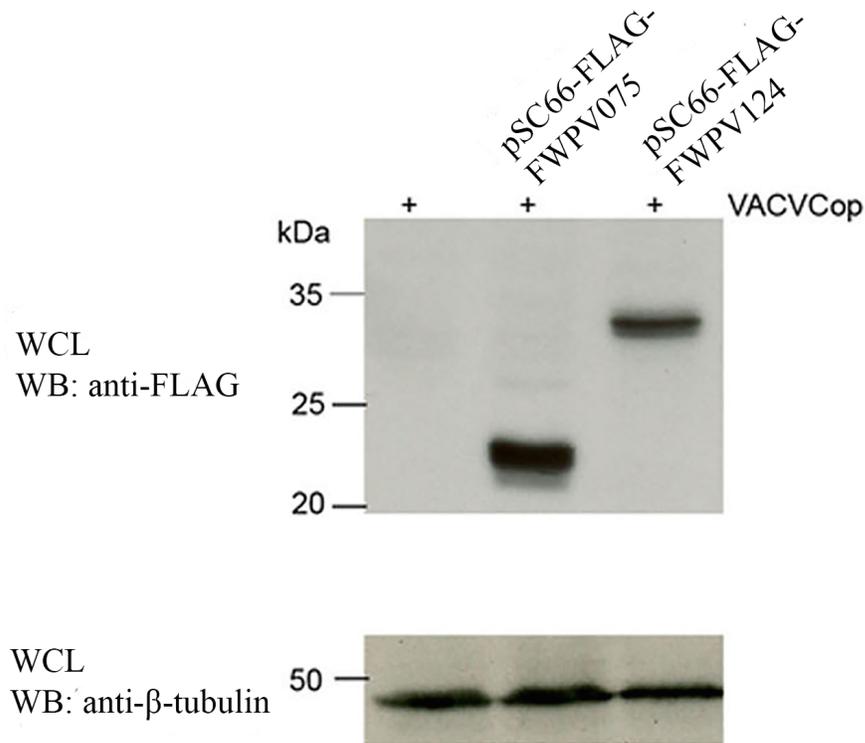
Figure 3.10 Avipoxviruses encode multiple KilA-N domain proteins that are expressed early during FWPV infection.

(A) QM5 cells were infected with FWPV at an MOI of 5 in the absence or presence of 80µg/ml AraC. At 4, 12, and 24 hrs post infection, total RNA was isolated. Following DNAse treatment, reverse transcription was performed to obtain cDNA, which was subsequently used as a template for PCR with specific primers for FWPV037, the KilA-N only genes FWPV075, FWPV159, FWPV161, FWPV163, FWPV236, and FWPV248, or the KilA-N-DUF genes FWPV124 or FWPV155, or GAPDH. PCR reactions were visualized by electrophoresis on 1% agarose gels stained with ethidium bromide. **(B)** QM5 cells were mock infected, or infected with FWPV at an MOI of 5 and transfected with pSC66-FLAG-FWPV075 (KilA-N-only) and pSC66-FLAG-FWPV124 (KilA-N-DUF). Cells were fixed and treated with DAPI to visualize the nucleus and virus factories, and stained with anti-FLAG to visualize FLAG-tagged proteins and anti-FK2 to recognize conjugated ubiquitin, and analysed by confocal microscopy. This experiment was performed three independent times and we observed on average over 100 cells per sample, which were evaluated across the entire cover slips of the samples. We showed one representative cells per sample in this Figure. **(C)** QM5 cells were infected with FWPV at an MOI of 5 and transfected with pSC66-FLAG-FWPV075 or pSC66-FLAG-FWPV124. Whole cell lysates were subjected to western blot analysis with anti-FLAG, or anti β-tubulin as a loading control.

ubiquitin ligase that is expressed early during infection. The ubiquitin ligase activity of p28 lies in the C-terminal RING domain, while the N-terminal KilA-N domain is crucial for its localization to virus factories. Here we demonstrate, that the double cysteine mutants in the RING domain of FWPV150 and FWPV157 abolish the ubiquitin ligase activity. Moreover, we demonstrate that FWPV150 and FWPV157 co-localize with K48 at the virus factories, which typically leads to proteasomal degradation of target proteins. Surprisingly, the double cysteine mutants FWPV150(C196S/C199S) and FWPV157(C218S/C221S) still co-localize with K48 ubiquitin chains. It is possible, that other ubiquitin ligases or ubiquitinated proteins are enriched in the presence of the double cysteine mutants.

There was only a modest localization of conjugated ubiquitin at the virus factory during FWPV infection **(Figure 3.4)**. We suspect that overexpression of the FWPV proteins FWPV150 and FWPV157 exaggerate the phenotype of complete co-localization of conjugated ubiquitin at the virus factories. We expected a modest co-localization of conjugated ubiquitin with endogenous FWPV150 or FWPV157 at the virus factory during wildtype FWPV infection. Furthermore, RING domain mutants FWPV150(C196S/C199S) and FWPV157(C218S/C221S) demonstrated co-localization with conjugated ubiquitin at the virus factory. However, there was a loss of complete localization at the virus factory and in addition punctate staining of ubiquitin was found in the cytoplasm. We speculate that this phenotype is due to the loss of ubiquitin ligase activity of the FWPV proteins.

The p28 ubiquitin ligase functions as a virulence factor during ECTV virus infection (37, 245). In order to test if FWPV150 and FWPV157 are virulence factors

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during FWPV infection, an animal model will need to be established. FWPV cannot infect mammalian cells due to species tropism (262). FWPV can infect, amongst other birds, chickens and turkeys, and FWPV infection typically leads to proliferative lesions in the skin (cutaneous form) that progress to thick scabs (2, 24). In more severe cases, FWPV can lead to lesions in the upper GI and respiratory tracts (diphtheritic form) (24). Overall, future studies should investigate the importance of FWPV150 and FWPV157 *in vivo* to determine if the lack of one or both FWPV p28-homologues would alter the virulence of FWPV.

One distinguishing feature of the Avipoxviruses is the large genome compared to other poxviruses, as exemplified by FWPV (288 kbp) and CNPV (365 kbp) (22, 23). While FWPV and CNPV are the prototypical members of Avipoxviruses, they exhibit some interesting genomic differences. Out of the 328 open reading frames in CNPV, FWPV contains only 209 predicted homologues. Furthermore, Avipoxviruses encode a number of novel proteins, which might be important as host-range factors or immune evasion strategies in birds. In addition to the KilA-N domain containing ubiquitin ligases, FWPV150 and FWPV157, there are six KilA-N only and two KilA-N with C-terminal domains of unknown function (DUF) in the FWPV genome (22). CNPV contains twenty-five KilA-N only ORFs. We demonstrated here, that all eight KilA-N domains and KilA-N-DUF are actively transcribed early during FWPV infection **(Figure 3.9)**. Future studies should investigate the role of these novel FWPV proteins during infection and interaction with host cells.

CHAPTER 4: THE POXVIRUS ENCODED UBIQUITIN LIGASE P28 IS REGULATED BY PROTEASOMAL DEGRADATION AND AUTOUBIQUITINATION

A version of the data presented in this chapter has been published:

Mottet K. [Δ], Bareiss B. [Δ], Milne C. D., Barry M. (2014) The poxvirus encoded ubiquitin ligase, p28, is regulated by proteasomal degradation and autoubiquitination. *Virology*. 468-470: 363-78.

[Δ] These authors contributed equally.

All experiments presented in this chapter were performed by myself, except for the flow cytometry assay in **Figure 4.6**, which was performed by Kelly Mottet. Parts of **Figure 4.3A**, **4.4** and **4.7** were initially performed by K. Mottet, but repeated by myself; the replications shown here were performed by myself. The original manuscript was written by myself with editorial contributions from Kelly Mottet, Robyn-Lee Burton, Dr. Thibault, Dr. Noyce, and Dr. Barry.

4.1 Introduction

This chapter focuses on the ubiquitin-proteasomal regulation of the poxvirus-encoded ubiquitin ligase p28 (139, 242). We suggest here that p28 is regulated by proteasomal degradation, initiated by both autoubiquitination and ubiquitination by another ubiquitin ligase. This feedback regulation has been seen in other ubiquitin ligases (171, 263-266). Our previous work demonstrated that p28 encoded by vaccinia virus strain IHDW is a functional ubiquitin ligase that localizes to cytoplasmic regions of virus replication, referred to as virus factories (243). In the presence of p28, conjugated ubiquitin localizes to virus factories during vaccinia infection (242, 243). Furthermore, *in vivo* studies indicate that p28 is a critical virulence factor during ectromelia virus infection (244, 245). Given that p28 is an important virulence factor, we investigated the regulation of p28 during virus infection. Here we show that p28 homologues localized with conjugated ubiquitin at the virus factories. We also demonstrate that p28 is regulated by proteasomal degradation. Upon treatment with proteasome inhibitors, p28 was dramatically stabilized and highly ubiquitinated, indicating that p28 is regulated by ubiquitination and proteasomal degradation. Furthermore, we suggest that p28 may regulate itself through autoubiquitination. Moreover, when p28 was disrupted by mutating the RING domain, conjugated ubiquitin still localized to the virus factories, indicating that an unknown ubiquitin ligase(s) was responsible. Taken together, we demonstrate that, like many cellular ubiquitin ligases, p28 is subjected to proteasomal degradation and highly regulated by ubiquitination.

4.2 Results

4.2.1 Mammalian poxviruses and *Avianpoxvirus* FWPV do not inhibit the proteasomal system

The ubiquitin-proteasome system has emerged as an important mechanism to control protein degradation (246, 267). Recently, it has become apparent that poxviruses use the UPS in order to manipulate host responses (30, 139, 233, 243, 268). Furthermore, it has been shown that a functioning proteasome is crucial for poxvirus replication (269, 270). Therefore, we sought to determine if the proteasome was fully functional during poxvirus infection. To perform these experiments, HeLa cells were mock-infected or infected with VACVCop, ECTV strain Moscow, CPXV strain Brighton red, or one *Avipoxvirus* member, FWPV **(Figure 4.1A and 4.1B)**. The chymotrypsin-like activity of the proteasome was assessed using a cell-based luciferase Proteasome-Glo assay (Promega) (271). Mock-infected HeLa cells demonstrated high luminescence, corresponding to the amount of chymotrypsin-like activity of the proteasome and graphed as RLU **(Figure 4.1A)**. Similarly, HeLa cells infected with VACVCop, CPXV, or ECTV demonstrated considerable proteasome activity with only a small difference between mock and infected cells **(Figure 4.1A)**. However, infected cells treated with MG132 resulted in less than 1% proteasome activity compared to infected cells without MG132 treatment **(Figure 4.1A)**. Although some decrease in proteasome function was evident late during VACV, CPXV and ECTV infection, the chymotrypsin-like activity of the proteasome remained largely functional **(Figure 4.1A)**. To further investigate the proteasomal activity during poxvirus infection,
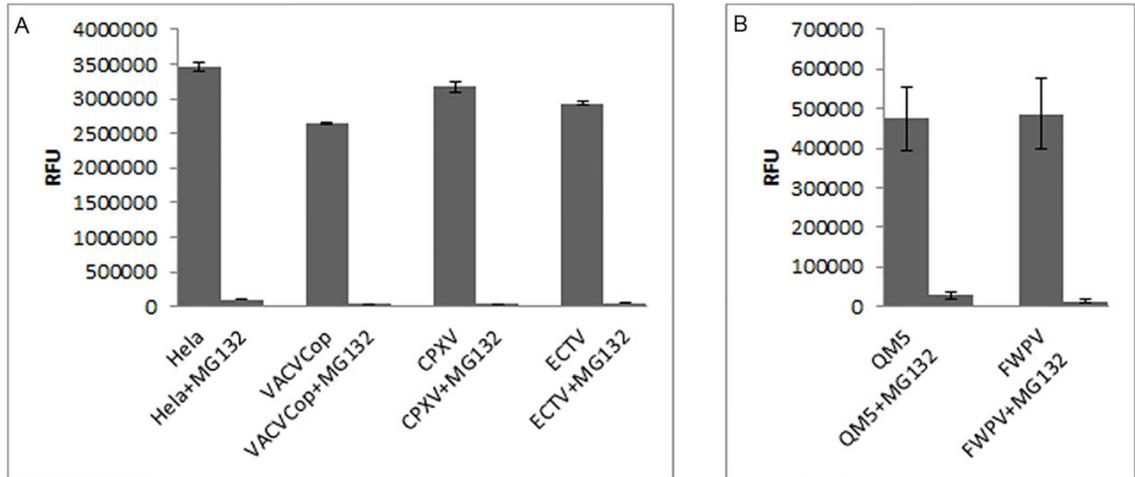
Figure 4.1 Mammalian poxviruses and *Avianpoxvirus* FWPV do not inhibit the proteasomal system.

(A) HeLa cells were mock-infected or infected with VACVCop, ECTV, or CPXV at an MOI of 10 for fourteen hours and at twelve hours post-infection treated with or without 10μM MG132. Proteasomal function was quantified by Proteasome-Glo assay (Promega), and standard deviations were calculated from three independent experiments. **(B)** QM5 cells were infected with FWPV at an MOI of 10 for fourteen hours and at twelve hours post-infection treated with or without 10μM MG132. Proteasomal function was quantified by Proteasome-Glo assay.

we focused on FWPV, a divergent member of the *Avipoxvirus* family **(Figure 4.1B)**. Mock-infected QM5 quail cells demonstrated proteasomal activity that was significantly inhibited in the presence of MG132 (p<0.0001) **(Figure 4.1B)**. FWPV infection demonstrated a non-significant loss of proteasomal function in QM5 cells compared to uninfected cells (p<0.1). Overall, these results demonstrate that the proteasome is functional during VACV, CPXV, ECTV and FWPV infection, which would allow poxviral encoded-ubiquitin ligases to make complete use of the cellular 26S proteasome system. Furthermore, the addition of MG132 inhibited the proteasome, as expected **(Figure 4.1)**. We next investigated whether the poxviral encoded ubiquitin ligase p28 and its homologues effect on conjugated ubiquitin.

4.2.2 p28 homologues co-localize with conjugated ubiquitin at the virus factory

p28 homologues are encoded in a wide range of poxviruses including VACV(IHDW), ECTV, MYXV and FWPV, while VACVCop is missing endogenous p28. p28 encoded by VACV(IHDW) and ECTV share 95% sequence identity **(Figure 4.2)**. In contrast, p28 encoded by VACV(IHDW) shared only 20% and 16% sequence identity and 48% and 40% sequence similarity with the MYXV homologue M143R, and the FWPV homologue, FWPV150, respectively **(Figure 4.2)** Notably, the cysteine and histidine residues critical for ubiquitin ligase function were highly conserved among the four proteins. Residues 44 to 51 within the KilA-N domain that are crucial for DNA binding, are also conserved between the four proteins **(Figure 4.2)** (246). Given the conservation

```
p28-IHD-W  :  MEFDPAKINTSSIDHVTILQYIDEPNDERLTVCIIRNINNITYYINITKINTHIANQF  :  58
p28-ECTV   :  MEFDPAKINTSSIDHVTILQYIDEPNDIRLPVCIIRNINNITYFINITKINPDIANQF  :  58
M143R      :  --MDH---------NVKILD-----NDYGINIVFLRS----NHYINITRLCAPMKKSF  :  38
FWPV150    :  --MSHLHLNNGDTEYRVI-------EDNGFSIILLKH---TEYINVTKLCKIHNKEF  :  45


p28-IHD-W  :  RAWKKRIAGRDYMTNLSRDTGIQQSKLTETIRNCQKNRNIYGLYIHYNLVINVVIDWI  :  116
p28-ECTV   :  RAWKKRIAGRDYMTNLSRDTGIQQSKLTETIRNCQKNRNIYGLYIHYNLVINVVIDWI  :  116
M143R      :  TNWKALKNSKYIMNSISIEENLDIDDLTFRIYKNKYSVYYHGIFVHPKL-IKYVISWI  :  95
FWPV150    :  YRWKRLISAGRITETVSRDISNQGFESPLVYVNRKGNKEFYGFYAHPQIAL-YIAKWI  :  102
```

KilA-N DNA binding domain

```
p28-IHD-W  :  TDVIVQSILRGLVNWY---IANNT----------------------------------  :  137
p28-ECTV   :  TDVIVQSILRGLVNWY---IANNT----------------------------------  :  137
M143R      :  SDEYYAKV-YSIINAYDENILKNTVLTLYVNYIYCLKQEDML----------------  :  136
FWPV150    :  SEDIFNKI-KHLINSYT--ISLKTVV--IKDFSYC----DELCPDAIIGKCCKTKSSC  :  151


                                                        *      *
p28-IHD-W  :  -----------YTPNTPNNTTIISELD--IIKILNKYEDVYRVSKEKECGICYEVVY  :  181
p28-ECTV   :  -----------YNPNTPNS-TIISELD--IIKILDKYEDVYRVSKEKECGICYEVVY  :  180
M143R      :  -----------YKAIHHRN-KLYHRILKTIPNVVNEYETLYDSYKGEECAICMEPVY  :  181
FWPV150    :  EYVHGDICDICGFEALHPTDIDKRLTHEKVCMQLLCKEDIKYD-----KCGICLDAIK  :  204
```

RING domain

```
                      *   *    *   *                    *    *
p28-IHD-W  :  SKRLENDRYFGLLDSCTHIFCITCINIWHKTRRETGASDNCPICRTRFRNIIMSKFYK  :  239
p28-ECTV   :  SKRLENDRYFGLLDSCNHIFCITCINIWHRTRRETGASDNCPICRTRFRNIIMSKFYK  :  238
M143R      :  AKPI-KSSEFGVLSHCNHVFCIECIDRWKKQ------NNKCPVCRTIEVSVIKSREFY  :  232
FWPV150    :  G----NKKPYGILSDCNHMFCINCIKTWMTI---INSKKQCPECRVPSKYIIQSPIWT  :  255


p28-IHD-W  :  LVN------------------  :  242
p28-ECTV   :  LVN------------------  :  241
M143R      :  KG-------------------  :  234
FWPV150    :  VDKVSKNQLSVSYKTVYIKSC  :  276
```

Figure 4.2 Alignment of p28 homologues.

p28 protein alignments for VACV strain IHDW, ECTV strain Moscow, MYXV strain
Lusanne,   and   FWPV   were   performed   via   ClustalW   (1.82)
(http://www.ebi.ac.uk/clustalW/#) (257). The KilA-N domain is underlined in black and
the RING domain is underlined in grey. Asterisks highlight the conserved cysteines and
histidine within the RING domain. The thick black line marks the motif in p28, which is
important for DNA binding. Conserved amino-acid residues were shaded using GeneDoc
(258, 259); The Genedoc shading mode was set to conservation, with shading level 4 and
the conserved percent was primary 100% (black), secondary 80% (dark grey), and
tertiary 60% (light grey). The shade style was set to amino acid identity.

of p28 throughout the poxvirus family, we sought to determine whether conjugated ubiquitin co-localized with other p28 homologues. HeLa cells were infected with VACVCop, VACV-FLAG-p28(IHDW), and VACV-FLAG- M143R that expresses the p28 homologue MYXV (**Figure 4.3; panel a-h and m-p**). We further included the p28 double cysteine mutant expressing virus VACV-FLAG-p28(C173S/C176S), which lost its ubiquitin ligase activity, to examine its effect on conjugated ubiquitin in HeLa cells (**Figure 4.3; panel i-l**). To express the p28 homologue FWPV150 encoded by FWPV, cells were infected with VACVCop and transfected with pSC66-FLAG-FWPV150 under the control of a poxvirus promoter (**Figure 4.3; panel q-t**). Cells were stained with DAPI to visualize both the nucleus and cytoplasmic viral factories, and co-stained with anti-FLAG to visualize FLAG-p28, FLAG-p28(C173S/C176S), FLAG-M143R, or FLAG-FWPV150. Using this approach, we could demonstrate that p28 homologues M143R and FWPV150, encoded by MYXV and FWPV, localized to viral factories (**Figure 4.3; panel g, k, o, s**). Furthermore, FK2 conjugated ubiquitin co-localized to viral factories upon expression of each p28 homologue (**Figure 4.3; panel h, l, p, t**). As expected, upon VACV infection only a minor accumulation of conjugated ubiquitin was observed at the virus factory, due to the lack of p28 (**Figure 4.3; panel d**), while the p28 mutant p28(C173S/C176S) still co-localized with conjugated ubiquitin at the virus factory (**Figure 4.3; panel l**) (245). Taken together, the data indicate that the accumulation of conjugated ubiquitin at the viral factories is conserved among close and distant relatives of the p28 family of poxvirus proteins. We further confirmed that the p28 mutant p28(C173S/C176S) still co-localized with FK2 at the virus factories in HeLa cells (243).
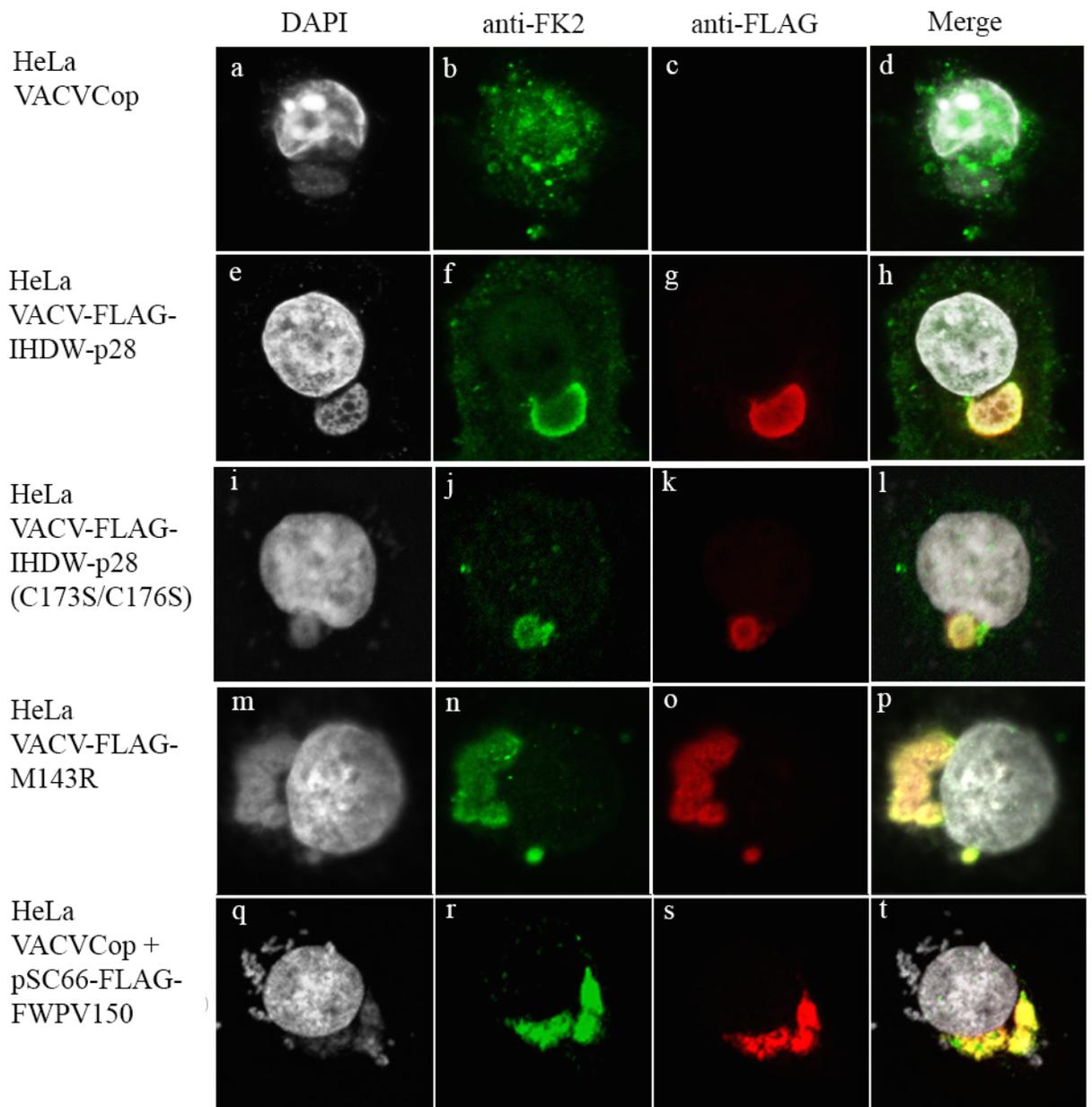
Figure 4.3 p28 homologues co-localize to the virus factory with conjugated ubiquitin in HeLa cells.

HeLa, were infected with VACVCop, VACV-FLAG-IHDW-p28, VACV-FLAG-p28(C173S/C176S), or VACV-FLAG-M143R, or infected with VACVCop and transfected with pSC66-FLAG-FWPV150 at an MOI of 5. Cells were fixed and treated with DAPI, to visualize the nucleus and virus factories, and stained anti-FLAG to detect FLAG-tagged proteins, and anti-FK2 to detect conjugated ubiquitin. This experiment was performed three independent times and we observed on average over 100 cells per sample, which were evaluated across the entire cover slips of the samples. We showed one representative cells per sample in this Figure.

Previous studies demonstrated that the p28 homologue in ECTV plays a crucial role for ECTV growth in macrophages (245). We started isolating bone marrow derived macrophages (BMDM) from mice and differentiated them into primary macrophages where ECTV lacking p28 fails to form virus factories in primary macrophages (245). The goal was to investigate, if a similar effect was seen to Senkevich *et.al*. Unfortunately, this project was terminated before we could conduct infection studies on primary macrophages, which is further discussed in Chapter 6. Instead, we characterized the co-localization of p28, and homologue M143R with FK2 in the readily available ATCC mouse macrophage cell lines J774 and Raw 264.7 **(Figure 4.4 A and B)**. We further investigated, if co-localization of the double cysteine mutant p28(C173S/C176S) with FK2 at the virus factory is also observed in mouse macrophage cell lines **(Figure 4.4A and B; panel m-p)**. p28, p28(C173S/C176S) and M143R co-localized with conjugated ubiquitin at the virus factories in mouse macrophage cell lines J774 and Raw 264.7, similar to the effect seen in HeLa cells **(Figure 4.4A and B; panel l, p, t)**. Since we could not detect a different phenotype in mouse macrophage cell lines for p28 homologues and the p28 double cysteine mutant, we continued to use HeLa cells in the following experiments due to its superior transfection efficacy.

4.2.3 The KilA-N DNA binding domain plays a critical role in localizing p28 to the virus factory, but does not affect the accumulation of conjugated ubiquitin at the virus factory

A

	DAPI	anti-FK2	anti-FLAG	Merge
J774 Mock	a	b	c	d
J774 VACVCop	e	f	g	h
J774 VACV-FLAG p28-IHDW	i	j	k	l
J774 VACV-FLAG-p28 (C173S/C176S)	m	n	o	p
J774 VACV-FLAG-M143R	q	r	s	t
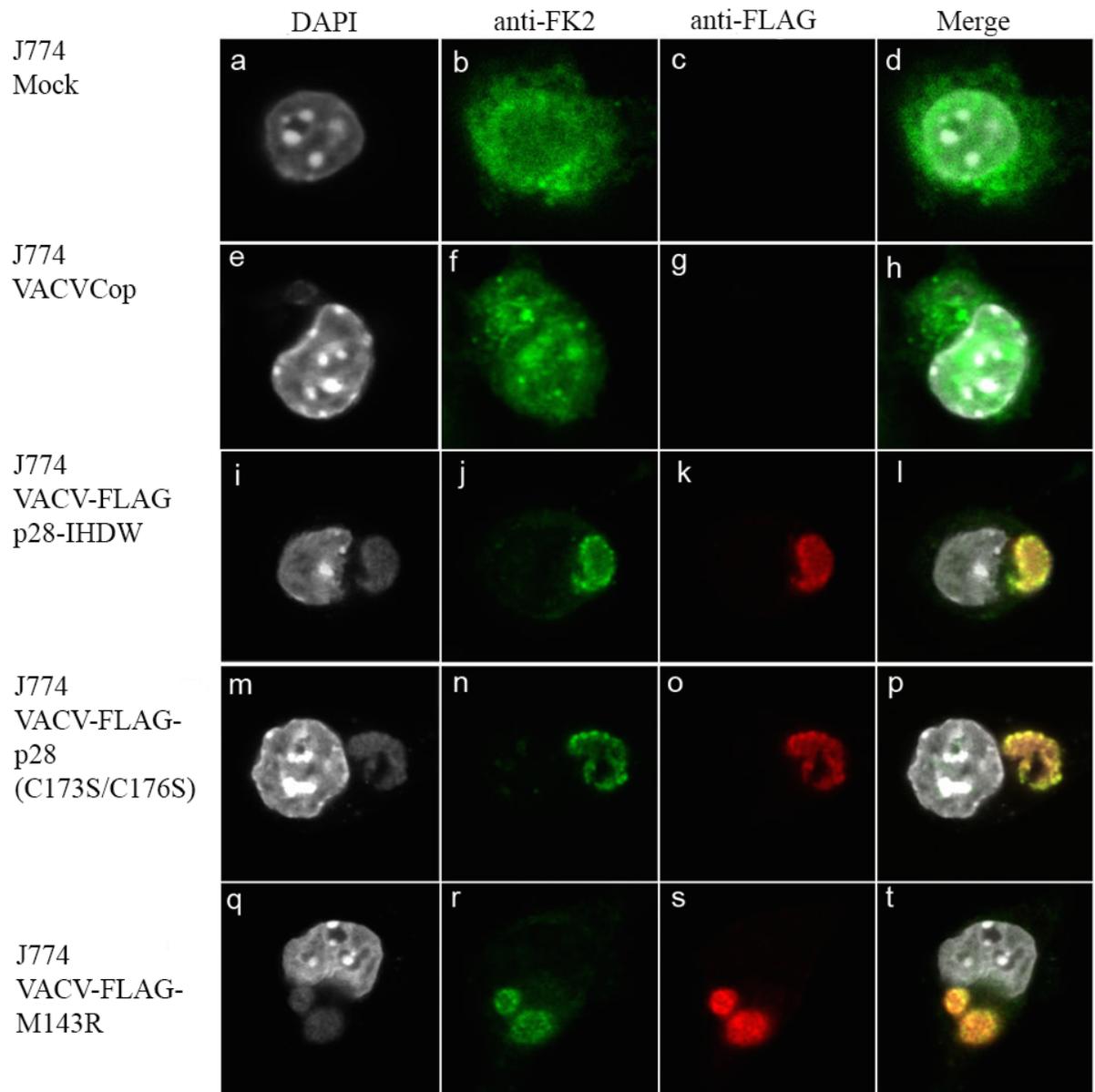
B
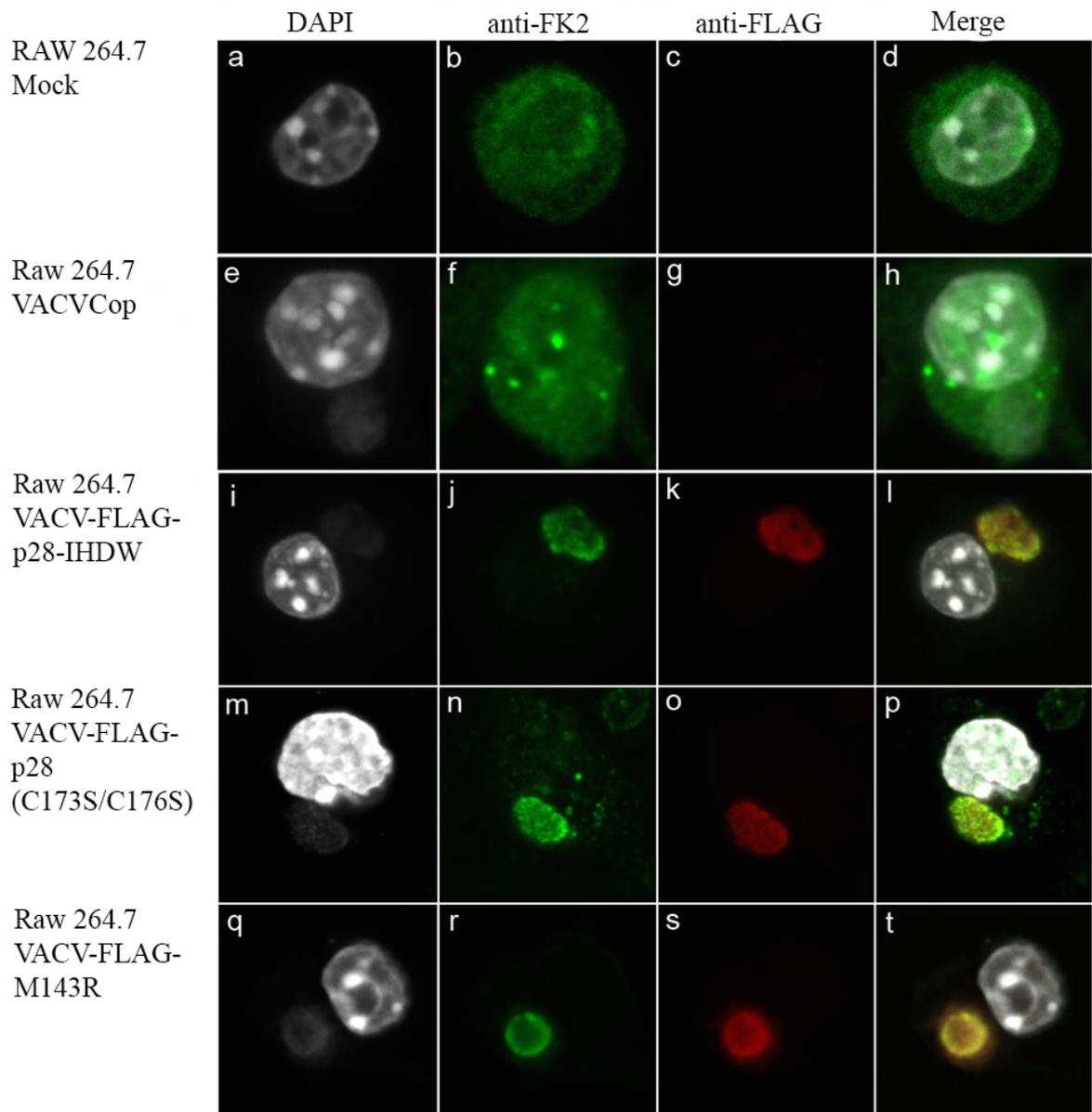
Figure 4.4 p28 homologues co-localize to the virus factory with conjugated ubiquitin in macrophage cell lines.

(A) J774, or **(B)** Raw264.7 were infected with VACVCop, VACV-FLAG-IHDW-p28, or VACV-FLAG-p28(C173S/C176S), or VACV-FLAG-M143R at an MOI of 5. Cells were fixed and treated with DAPI, to visualize the nucleus and virus factories, and stained anti-FLAG to detect FLAG-tagged proteins and anti-FK2 to detect conjugated ubiquitin. This experiment was performed three independent times and we observed on average over 100 cells per sample, which were evaluated across the entire cover slips of the samples. We showed one representative cells per sample in this Figure.

The N-terminus of p28 contains a KilA-N DNA binding domain (246), and deletions within this domain have previously been reported to prevent localization of p28 to viral factories (247). Therefore, we sought to determine if the KilA-N domain played a critical role in the co-localization of conjugated ubiquitin at the viral factory. To examine the co-localization of p28 with conjugated ubiquitin, HeLa cells were infected with VACVCop and transfected with pSC66-p28, pSC66-FLAG-p28(1-152), containing the KilA-N domain, pSC66-FLAG(153-242), containing the RING-only domain, or pSC66-FLAG(Δ44-51), missing eight amino acids within the KilA-N domain **(Figure 4.5A and 4.5B)**. Cells were stained with DAPI to visualize the virus factories and co-stained with anti-FK2 and anti-FLAG to visualize conjugated ubiquitin and p28, respectively. As expected, pSC66-FLAG-p28(IHDW) and pSC66-FLAG-p28(1-152), each containing a functional KilA-N domain, localized to the virus factory **(Figure 4.5B; panel g and k)**. In contrast, pSC66-FLAG-p28(153-242), containing the RING-only domain, and pSC66-FLAG-p28(Δ44-51), missing eight amino acids within the KilA-N domain, failed to accumulate at viral factories and instead were distributed throughout the cell and nucleus **(Figure 4.5B; panel o and s)**. The data indicate that the KilA-N DNA binding domain was critical for localization to the viral factories, as previously demonstrated (247). Given the obvious differences in localization, we next determined whether conjugated ubiquitin was present at the factory. Conjugated ubiquitin was enriched at the virus factory upon expression of FLAG-p28 **(Figure 4.5B; panel f)**. Significantly, pSC66-FLAG-p28(1-152), containing only the KilA-N domain, failed to accumulate significant amounts of conjugated ubiquitin at the virus factory, even though pSC66-FLAG-p28(1-152) localized predominantly to the virus factory **(Figure 4.5B; panel j)**. Expression of the

A

KilA-N DNA Binding   RING

YINITKI

p28

1   19 44 51   132   167   228   242

p28(1-152)

1   152

p28(153-242)

153   242

p28(Δ44-51)

1   242

B

	DAPI	anti-FK2	anti-FLAG	Merge
VACVCop	a	b	c	d
VACVCop + pSC66-FLAG-p28	e	f	g	h
VACVCop + pSC66-FLAG-p28(1-152)	i	j	k	l
VACVCop + pSC66-FLAG-p28(152–242)	m	n	o	p
VACVCop pSC66-FLAG-p28(Δ44–51)	q	r	s	t
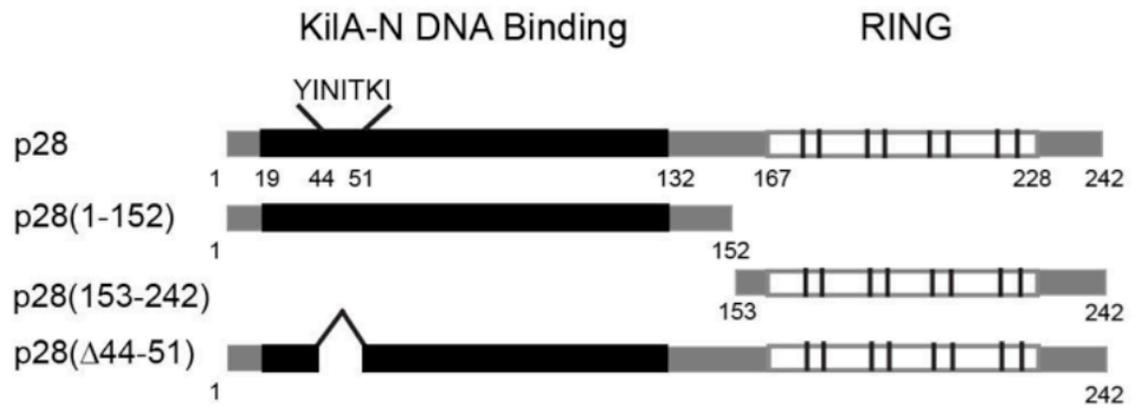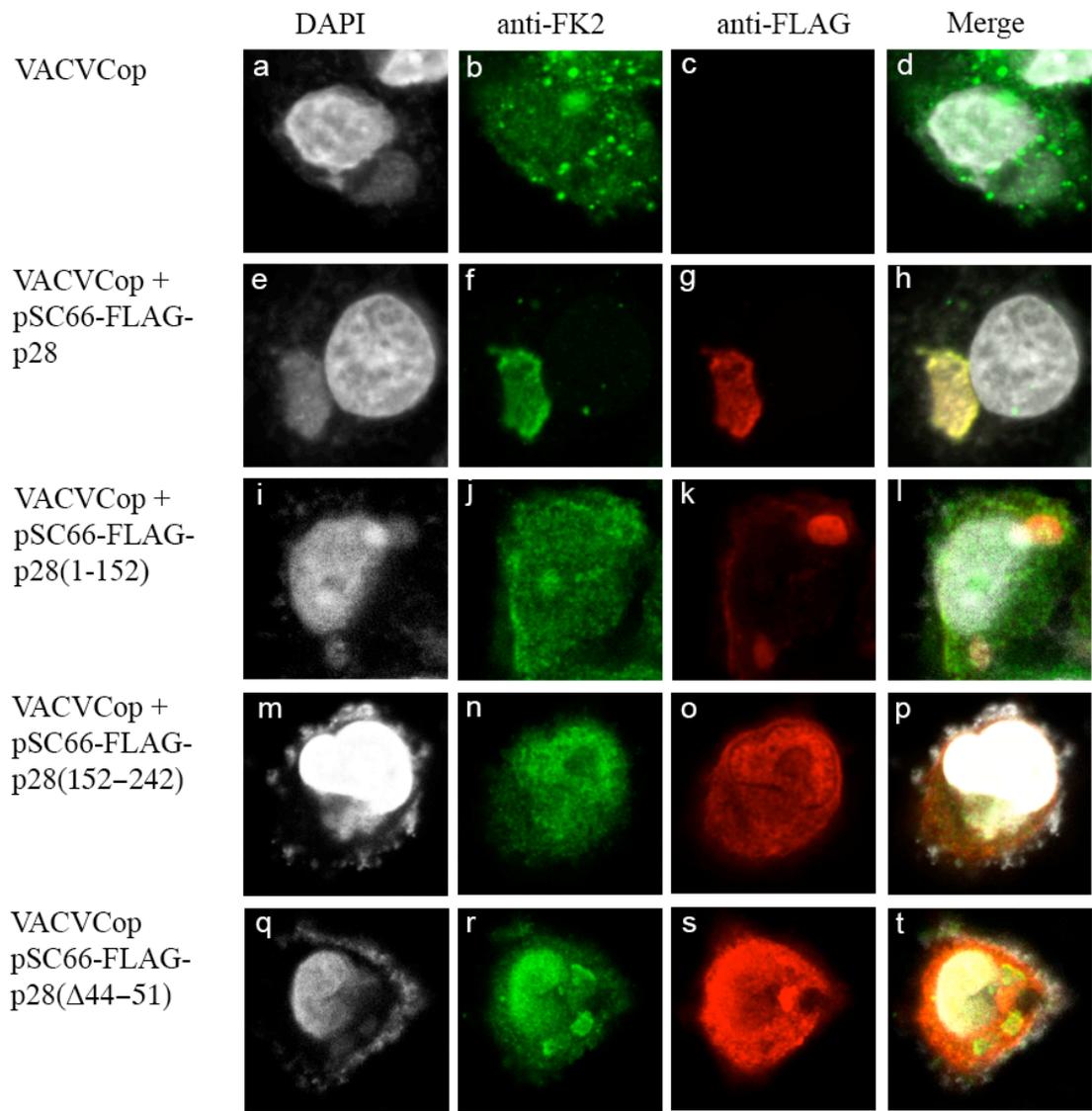
Figure 4.5 The KilA-N DNA binding domain plays a critical role in p28 localization to the virus factory, but does not affect the accumulation of conjugated ubiquitin at the virus factory.

(A) A schematic of wild type p28 and mutant versions of p28. The KilA-N DNA binding domain is highlighted in black and the RING domain is shown in white. **(B)** HeLa cells were infected with VACVCop at an MOI of 5 and transfected with pSC66-FLAG-p28, pSC66-FLAG-p28(1-152), pSC66-FLAG-p28(153-242) and pSC66-FLAG-p28(Δ44-51). Cells were fixed, stained with DAPI, and p28 was detected by anti-FLAG staining. Conjugated ubiquitin was detected by anti-FK2 staining. This experiment was performed three independent times and we observed on average over 100 cells per sample, which were evaluated across the entire cover slips of the samples. We showed one representative cells per sample in this Figure.

RING-only domain, pSC66-FLAG-p28(153-242), demonstrated only a minor enrichment of ubiquitin at the virus factory **(Figure 4.5B; panel n)**. In cells expressing FLAG-p28(Δ44-51), missing residues within the KilA-N domain, there was also only a minor accumulation of conjugated ubiquitin at the virus factory **(Figure 4.5B; panel r)**. Overall, the data indicate that wild type p28 co-localized at the virus factory with conjugated ubiquitin, whereas the p28 mutants failed to accumulate conjugated ubiquitin at the virus factory **(Figure 4.5B)**. This suggests that the localization of the RING domain at the virus factory, which is mediated by the KilA-N domain, is required for enrichment of conjugated ubiquitin at the virus factory. We next investigated the ubiquitin ligase activity of the p28 mutants to confirm their loss of catalytic activity.

4.2.4 p28 RING mutants lost ubiquitin ligase activity

We performed an *in vitro* ubiquitination assay to demonstrate the E3 ligase activity or catalytic loss of the p28 mutants and exclude residual ubiquitin ligase activity. Cells were infected with VACVCop, transfected with pSC66-FLAG-p28, pSC66-FLAG-p28(1-152), pSC66-FLAG-p28(153-242), pSC66-FLAG-p28(Δ44-51), pSC66-FLAG-p28(C173S/C176S), pSC66-FLAG-p28(1-184), or pSC66-FLAG-p28(1-204) and were subjected to immunoprecipitation using an anti-FLAG antibody. We used the very stringent RIPA lysis buffer. The resulting immune complexes were immobilized on Protein G beads, and were used as the source of ubiquitin ligase and tested in an *in vitro* ubiquitination assay (255). The formation of ubiquitin conjugates was detected by western blotting for biotinylated ubiquitin adducts. Cell lysates stained with rabbit anti-

FLAG showed the presence of all FLAG immunoprecipitated proteins. We previously demonstrated the ubiquitin ligase activity of p28 in an *in vitro* ubiquitination assay; at the same time we showed that the double cysteine mutant p28(C173S/C176S) lost its ubiquitin ligase activity (243). Immune complexes generated from infected/transfected cells expressing FLAG-p28, and p28 mutants containing an intact RING domain FLAG-p28(153-242), and FLAG-p28(Δ44-51) showed the presence of both free ubiquitin, as well as high molecular weight ubiquitin conjugates after being subjected to an *in vitro* ubiquitination assay **(Figure 4.6)**. These high molecular weight ubiquitin conjugates were absent in the p28 double cysteine mutants and p28 mutants, which lack the RING domain (pSC66-FLAG-p28(1-152)), or have truncated versions of the RING domain (pSC66-FLAG-p28(1-184) and pSC66-FLAG-p28(1-204)) **(Figure 4.6)**. This confirms that an intact RING domain is crucial for ubiquitin ligase activity in p28. We next investigated the ubiquitination pattern of the p28 mutants to see which domains are regulated by ubiquitination.

4.2.5 Both the KilA-N and RING domains of p28 are ubiquitinated in the presence of MG132

To further investigate which domains of p28 are ubiquitinated during infection, HeLa cells were infected with a VACV-HA-Ub strain, which expresses HA-tagged ubiquitin, and transfected with p28 mutants described in **Figure 4.5**. Ubiquitination was detected by blotting with anti-FLAG and anti-HA in the presence and absence of MG132 **(Figure 4.7A)**. Significantly, FLAG-p28(IHDW), FLAG-p28(1-152), FLAG-p28(153-242) and
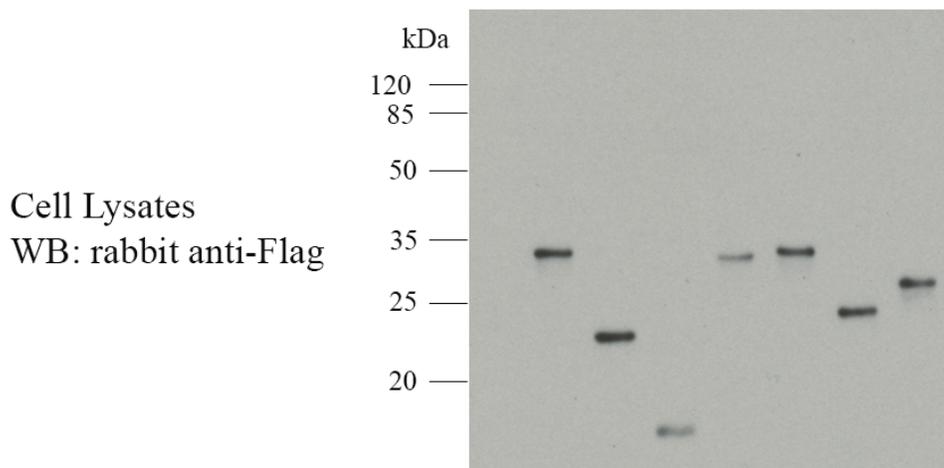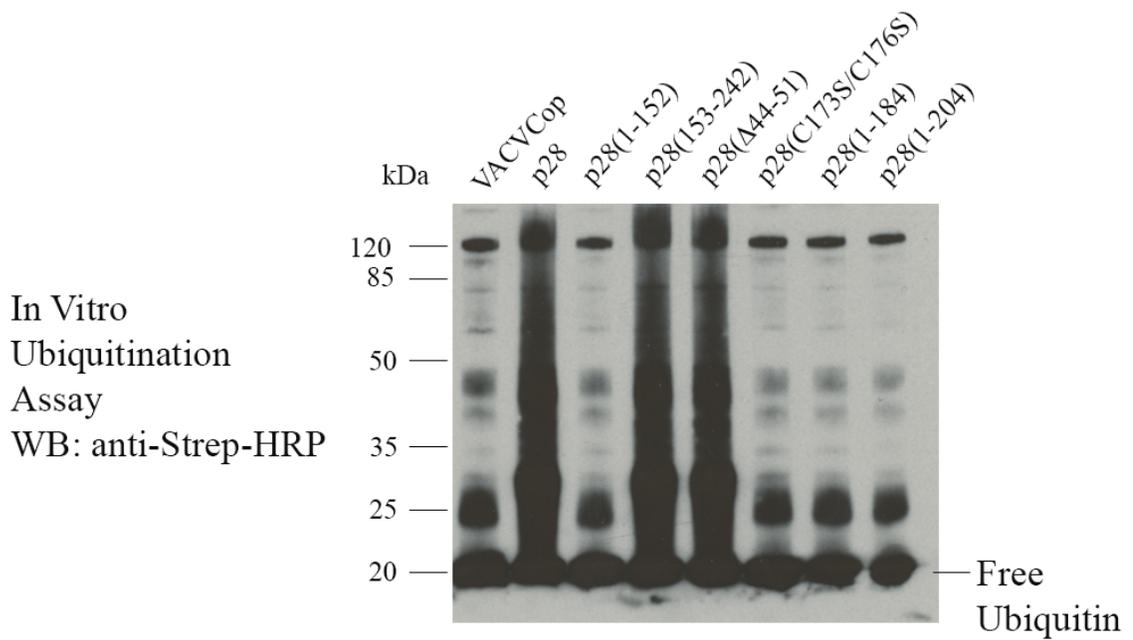
Figure 4.6 *In vitro* ubiquitin ligase activity of p28 and p28 mutants.

Protein G beads containing FLAG tagged proteins were mixed in an *in vitro* ubiquitination reaction with E1, (E2), ATP, and biotin-labeled ubiquitin (BIOMOL International). Reactions were separated by SDS-PAGE and were probed with streptavidin-tagged HRP. Western blotting shows the appearance of high-molecular mass ubiquitin adducts in reactions containing either p28, or p28 mutants containing the RING domain.

A
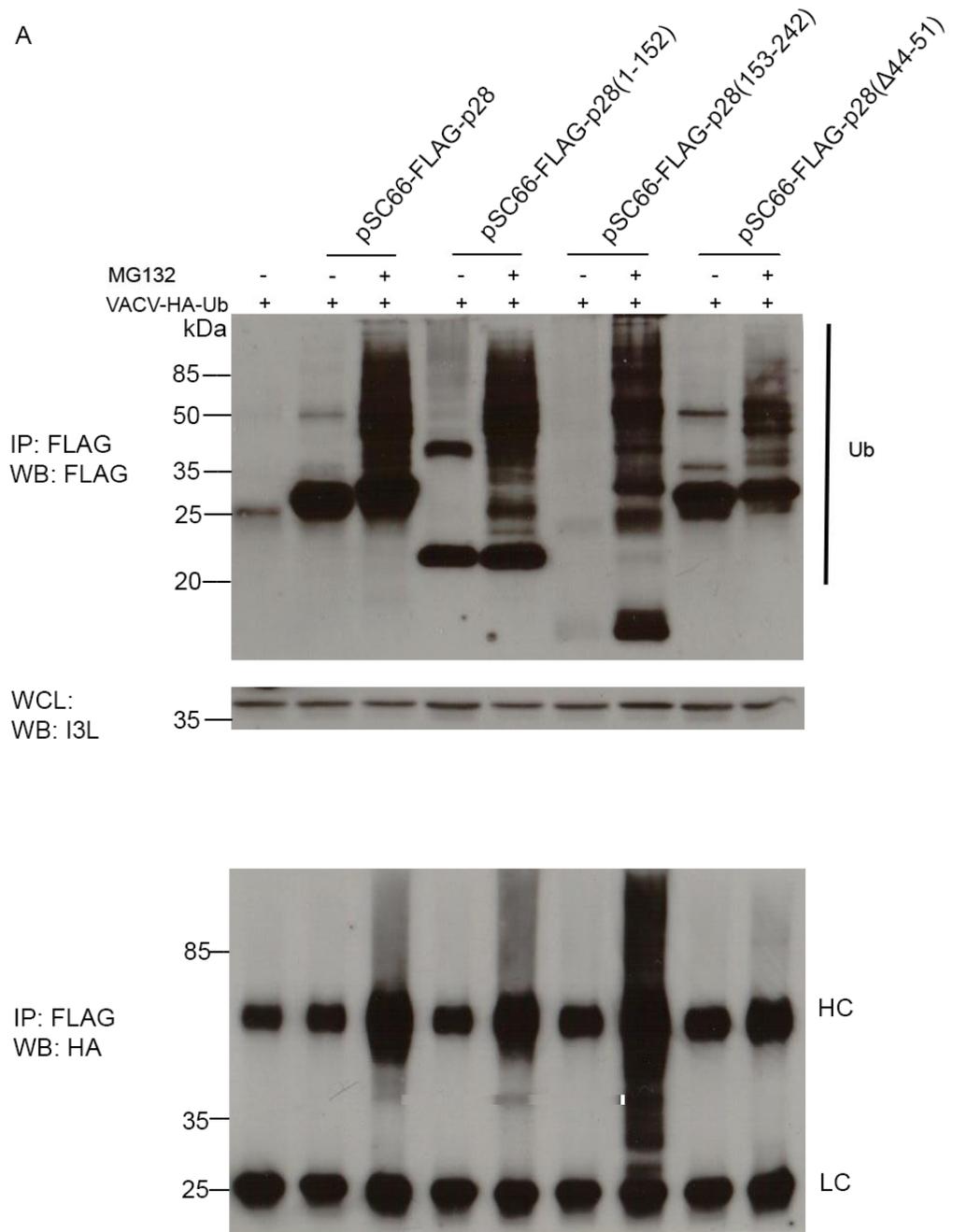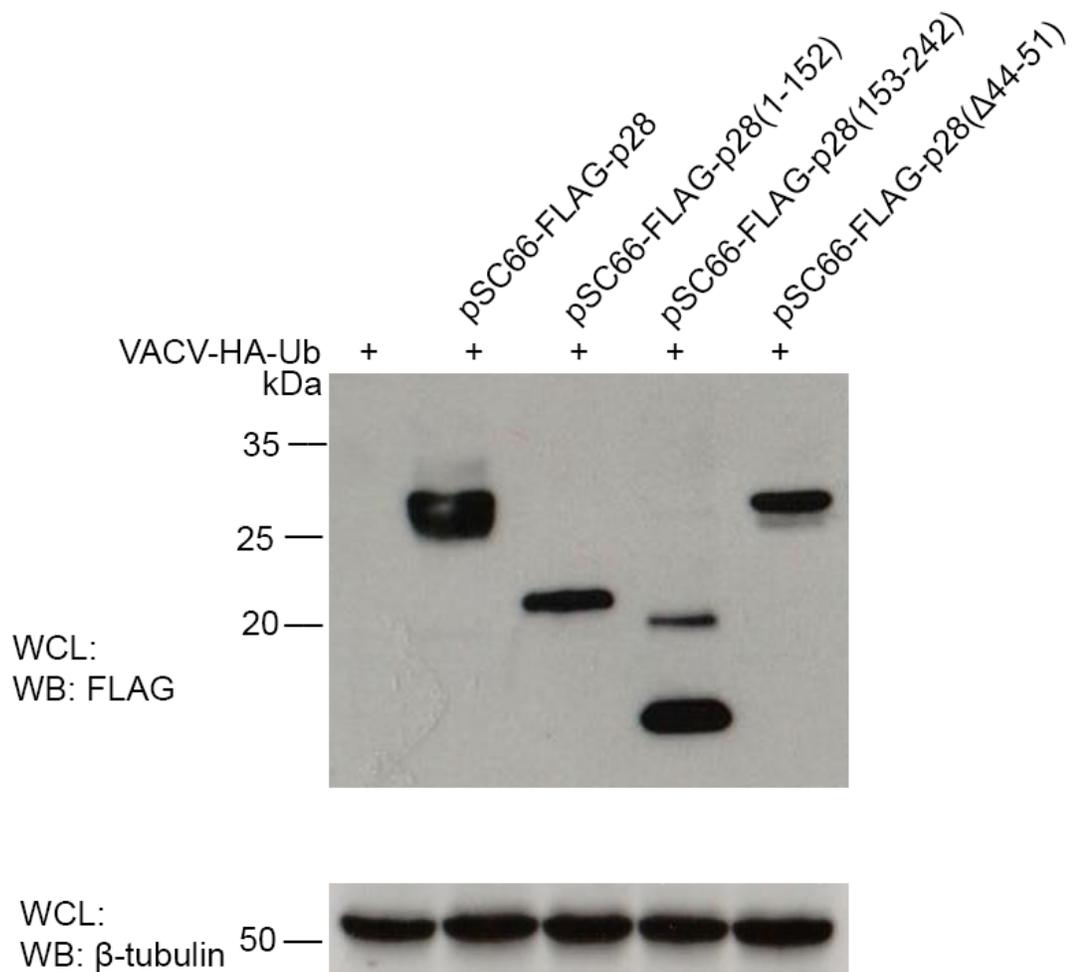
Figure 4.7 p28 is regulated by ubiquitination.

(A) To detect ubiquitination, HeLa cells (2 x 10^6) were infected with VACV-HA-Ub and transfected with pSC66-FLAG-p28, pSC66-FLAG-p28(1-152), pSC66-FLAG-p28(153-242) or pSC66-FLAG-p28(Δ44-51). Cells treated with or without MG132 (10mM) for six hours were lysed in RIPA buffer and p28 constructs were immunoprecipitated with mouse anti-FLAG M2 and western blotted with rabbit anti-FLAG and anti-HA to detect ubiquitination **(B)** Whole cell lysates were ubiquitinated in the presence of MG132 **(Figure 4.7A)**. The next section evaluated the role of a functional RING domain for the ubiquitination-mediated regulation of p28. were harvested and western blotted with anti-FLAG and anti-β-tubulin as a loading control.

FLAG-p28(Δ44-51) were all ubiquitinated upon treatment with MG132 (Figure 4.7A).

Despite obvious differences in viral factory localization (Figure 4.5B), all four proteins

were ubiquitinated in the presence of MG132 (Figure 4.7A). The next section evaluated

the role of a functional RING domain for the ubiquitination-mediated regulation of p28.

4.2.6 p28 catalytic non-functional RING mutants are still stabilized in the presence

of MG132

p28 is controlled by proteosomal degradation and many ubiquitin ligases regulate

themselves through autoubiquitination (139, 272, 273). Given this, we sought to

determine the role of the RING domain in autoubiquitination. We generated three p28

RING mutants (243), pEGFP-p28(C173S/C176S), containing two point mutations critical

for ubiquitin ligase function, pEGFP-p28(1-184), a truncated version lacking the majority

of the RING domain, and pEGFP-p28(1-204), lacking amino acids 205-242 of the RING

domain, thereby disrupting the ubiquitin ligase activity of p28 **(Figure 4.8A)** (243). Each

of the three mutant proteins were analyzed for expression in the presence or absence of

MG132 via flow cytometry **(Figure 4.8B)**. Furthermore, cells were incubated in TMRE

containing media. TMRE is a cell permeable dye, which is readily accumulated within

healthy, respiring mitochondria (274). The loss of mitochondrial membrane potential in

unhealthy cells is associated with a decrease in TMRE uptake (274). Here, we used

TMRE as an indication for healthy cells. As shown previously, EGFP expression was

unaltered upon MG132 treatment **(Figure 4.8B; panel a and b)**, while EGFP-

p28(IHDW) was stabilized upon the addition of MG132 suggesting it is regulated by

proteasomal degradation **(Figure 4.8B; panels c and d)**. In the absence of MG132,

A

KilA-N DNA Binding   RING
                                          C173 C196 C202 C222
                                          C176 H198 C205 C225

p28
1 19   44 51   132   167   228 242

YINITKI

                                          C173S
                                          C176S
p28(C173S/C176S)
1   242

p28(1-184)
1   184

p28(1-204)
1   204
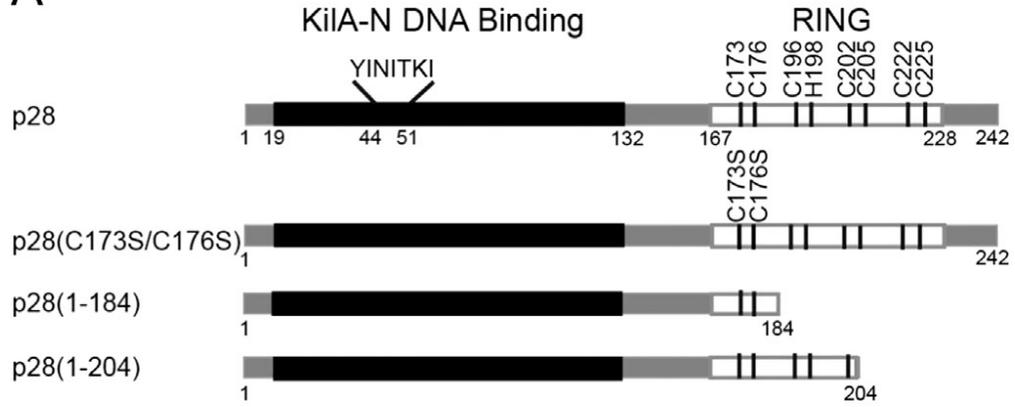
B
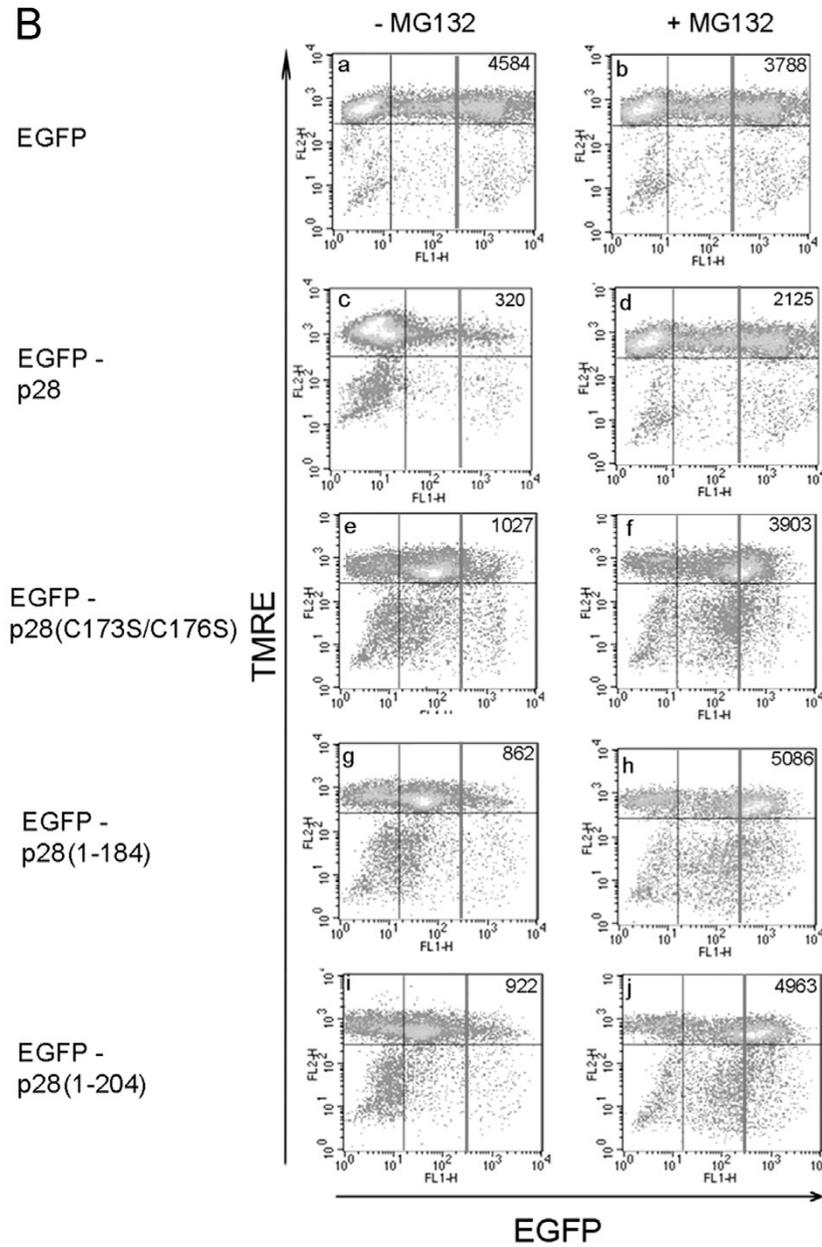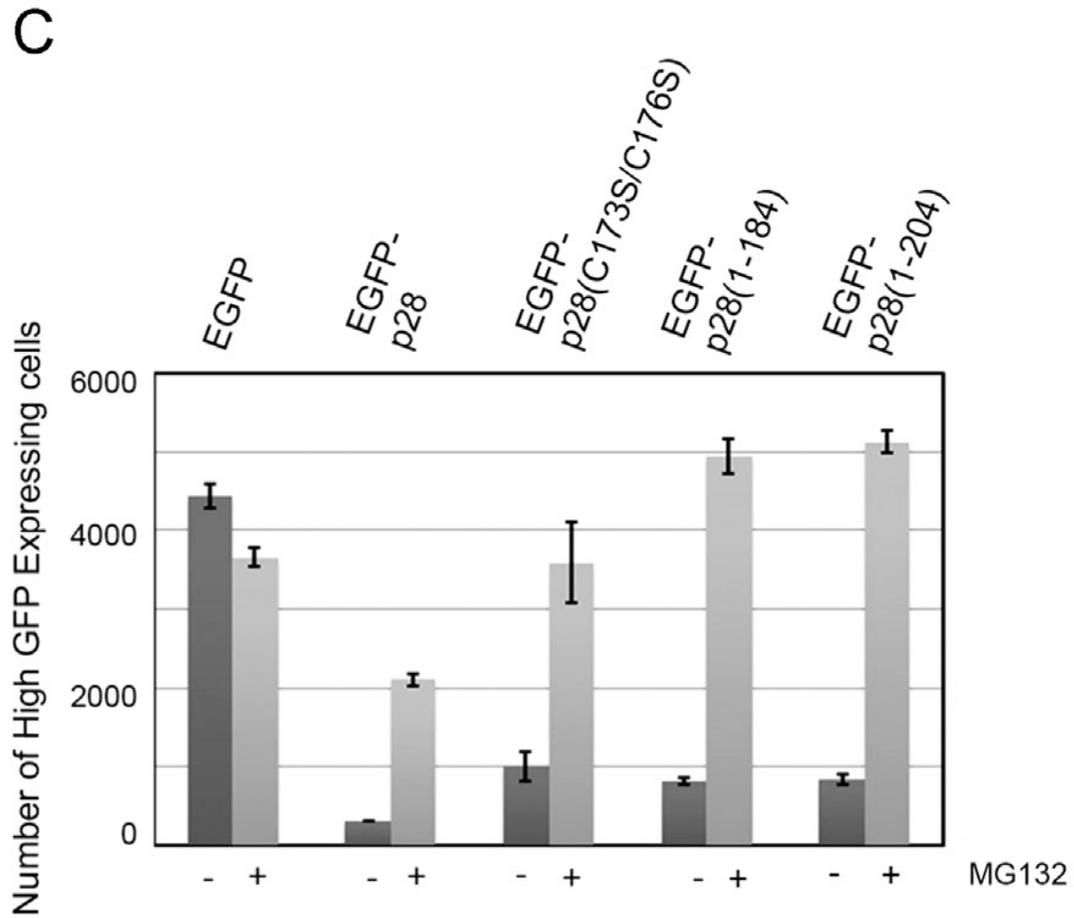
Figure 4.8 Mutations in the RING domain stabilize p28 (Kelly Mottet).

(A) Schematic representation of the p28 RING mutants. The data shown in **(B)** was collected by Kelly Mottet and repeated in triplicate and graphed as the number of high GFP-expressing cells in the upper right quadrant **(C). (B)** HeLa cells were transfected with pEGFP-p28(C173S/C176S), pEGFP-p28(1-184) or pEGFP-p28(1-204) for 16 hours and treated with or without 10μM MG132 for 6 hours. Cells were labeled with 0.2μM, washed and resuspended in PBS containing 1% FBS and analyzed by two-color flow cytometry **(C)** The number of EGFP cells were quantified in triplicate in the presence and absence of MG132.

EGFP-p28(C173S/C176S), EGFP-p28(1-184), and EGFP-p28(1-204) were stabilized due to inactivation or absence of the RING domain **(Figure 4.8B)**, and were further stabilized upon MG132 treatment **(Figure 4.8B)**, indicating that wild type p28 was regulated by proteasomal degradation **(Figure 4.8B).** The data indicated that mutation of the RING domain results in increased

stabilization of the p28 mutant proteins, further suggesting that wild type p28(IHDW) uses its RING domain to regulate itself by autoubiquitination. However, since the RING mutants demonstrated an increase in stabilization this suggests that p28 is further regulated by another ubiquitin ligase.

4.2.7 An additional ubiquitin ligase promotes the accumulation of conjugated ubiquitin at the virus factory

Since the p28(IHDW) RING mutants from the previous section were subjected to proteasomal degradation **(Figure 4.8)**, we sought to determine if conjugated ubiquitin localized to viral factories in their presence **(Figure 4.9)**. HeLa cells were mock-infected or infected with VACVCop and transfected with pSC66-FLAG-p28(IHDW), pSC66-FLAG-p28(C173S/C176S), pSC66-FLAG-p28(1-184) and pSC66-FLAG-p28(1-204). As expected, conjugated ubiquitin co-localized with FLAG-p28(IHDW) at the viral factory **(Figure 4.9)**. Conjugated ubiquitin was still present at the virus factory when FLAG-p28(C173S/C176S) was expressed in cells **(Figure 4.9)**, suggesting that an additional ubiquitin ligase was responsible since FLAG-p28(C173S/C176S) lacks intrinsic ubiquitin ligase activity (243). Similarly, conjugated ubiquitin was also present at the viral factory upon expression of FLAG-p28(1-204) **(Figure 4.9)**. In contrast, little ubiquitin was
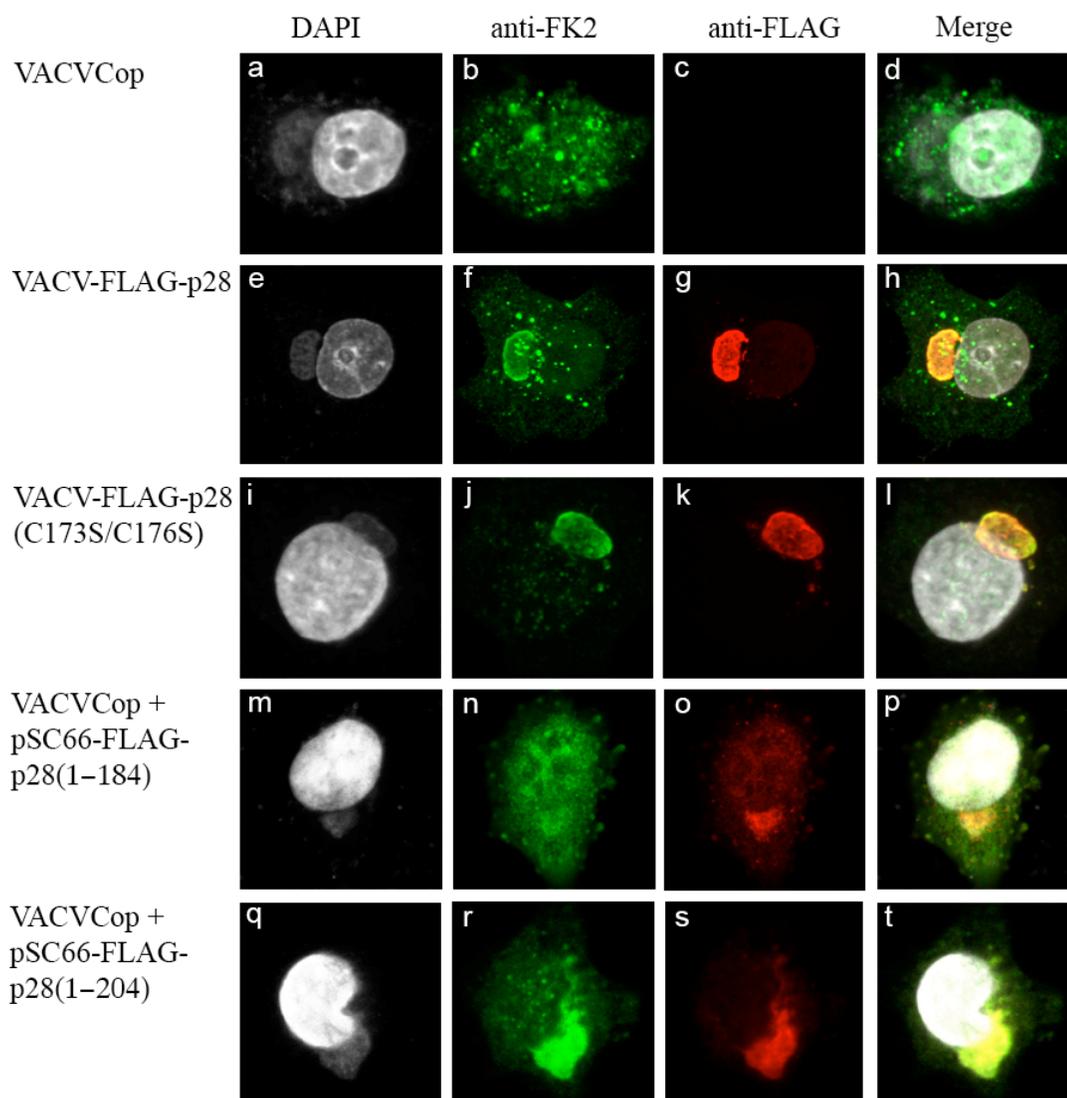
(A) HeLa cells were mock infected or infected with VACV at an MOI of 5 and transfected with pSC66-FLAG-p28, pSC66-FLAG-p28(C173S/C176S), pSC66-FLAG-p28(1-184), or pSC66-FLAG-p28(1-204). Cells were fixed, stained with DAPI, to visualize the nucleus and cytoplasmic virus factories, and stained with anti-FK2 and anti-FLAG to detect conjugated ubiquitin and p28 protein expression, respectively. This experiment was performed three independent times and we observed on average over 100 cells per sample, which were evaluated across the entire cover slips of the samples. We showed one representative cells per sample in this Figure.

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A
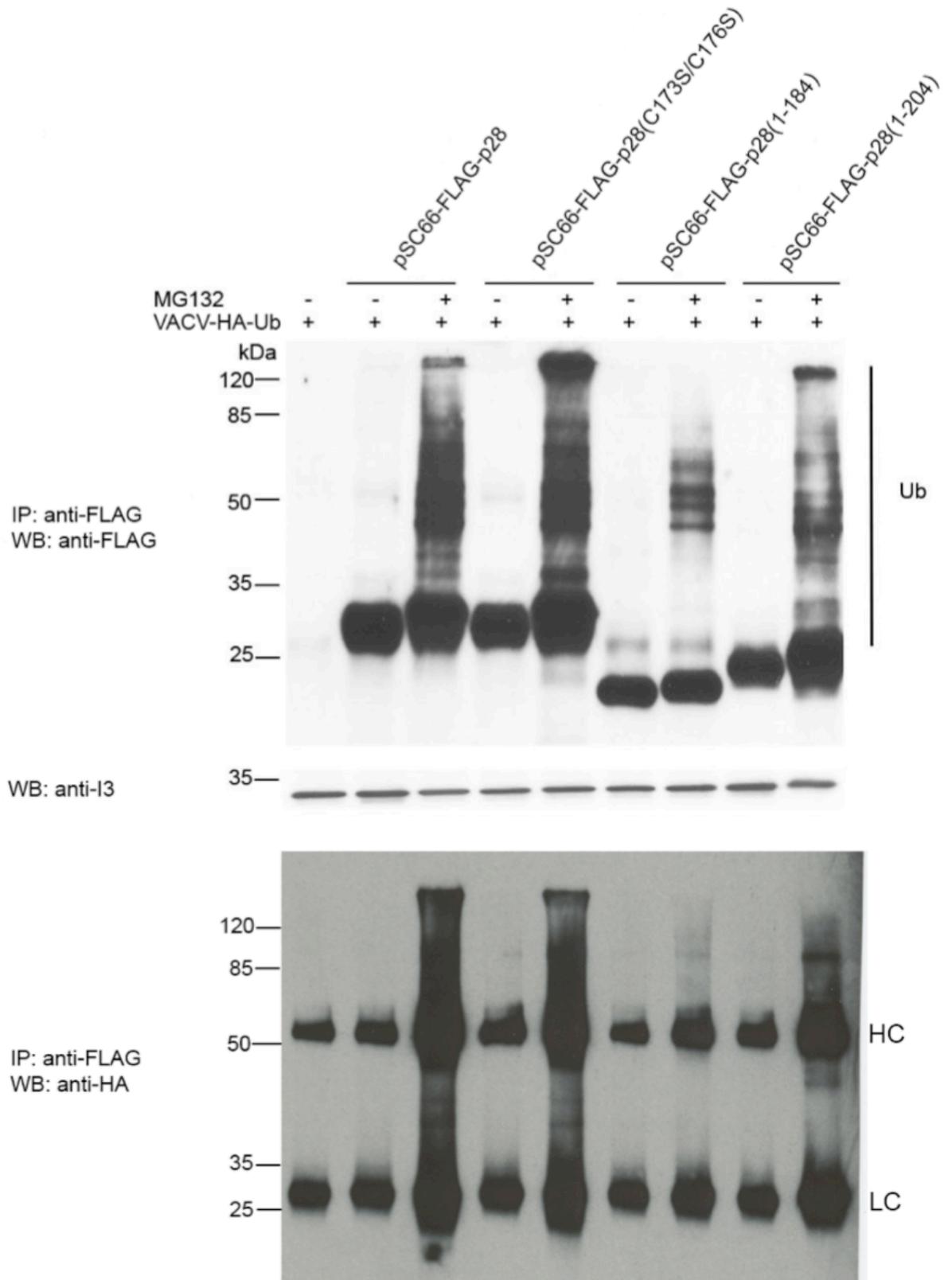
B
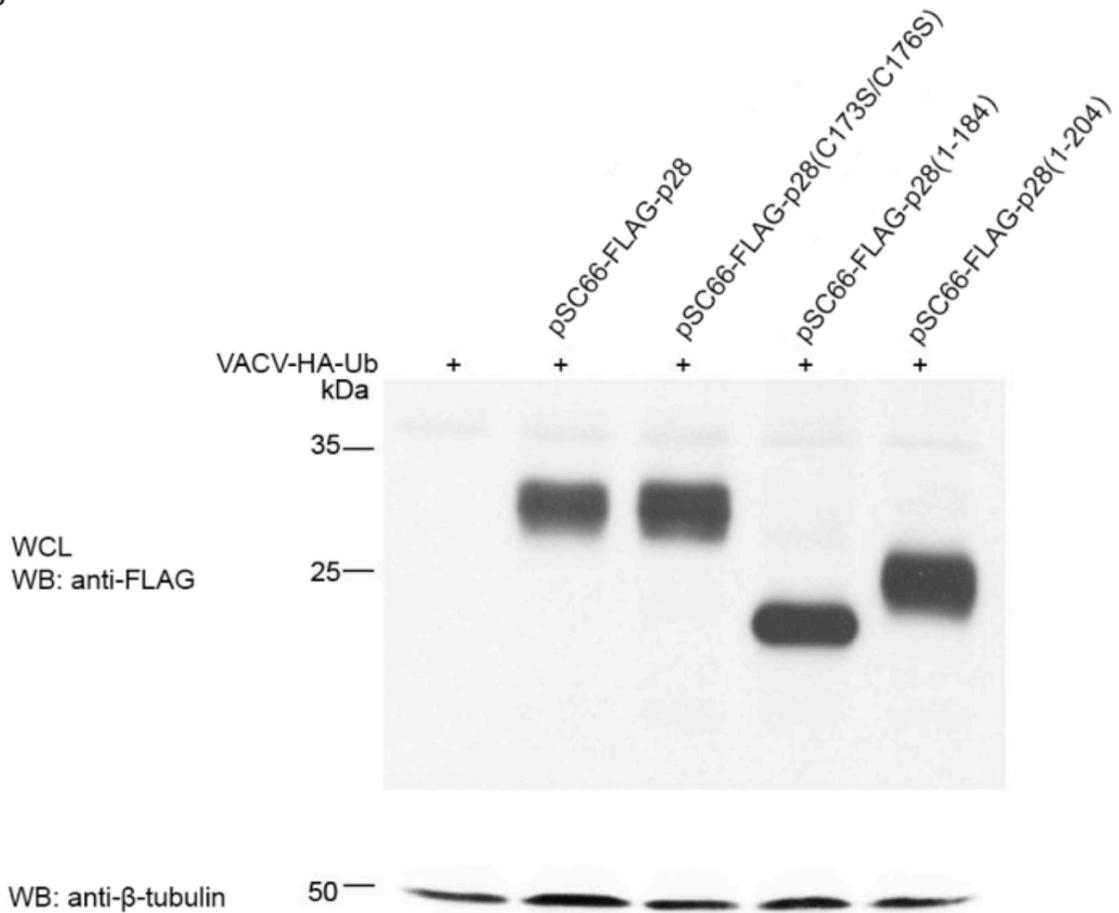
Figure 4.10 p28 RING mutants demonstrate variable patterns of ubiquitination.

(A) To detect ubiquitination, HeLa cells (2 x 10^6) were infected with VACV and transfected with pSC66-FLAG-p28, pSC66-FLAG-p28(C173S/C176S), pSC66-FLAG-p28(1-184), or pSC66-FLAG-p28(1-204). Cells treated with or without MG132 (10mM) for six hours were lysed in RIPA buffer and p28 constructs were immunoprecipitated with mouse anti-FLAG M2 and western blotted with rabbit anti-FLAG and anti-HA to detect ubiquitination. **(B)** Whole cell lysates were harvested and western blotted with anti-FLAG and anti-beta-tubulin as a loading control.

detected at the virus factories in FLAG-p28(1-184)-expressing cells **(Figure 4.9)**, suggesting that the region between amino acids 184 and 204 may be critical for the ubiquitination of p28 by another ubiquitin ligase. The data indicate that at least one unknown additional ubiquitin ligase is responsible for the presence of conjugated ubiquitin at viral factories. To further investigate the ubiquitination of the p28 RING mutants we infected HeLa cells with VACVCop and transfected cells with p28 RING mutants in the absence and presence of MG132. Ubiquitination was detected by blotting with anti-FLAG and anti-HA **(Figure 4.10A and B)**. pSC66-FLAG-p28(IHDW) and pSC66-FLAG-p28(C173/176S) demonstrated obvious ubiquitination upon treatment with MG132 **(Figure 4.10A)**. In contrast, pSC66-FLAG-p28(1-184) and pSC66-FLAG-p28(1-204) showed less obvious ubiquitination upon treatment with MG132 **(Figure 4.10A)**.

4.3 Summary and Brief Discussion

Poxviruses survive and replicate within host cells through the manipulation of cellular pathways (20, 30). Recently, poxvirus manipulation of the host ubiquitin-proteasome system has become increasingly apparent (229). Since the ubiquitin-proteasome system plays an important role in cellular homeostasis, many viruses have evolved strategies to regulate the process of ubiquitination to their own advantage, and poxviruses are no exception (211, 212). Recent evidence indicates that poxviruses encode a family of BTB/kelch proteins and ankyrin/F-box proteins that likely function as substrate adaptors for cellular cullin-3 and cullin-1 based ubiquitin ligases (235, 240, 269, 270, 275-281). Additionally, poxviruses have also been shown to encode proteins with intrinsic ubiquitin ligase activity (242, 243). These include M153R, a membrane associated RING-CH

ubiquitin ligase encoded by MYXV, and p28, a RING encoded ubiquitin ligase (73, 242, 243). p28 is a highly conserved ubiquitin ligase that has been shown to be a critical virulence factor (242-245). Here we further investigate the regulation of p28.

Since many cellular and viral ubiquitin ligases are regulated through ubiquitination and autoubiquitination, we investigated the ubiquitin-proteasomal dependent regulation of p28. We first set out to determine if the ubiquitin-proteasome-system was functional during infection with a number of poxviruses, by measuring the chymotrypsin-like activity of the proteasome **(Figure 4.1)**. These include VACV, CPXV, ECTV and FWPV in the presence and absence of MG132. Overall, we detected a functional proteasome during all types of poxvirus infections. This is in agreement with the literature, where an active proteasome was reported during VACV infection (282). The fact that the ubiquitin-proteasome system remains functional indicates a great advantage for poxviruses in using the cellular systems and encoding their own ubiquitin ligases. However, there is a potential disadvantage, since cellular ubiquitin ligases with anti-viral features can still function, as well.

In addition to p28(IHDW), other poxvirus encode p28-like ubiquitin ligases, as well. For example, p28 is conserved throughout the *Orthopoxviridae*; distantly related p28 proteins are found in other genera, including M143R encoded by MYXV, and FWPV150 encoded by FWPV (22, 283). Attenuated poxviruses, such as VACV strain Copenhagen and Western Reserve, contain deleted or truncated versions of p28, suggesting that p28 plays an important role in virulent viruses (284). Despite the lower sequence identity compared to p28-IHDW, both M143R and FWPV150 co-localized with conjugated ubiquitin at the virus factory indicating that p28 function is highly conserved

among members of the poxvirus family **(Figure 4.3)**. Our data indicate that localization of p28 at the viral factories was dependent on the KilA-N DNA binding domain **(Figure 4.5)** that is found in bacteria, bacteriophage and large DNA viruses (246, 247). As suspected, the KilA-N domain alone was not sufficient to trigger the enrichment of conjugated ubiquitin at the virus factories **(Figure 4.5)**. This is in accordance with our data in Chapter 3, where the KilA-N only protein FWPV075 in FWPV localized to the virus factory, without effect on accumulating conjugated ubiquitin at the virus factory **(Figure 3.9)**.

We demonstrated the catalytic loss of ubiquitin ligase activity in p28 mutants lacking the RING domain, or containing mutations and truncations within the RING domain **(Figure 4.6).** Bearing this in mind, confocal imaging demonstrated conjugated ubiquitin at the virus factories in the presence of two of the RING mutants, FLAG-p28(C173S/C176S) and FLAG-p28(1-204) **(Figure 4.7)**. In contrast, it appears that FLAG-p28(1-184) demonstrated only minor amounts of ubiquitination **(Figure 4.8)**. Again, all three RING mutants lacked intrinsic ubiquitin ligase activity **(Figure 4.9)**, indicating that the conjugated ubiquitin present at the factory was due to the activity of another ubiquitin ligase.

Since many cellular and viral ubiquitin ligases are regulated through ubiquitination and autoubiquitination, we investigated the regulation of p28 by the UPS (139). We used a flow cytometry assay to determine if p28 was regulated by the proteasome. In the absence of a proteasome inhibitor, EGFP-p28 was detected at low levels. However, upon MG132-mediated inhibition of the proteasome, p28 was significantly stabilized and detected at high levels **(Figure 4.6)**, suggesting that p28 is

regulated by proteasomal degradation. This might be a mechanism to temporally control the activity of p28 during different stages of infection.

Given that our data indicates that p28 is tightly controlled by proteasomal degradation, we further examined the three p28 RING mutants p28(C173S/C176S), p28(1-184), and p28(1-204), which lost the ability of auto-ubiquitination **(Figure 4.6)**. In the absence of MG132, all three of the RING mutants were detected at low levels, and were stabilized in presence of MG132. Interestingly, in the presence of MG132, the RING mutants were detected at even higher levels compared to wildtype p28 **(Figure 4.6B)**. This suggests that the RING domain of p28 plays a role in ubiquitinating itself, and is further regulated by another ubiquitin ligase, leading to subsequent degradation via the proteasome. A large number of ubiquitin ligases are known to be autoubiquitinated in order to regulate themselves (139, 285). For example, Mdm2 is known to mediate its own degradation through autoubiquitination (286, 287). Similar to p28, mutating the RING domain of Mdm2 inhibits ubiquitin ligase activity stabilizing Mdm2 (139). Importantly, upon MG132 treatment, we found that all three p28 RING mutants were further stabilized **(Figure 4.5B)**. Similar to p28, Mdm2 stability is also regulated by other ubiquitin ligases. This event appears to be common within the ubiquitin-proteasome pathway (288). Surprisingly, there was no significant difference between the stabilization of p28(1-184) and p28(1-204) in our transfection model. It is possible, that the ubiquitin ligase, which is ubiquitinating p28 is of poxviral origin and was missing in this model. Future experiments will investigate the MG132-mediated stabilization of p28(1-184) and p28(1-204) in presence of VACVCop infection.

Our data suggests that p28 is a dually regulated ubiquitin ligase, which regulates itself through autoubiquitination in addition to being regulated by another ubiquitin ligase or ligases. Identification of this unknown ubiquitin ligase or ligases will provide further insight into the regulation of p28.

CHAPTER 5: IDENTIFICATION OF P28 INTERACTION PARTNERS

This work represents a publication in progress. I performed all experiments presented here with input from Dr. Ingham, except for the Mass Spectrometry, which was performed by Jack Moore in a collaboration with the Dr. Richard Fahlman's group (Department of Biochemistry, University of Alberta).The first draft of this chapter was written by myself, while the final draft had editorial contributions from Dr. Ryan Noyce and Dr. Ingham.

5.1 Introduction

p28 is a E3 ubiquitin ligase, found in diverse members of the poxvirus family, including VACV, ECTV, MYXV, Shope fibroma virus, variola virus and avipoxviruses (22, 23, 289). The identification and characterization of both cellular and viral protein substrates targeted by p28 is important to understand its role as a virulence factor during infection and is the focus of this chapter.

Over the years a significant amount of effort has been devoted to identifying ubiquitinated target proteins as well as to understanding the role of ubiquitin ligases during cellular processes (290-295). Identification of ubiquitinated substrates, however, has generally been difficult due to the transient interaction of ubiquitin ligases with their substrates (296). Furthermore, substrates of ubiquitin ligases can also be of low abundance within cells, making their detection even more difficult (297).

In this study, we used the traditional interaction-based method of co-immunoprecipitating FLAG-tagged p28 during VACV infection to detect both cellular and viral protein substrates of the ubiquitin ligase p28. Cells were treated with MG132, a proteasomal inhibitor, to prevent the degradation of ubiquitinated substrates and allow for easier detection. A p28-FLAG expression construct lacking its ubiquitin ligase activity (C173S/C176S) was included as well since this protein should still likely interact with substrates without their subsequent ubiquitin-mediated degradation. Mass spectrometry identified over 100 cellular and viral proteins associated with the poxviral ubiquitin ligase p28 **(Appendix, Table A1 and A2)**. 56% of the hits were identified in both the p28 and the p28 mutant immunoprecipitations, while 29% of the hits were only present in the p28

immunoprecipitations and 15% were only associated with the p28 mutant. Many of the co-immunoprecipitated cellular proteins associated with p28 and the p28 mutant are known members of (i) DNA mismatch repair proteins and DNA stress sensors, including MSH2, MSH6 and ATM; (222) components of the nuclear import and export machinery; (iii) the heat shock protein (HSP) family, including HSP70 and HSP90; as well as (iv) poxviral proteins (**Figure 5.2**). A number of these cellular and poxviral proteins accumulate at virus factories following infection, indicating that these proteins might play a role in viral replication, morphogenesis, or viral DNA sensing. A number of experiments were carried out to confirm the identity of these proteins and the authenticity of their association with p28 and p28(C173S/C176S). MSH2, ATM, exportin-1, HSP70 and HSP90 were detected in anti-FLAG immunoprecipitates from lysates of HeLa cells infected with VACVCop expressing FLAG-p28. Furthermore, the colocalization of these proteins with p28 at the viral factories was monitored by immunofluorescence microscopy. Only HSP70 was enriched at virus factories in the presence of FLAG-p28. Interestingly, HSP70 was not ubiquitinated in the presence of p28, suggesting that it did not act as a substrate for p28. Furthermore, confocal analysis showed that there was a trend within p28 homologues in MYXV and FWPV towards triggering enrichment of HSP70 at the virus factory.

5.2 Results

5.2.1 Numerous cellular and viral proteins co-immunoprecipitate with FLAG-p28

To identify interaction partners and potential substrates of p28, anti-FLAG immunoprecipitates were collected from lysates in HeLa cells infected with VACVCop,

148
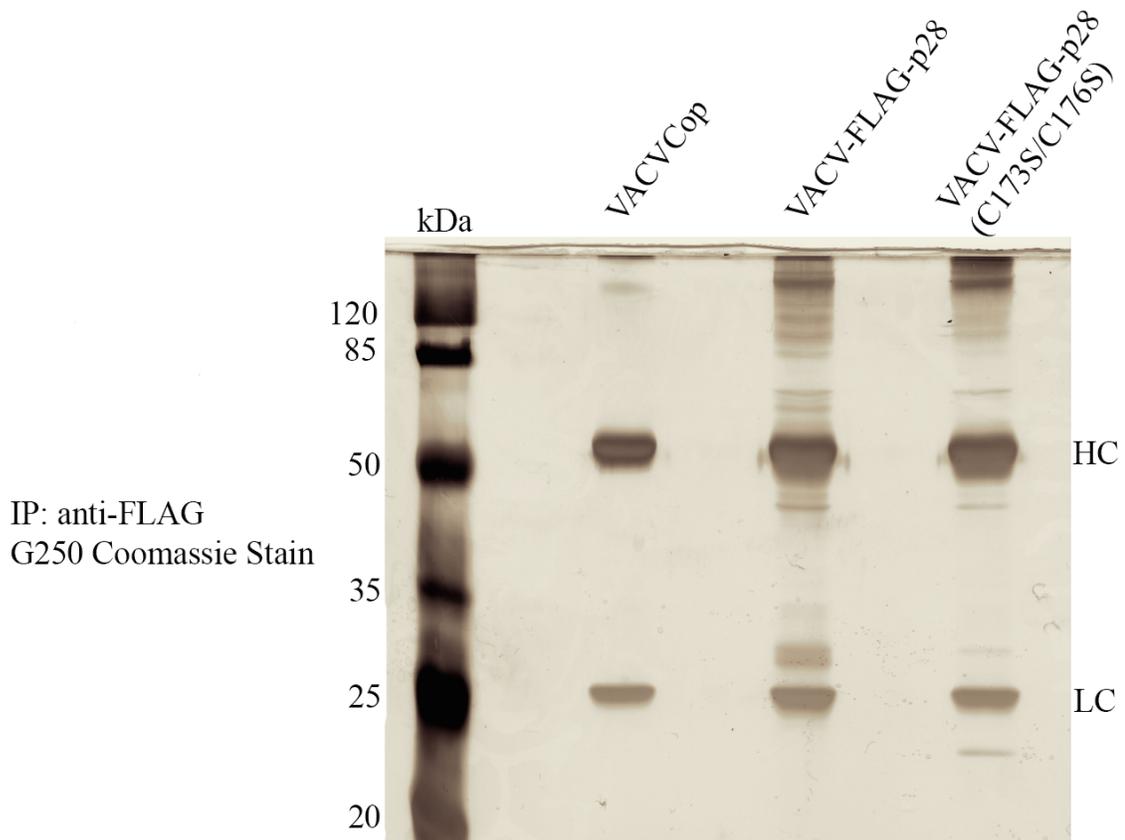
Figure 5.1 FLAG-p28 and FLAG-p28(C173S/C176S) mutant-interacting proteins.

Coomassie blue stained SDS-PAGE gel of immunoprecipitates from lysates of VACVCop, VACVCop expressing FLAG-p28, or VACVCop expressing FLAG-p28(C173S/C176S). Gel slices of the entire lane for each sample were digested and analysed by Mass spectrometry in collaboration with the Fahlman group. Heavy chain (HC), Light chain (LC).

VACVCop expressing FLAG-p28, and VACVCop expressing a mutant of p28 that lacks the ubiquitin ligase activity, FLAG-p28(C173S/C176S). We included the FLAG-p28(C173S/C176S) in these experiments because we postulated that this mutant may bind substrates but not target them for degradation. Immunoprecipitates were run on an SDS-PAGE gel and stained with Coomassie Brilliant Blue G250 **(Figure 5.1)**. In our negative control, immunoprecipitates from lysates of VACVCop-infected cells, the only bands observed were the heavy and light chain of the anti-FLAG antibody. In contrast, we detected multiple bands in the immunoprecipitates from the lysates of VACV-FLAG-p28-infected cells. The banding pattern observed in the immunoprecipitates from the VACV-FLAG-p28(C173S/C176S) infected cells were very similar to those observed in the immunoprecipitates of the VACV-FLAG-p28-infected cells. To identify the proteins present in the p28 immunoprecipitates, whole lanes were cut into smaller pieces and sent for identification using mass spectrometry in collaboration with Dr. Richard Fahlman's group (Department of Biochemistry, University of Alberta). The following subsection will cover bioinformatics analysis to group and highlight interesting p28 hits.

5.2.2 Identification and functional grouping of p28 mass spectrometry hits

This subsection deals with the bioinformatics analysis and decision-making, which mass spectrometry hits to follow up with in the next subsections. The cellular and viral proteins that co-immunoprecipitated with FLAG-p28 were identified using a LC-MS/MS instrument and Thermo Proteome Discoverer software **(Appendix, Table A1 and A2)**. Mass spectrometry hits were considered significant hits when the protein had at least two

A

p28
15 VACV Hits

Both
24 VACV Hits

p28
(C173S/C176S)
14 VACV Hits

B

p28
62 Human Hits

Both
124 Human Hits

p28
(C173S/C176S)
24 Human Hits

C

p28 Human Hits

- Ubiquitin-Proteasome system
- HSP/Chaperone protein folding
- Nuclear transport
- RNA binding
- DNA damage stress sensors
- others

28%, 8%, 20%, 18%, 16%, 10%

D

p28(C173S/C176S) Human Hits

- Ubiquitin-Proteasome system
- HSP/Chaperone protein folding
- Nuclear transport
- RNA binding
- DNA damage stress sensors
- others

26%, 8%, 21%, 17%, 14%, 14%

Figure 5.2 Analysis of functional annotation clustering for p28 mass spectrometry hits.

(A) Overlap of VACV Hits and **(B)** Human Hits in p28 and p28(C173S/C176S) samples. (**C**) DAVID Bioinformatics Resources 6.7 was used to analyze functional annotation clustering results from the mass spectometry hits for p28 cellular interaction partners and **(D)** p28(C173S/C176S) cellular interaction partners. Amongst others, we found clustering of hits belonging to the ubiquitin-proteasome system, DNA damage and stress response pathways, HSP family, and components of the nuclear transport machinery.

peptides identified at a medium confidence according to the common Molecular and Cellular Proteomics (MCP) guidelines. We excluded mass spectrometry hits, found in the

negative control of immunoprecipitates from lysates of HeLa cells infected with VACVCop. Interestingly, most of the proteins that were identified through co-immunoprecipitation with FLAG-p28 were also present in lysates co-immunopreciptated with FLAG-p28(C173S/C176S) following poxvirus infection **(Figure 5.2)**.

To extract biological features associated with the identified mass spectrometry protein hits, DAVID Bioinformatics Resources 6.7 (DAVID; National Institute of Allergy and Infectious Diseases [NIAID, NIH], http://david.abcc.ncifcrf.gov) was used for the analysis of functional annotation clustering (256). A number of the protein hits seemed to cluster into major cellular pathways, including the UPS, DNA damage and stress response pathways, the HSP family, as well as components of the nuclear transport machinery **(Figure 5.2)**. Many of these proteins played an important role in the life cycles of other viruses, but have not been well documented during poxvirus infection (298-316). Several protein hits identified during the mass spectrometry screen were further characterized to confirm whether any interaction with p28 could be detected.

5.2.3 Verification of viral and cellular p28 mass spectrometry hits with co-immunoprecipitation studies

To confirm mass spectrometry interaction partners and exclude false positive hits, we analyzed anti-FLAG immunoprecipitates from lysates of HeLa cells infected with VACVCop, VACVCop expressing FLAG-p28, or VACVCop expressing FLAG-

p28(C173S/C176S) by western blotting using antibodies specific for the identified p28 interaction partners. We included the VACVCop as a negative control and the FLAG-p28(C173S/C176S) control to keep samples consistent with the mass spectrometry experiment explained in section 5.2.1. WCL of HeLa cells infected with VACVCop expressing FLAG-p28 were also included to confirm that the interacting proteins were expressed in cells.

The viral proteins previously shown to interact with p28 in the mass spectrometry experiment were initially analyzed in these co-immunoprecipitation experiments using antibodies that recognize A34, A55, G1, I3, and E9 **(Figure 5.3.1; panel b-f)**. A34 was detected in both, p28 and p28(C173S/C176S) VACV Hits; A55 was only detected in p28 VACV Hits; G1 was detected in both, p28 and p28(C173S/C176S) VACV Hits; I3 and E9 were detected in both, p28 and p28(C173S/C176S) VACV Hits. We were unable to detect A34, A55, G1, I3, or E9 in anti-FLAG-immunoprecipitates of cells expressing FLAG-p28 or FLAG-p28(C173S/C176S) even though FLAG-p28 was readily detected in these lysates **(Figure 5.3.1; panel a)**. In addition, western blotting of WCL with anti-A34, anti-A55, anti-G1, anti-I3, and anti-E9 antibodies confirmed the expression and presence of these proteins in the lystates **(Figure 5.3.1 panel b-f)**. These viral proteins, which failed to be detected in anti-FLAG immunoprecipitates, were excluded from our list of potential p28 hits for the next validation steps in the subsections below. The anti-FLAG western blot is representative of one immunoprecipitation and demonstrates that the anti-FLAG immunoprecipitations worked. This representative blot is also shown in Figure 5.3.2 and A.2.1.
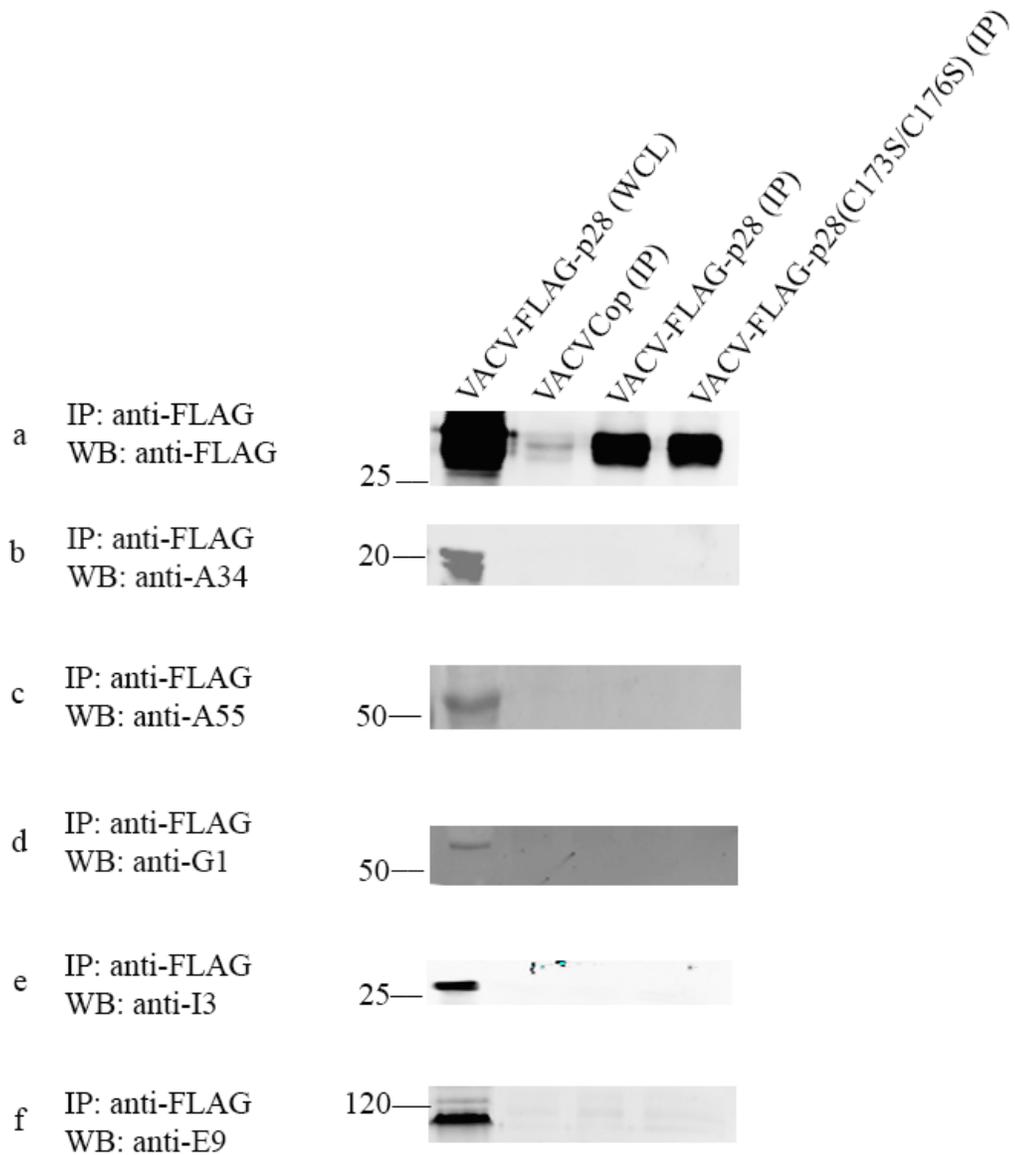
Figure 5.3.1 Verification of viral and cellular p28 mass spectrometry hits with co-immunoprecipitation studies.

HeLa cells were infected with VACVCop, VACV-FLAG-p28, or VACV-FLAG-p28(C173S/C176S) at an MOI of 5 and cells were treated with MG132 (20μM) for the last 4 hours of 16 hour infection. Cells were lysed in NP-40 lysis buffer and FLAG-p28, or FLAG-p28(C173S/C176S) were immunoprecipitated with mouse anti-FLAG M2 and western blotted with the indicated antibodies in panel a-f. Whole cell lysates (WCL). Note: All though some of these blots are from separate anti-FLAG IPs, we verified that the FLAG proteins were precipitated in all IPs. The anti-FLAG blot shown is representative from one IP. This representative blot is also shown in Figure 5.3.2 and A.2.1.
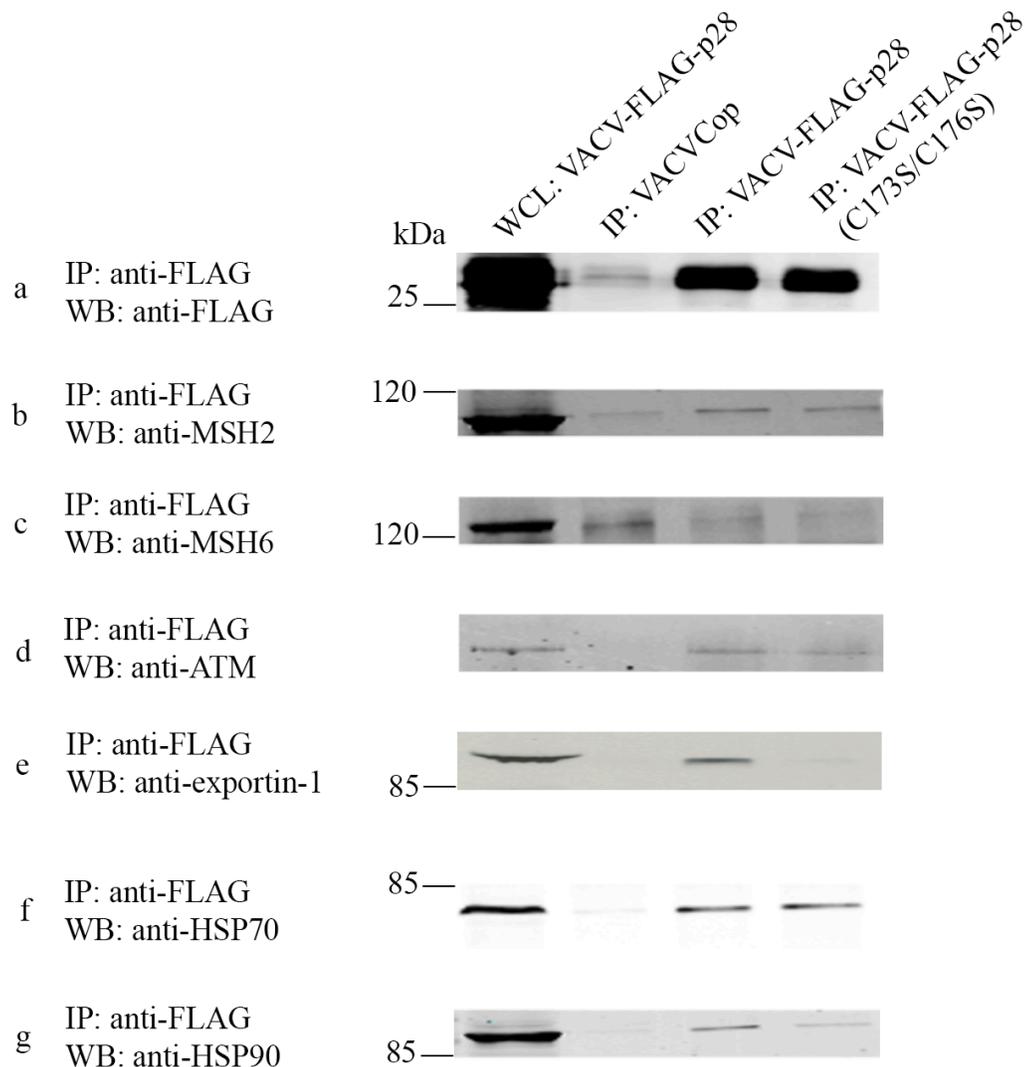
Figure 5.3.2 Verification of cellular p28 mass spectrometry hits belonging to functional annotation clustering.

HeLa cells were infected with VACVCop, VACV-FLAG-p28, or VACV-FLAG-p28(C173S/C176S) at an MOI of 5 and cells were treated with MG132 (20μM) for the last 4 hours of 16 hour infection. Cells were lysed in NP-40 lysis buffer and FLAG-p28, or FLAG-p28(C173S/C176S) were immunoprecipitated with mouse anti-FLAG M2 and immunoblotted with the indicated antibodies in panel a-g. Whole cell lysate (WCL). Note: All though some of these blots are from separate anti-FLAG IPs, we verified that the FLAG proteins were precipitated in all IPs. The anti-FLAG blot shown is representative from one IP. This representative blot is also shown in Figure 5.3.1 and A.2.1.

We further tested cellular hits from the functional cluster of DNA damage and stress sensor proteins (MSH2, MSH6, and ATM, which were all present in both, p28 and p28(C173S/C176S) immunoprecipitations)) **(Figure 5.3.2; panel b-d)**, components of the nuclear transport machinery (exportin-1, which was present in both, p28 and p28(C173S/C176S) immunoprecipitations)) **(Figure 5.3.2; panel e)**, and heat shock proteins (HSP70 and HSP90, which were both present in p28 and p28(C173S/C176S) Human Hits) **(Figure 5.3.2; panel f and g)** with specific antibodies. Western blotting with anti-MSH2, anti-MSH6, anti-ATM, anti-HSP70, and anti-HSP90 antibodies demonstrated that these proteins were present in anti-FLAG immunoprecipitates from lysates of both FLAG-p28 as well as FLAG-p28(C173S/C176S)-expressing cells **(Figure 5.3.2; panel b-d, f, and g)**. We could consistently detect a background band in the anti-FLAG immunoprecipitates from lysates from HeLa cells infected with VACVCop, and VACVCop expressing FLAG-p28 and FLAG-p28(C173S/C176S) for MSH6 **(Figure 5.3.2; panel c)**. We excluded MSH6 from further validation steps and focus on the hits, which were positive in our immunoprecipitation studies. Furthermore, we could detect an interaction between FLAG-p28 and exportin-1 in our western blots. However, we could only detect a weak interaction between the double cysteine mutant FLAG-p28(C173S/C176S) and exportin-1 **(Figure 5.3.2; panel e)**. Our results were consistent in three independent experiments.

In summary, we selected poxviral proteins and prominent cellular hits from the functional annotation clustering of DNA mismatch repair proteins, nuclear transport and HSPs for further validation. Our co-immunoprecipitation data indicated a protein-protein interaction between the HSP70 and HSP90 chaperone proteins, the MSH2 and ATM

DNA repair proteins, and exportin-1 from the nuclear transport cluster with FLAG-p28. None of the tested poxviral proteins were detected in immunoprecipitates from the lysates of VACV-FLAG-p28-infected cells, suggesting that our mass spectrometry approach comes with a high false positive rate and that further validation steps are required.

In the subsections below, I further investigated the subcellular localization of the confirmed co-immunoprecipitation hits during VACVCop infection and whether it is altered in the presence of VACVCop infected cells expressing FLAG-p28 or FLAG-p28(C173S/C176S).

5.2.4 Confocal studies reveal that p28 leads to the enrichment of HSP70 at virus factories

This group of experiments was designed to investigate whether and how p28 affected the cellular localization of the mass spectrometry hits, which were positive in co-immunoprecipitation studies in **section 5.2.3.2**. We compared the localization and the amount of MSH2, ATM, exportin-1, HSP70, and HSP90 at the virus factory where p28 resides. HeLa cells were infected with VACVCop, VACV-FLAG-p28, or VACV-FLAG-p28(C17S/C176S) at an MOI of 5 for 14 hours. Cells were fixed in 4% PFA and stained for anti-FLAG, anti-MSH2, anti-ATM, anti-exportin-1, anti-HSP70, anti-HSP90, and DAPI and were evaluated with confocal analysis **(Figure 5.4.1)**. We analyzed 60 cells from three independent experiments per sample and measured the raw fluorescence integrated densities of ROI at the virus factory and ROI of the entire cell. This allowed for calculation of the percentage of the substrate that is localized to the virus factory in

A

	DAPI	anti-MSH2	anti-FLAG	Merge
Mock				
VACVCop				
VACV-FLAG-p28				
VACV-FLAG-p28 (C173S/C176S)				

160

B

	DAPI	anti-ATM	anti-FLAG	Merge
Mock				
VACVCop				
VACV-FLAG-p28				
VACV-FLAG-p28 (C173S/C176S)				

C

| | DAPI | anti-Exportin-1 | anti-FLAG | Merge |

Mock

VACVCop

VACV-FLAG-p28

VACV-FLAG-p28 (C173S/C176S)

D

	DAPI	anti-HSP90	anti-FLAG	Merge

Mock

VACVCop

VACV-FLAG-p28

VACV-FLAG-p28
(C173S/C176S)
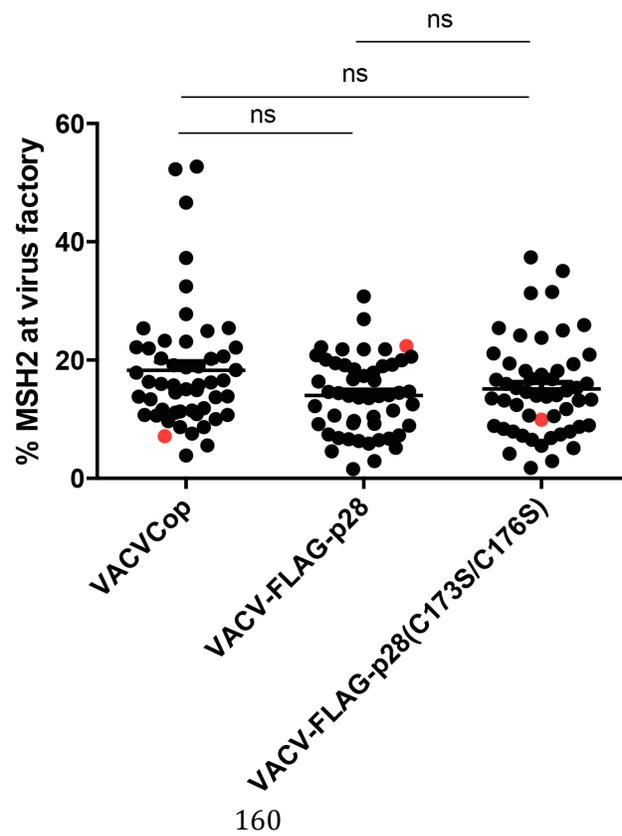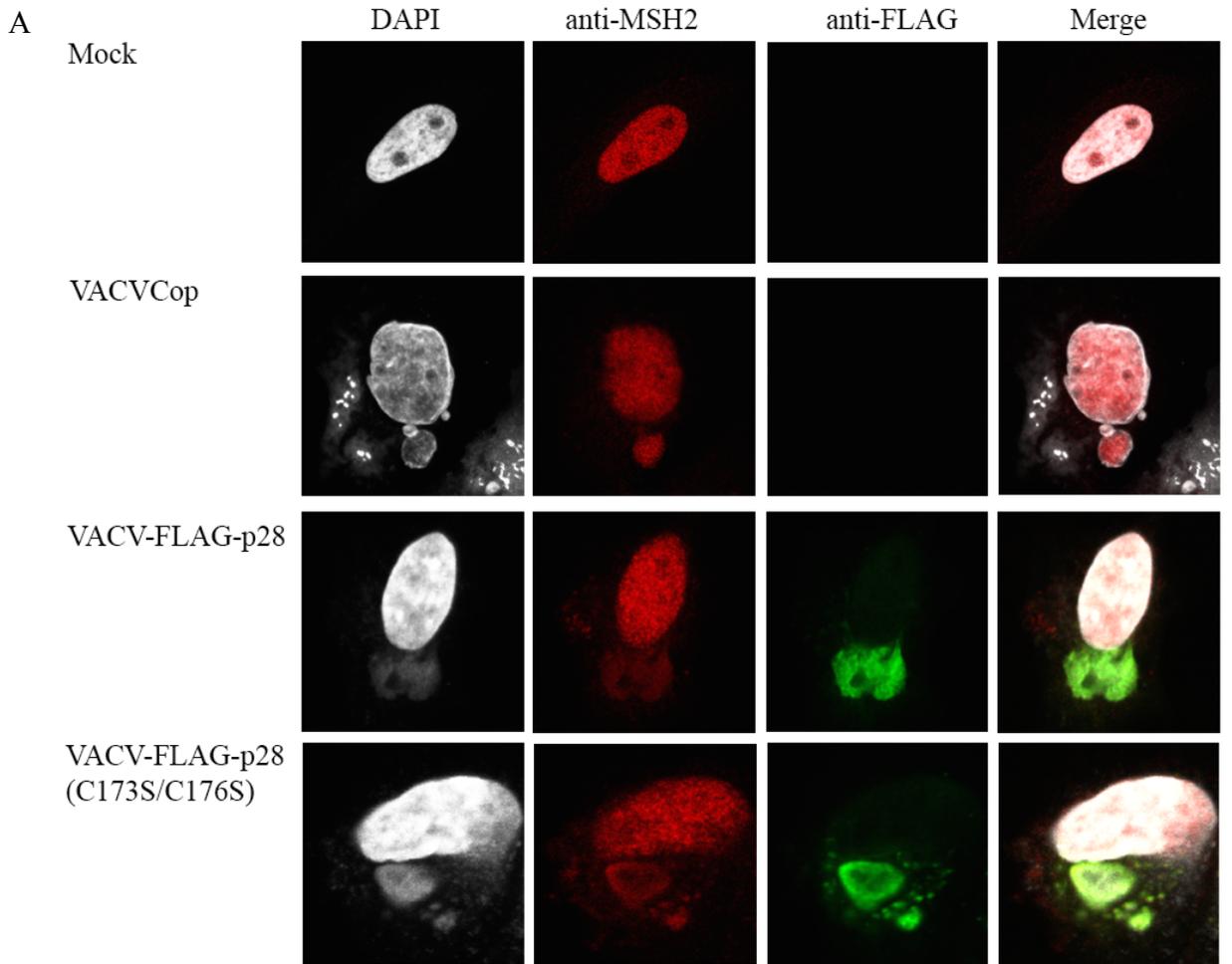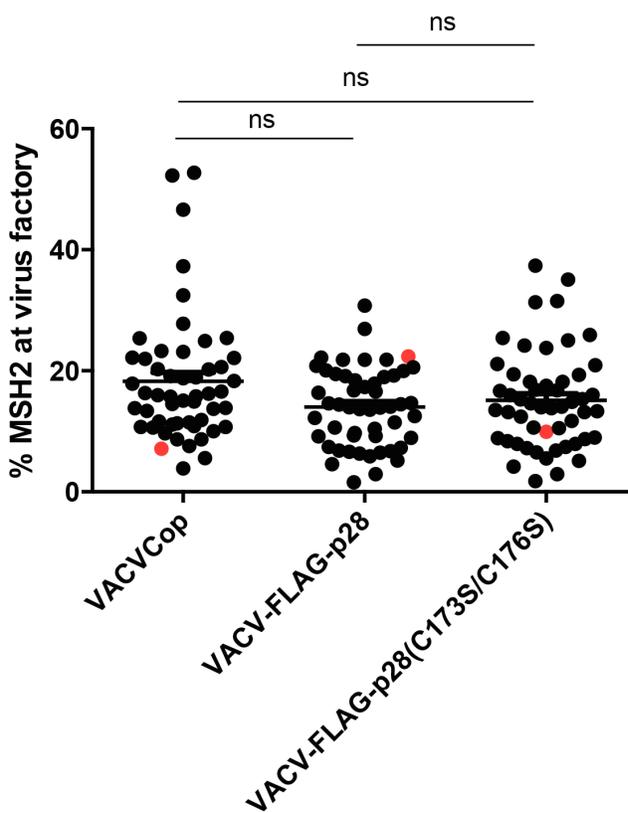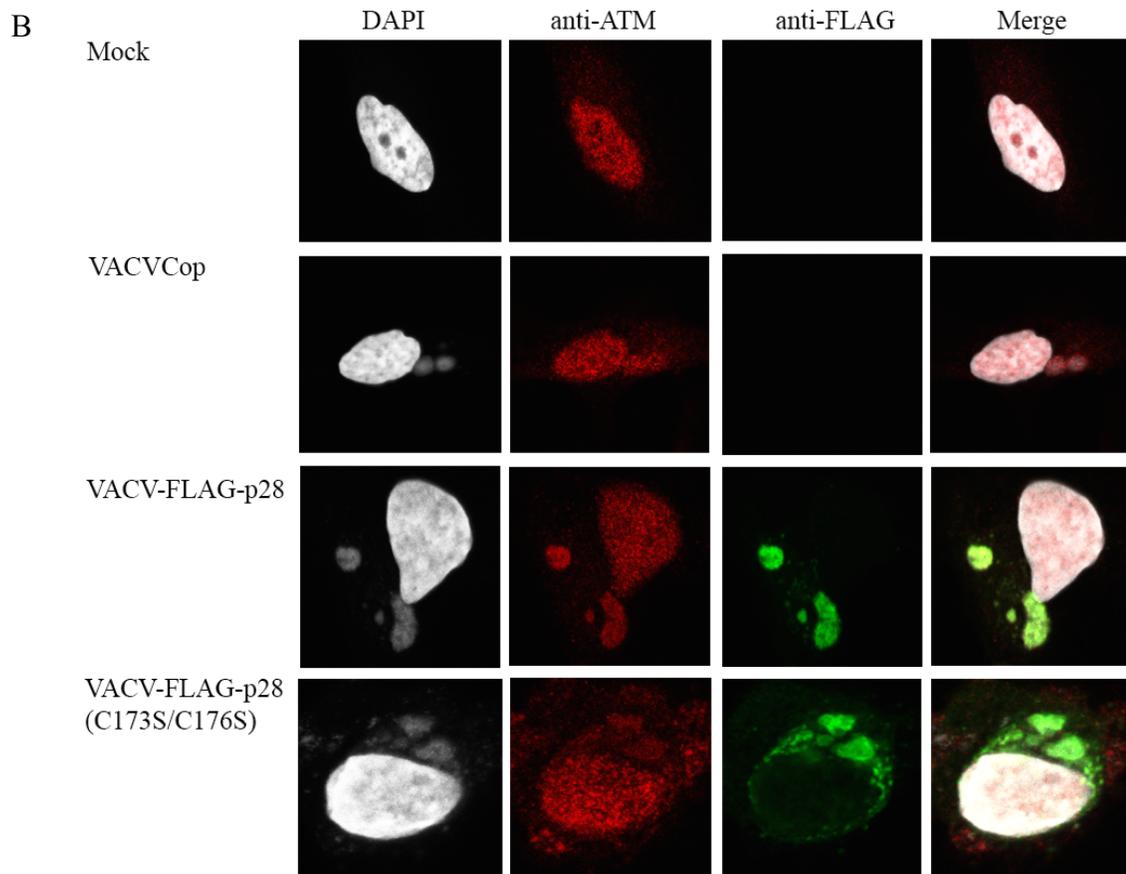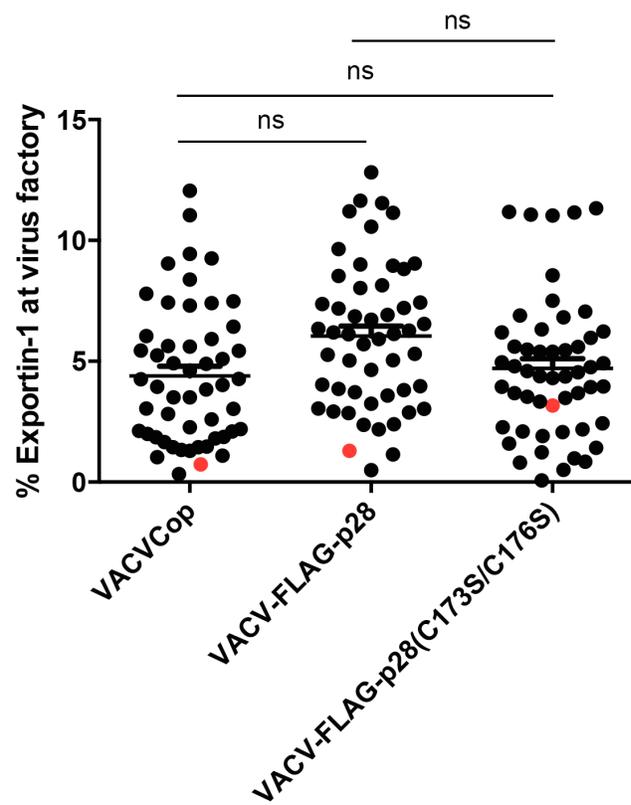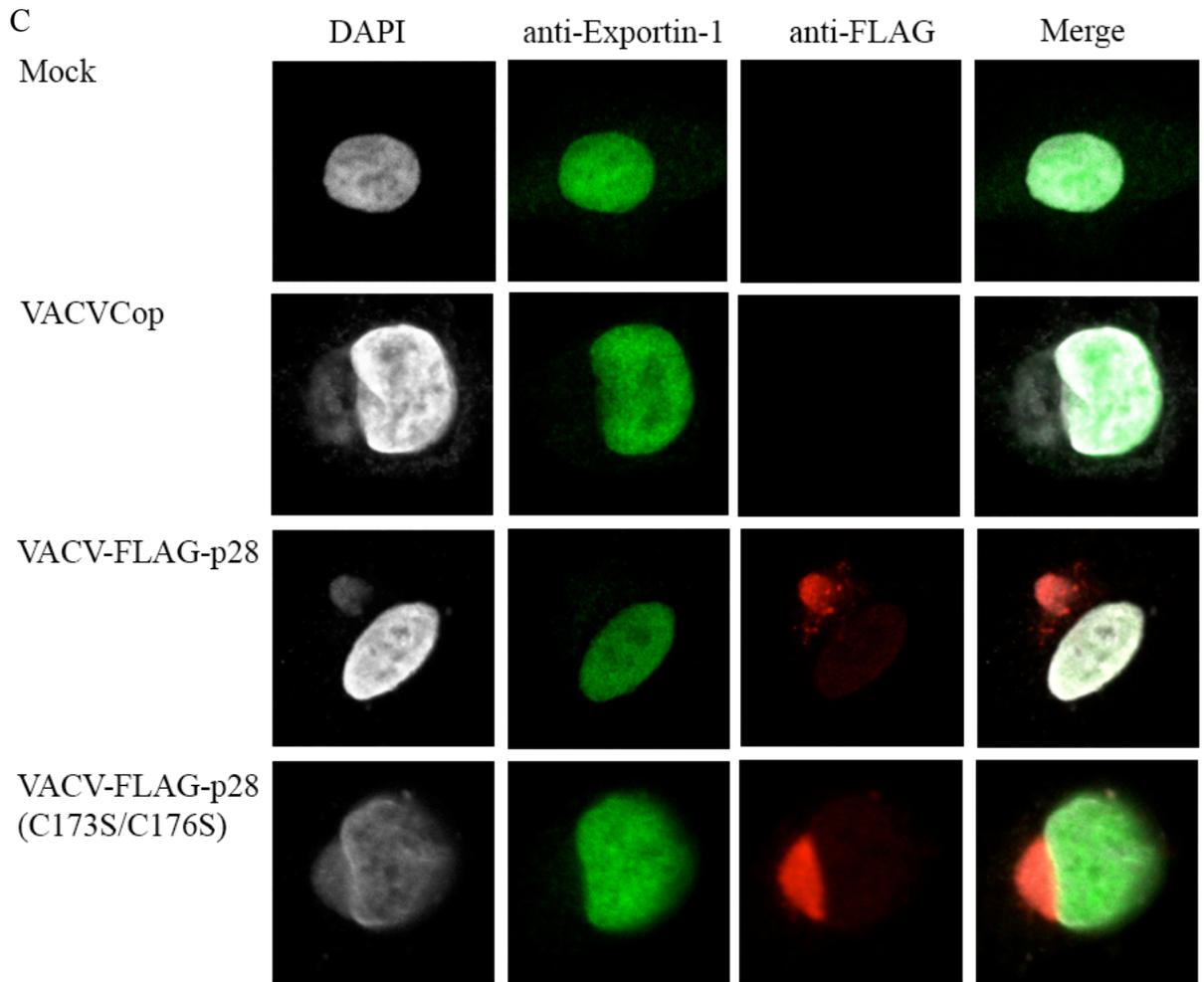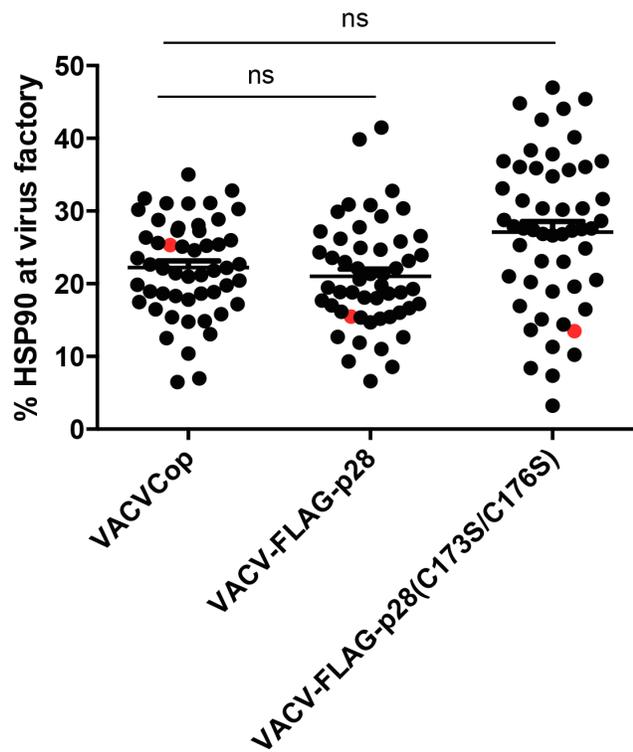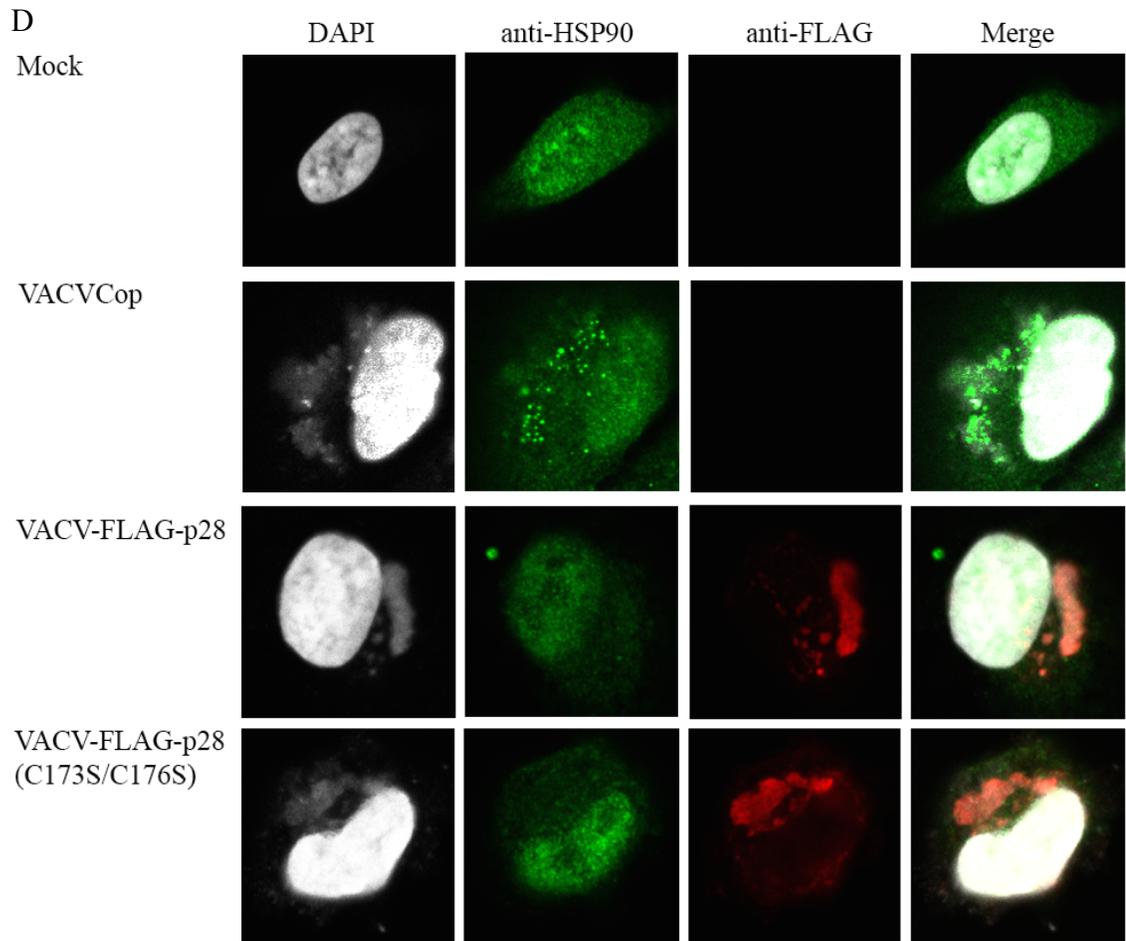
E
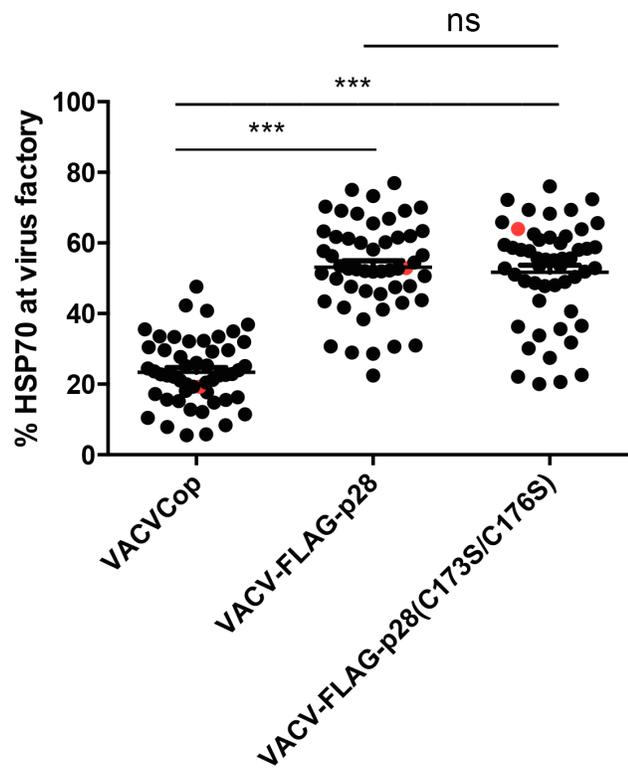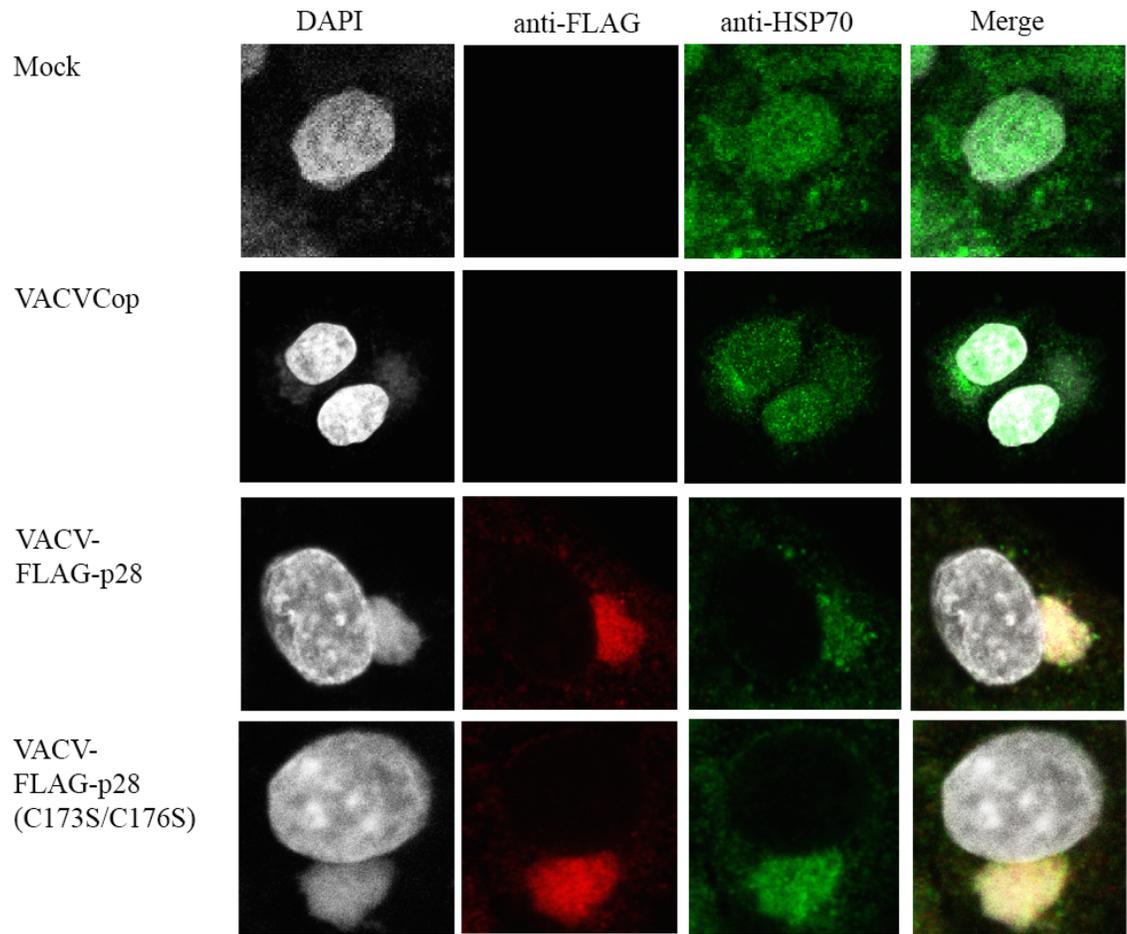
Figure 5.4.1 p28 does not affect the cellular localization of MSH2, ATM, Exportin-1, and HSP90, but leads to enrichment of HSP70 at the virus factory.

HeLa cells were infected with VACVCop, VACV-FLAG-p28, or VACV-FLAG-p28(C17S/C176S) at an MOI of 5 for 14 hours. Cells were fixed in 4% PFA stained for mouse anti-FLAG, DAPI, and (A) anti-MSH2, (B) anti-ATM, or (C) anti-Exportin-1, (D) anti-HSP90, and (E) anti-HSP70 and analyzed with confocal analysis and Fiji/Prism for statistical analysis. . One-way Analysis of variance (ANOVA) tests with Tukey post-hoc column comparison were used to compare calculate statistical significance. *** $p<0.001$ was considered statistically significant. Non significant (ns). The dots, which correspond to the cells in the panels, are marked in red. VACVCop dot corresponds to the right cell in the picture.

the presence or absence of functional p28. Both MSH2 and ATM, from the cluster of DNA damage sensors, and exportin-1, from the nuclear traffic cluster, were localized exclusively in the nucleus in mock infected cells **(Figure 5.4.1)**. During VACVCop infection, an average of 17% of MSH2 and 22% of ATM, but only 4% of exportin-1 was localized to the virus factory. The presence of p28 or p28(C173S/C176S) did not significantly change this cellular distribution **(Figure 5.4.1)**. HSP90 was enriched to an average of approximately 20% at the virus factory, which did not alter in the presence of p28. Therefore, this excludes the possibility that p28 recruits MSH2, ATM, exportin-1, or HSP70 to the virus factory. We further investigated the subcellular localization of HSP70. HSP70 was found speckled throughout the cytoplasm and in the nucleus in mock-infected cells **(Figure 5.4.1)**. During VACVCop infection, roughly 20% of cellular HSP70 was localized to the virus factory. Intriguingly, in the presence of FLAG-p28, the amount of cellular HSP70 co-localizing to the virus factory increased significantly to 50% (p-value<0.001(***)) **(Figure 5.4.1)**. A similar localization was observed for FLAG-p28(C173S/C176S) (p-value<0.001(***), and there was no significant difference between the presence of p28 and p28(C173S/C176S), indicating that HSP70 localization to the virus factory is independent of p28 ubiquitin ligase activity. To test if HSP70 enrichment at the virus factory is not merely an artifact during overexpression of a synthetic FLAG-tagged protein, we investigated the distribution of FLAG-A50, a poxviral DNA ligase **(Figure 5.4.2)**. The presence of FLAG-A50 did not alter the amount of HSP70 at the virus factory, compared to VACVCop infection, suggesting that the effect is due to the presence of p28. In summary, while there was no effect on the

166
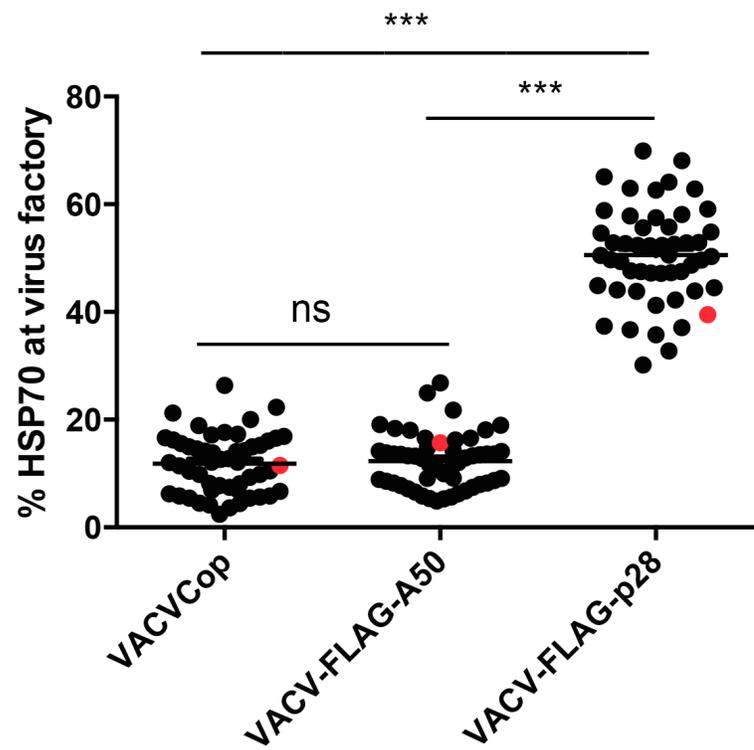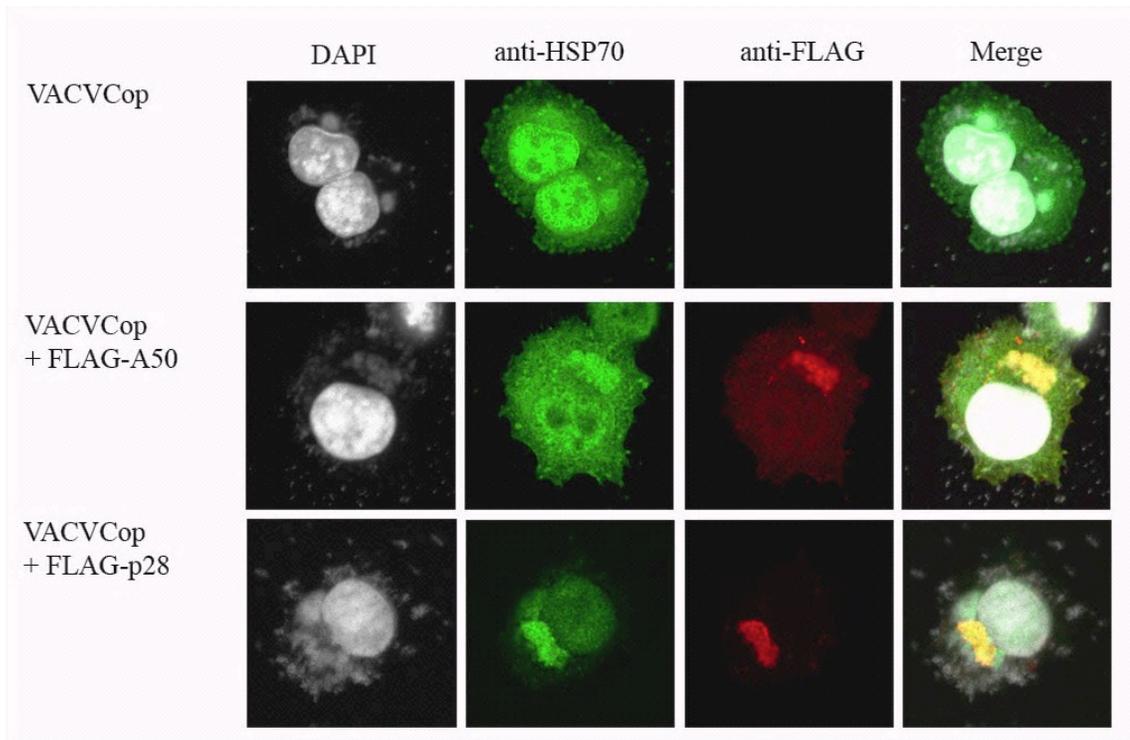
Figure 5.4.2 HSP70 does not co-localize with FLAG control protein.

HeLa cells were infected with VACVCop, VACVCop and transfected with pSC66-FLAG-A50 or VACVCop and transfected with pSC66-FLAG-p28 at an MOI of 5 for 14 hours. Cells were fixed in 4% PFA stained for mouse anti-FLAG, DAPI, and anti-HSP70 and analyzed with confocal analysis and Fiji/Prism for statistical analysis. One-way Analysis of variance (ANOVA) tests with Tukey post-hoc column comparison were used to compare calculate statistical significance. *** $p < 0.001$ was considered statistically significant. The dots, which correspond to the cells in the panels, are marked in red. VACVCop dot corresponds to the right cell in the picture.

subcellular localization of MSH2, ATM, exportin-1 and HSP90, the presence of p28 did lead to the enrichment of HSP70 at the virus factory. For this reason, the following subsections focus on HSP70 only.

5.2.5 p28 does not promote the ubiquitination of HSP70

This experiment followed up on the co-immunoprecipitation data from **section 5.2.3**. It investigated whether HSP70 was a substrate of p28, by determining its ubiquitination status in cells expressing p28. To do so, HeLa cells were infected with VACVCop or VACVCop expressing FLAG-p28 or FLAG-p28(C173S/C176S).

In addition, infected cells further expressed HA-tagged ubiquitin for the better detection of ubiquitination. Cells were also treated with proteasomal inhibitor MG132 to stabilize the ubiquitinated proteins (317). Cells were lysed in NP-40 lysis buffer containing 5mM NEM, an inhibitor of de-ubiquitinating enzymes **(Figure 5.5)**. Immunoprecipitation was then performed with antibodies against endogenous HSP70 and western blotted with anti-HA to detect incorporation of HA-tagged ubiquitin. As a positive control for laddering, which is consistent with ubiquitin modification, we examined the ubiquitination laddering of FLAG-p28 (318). Western blotting of anti-FLAG immunoprecipitates from lysates of HeLa cells infected with VACVCop expressing FLAG-p28 demonstrated that we could immunoprecipitate p28 in our positive control and detect higher molecular weight laddering, which is typical for ubiquitination. We were also able to immunoprecipitate endogenous HSP70, but failed to detect higher molecular weight laddering in the presence of FLAG-p28 and MG132, which would have been an indication of substrate ubiquitination via p28. As a negative control, we

kDa

Pos.ctr: IP anti-FLAG VACV-FLAG-p28

Neg. ctr: Isotype IP VACV-FLAG-p28

IP anti-HSP70 VACVCop

IP anti-HSP70 VACV-FLAG-p28

IP anti-HSP70 VACV-FLAG-p28 (C173S/C176S)

+   +   +   +   +   VACV-HA-Ub

WB: anti-HA

85 —

50 —

HC

WB: anti-HSP70

85 —

WB: anti-FLAG

35 —

HC

WB: Lysates anti-HA

WB: Lysates anti-HSP70

85 —

WB: Lysates anti-FLAG

35 —
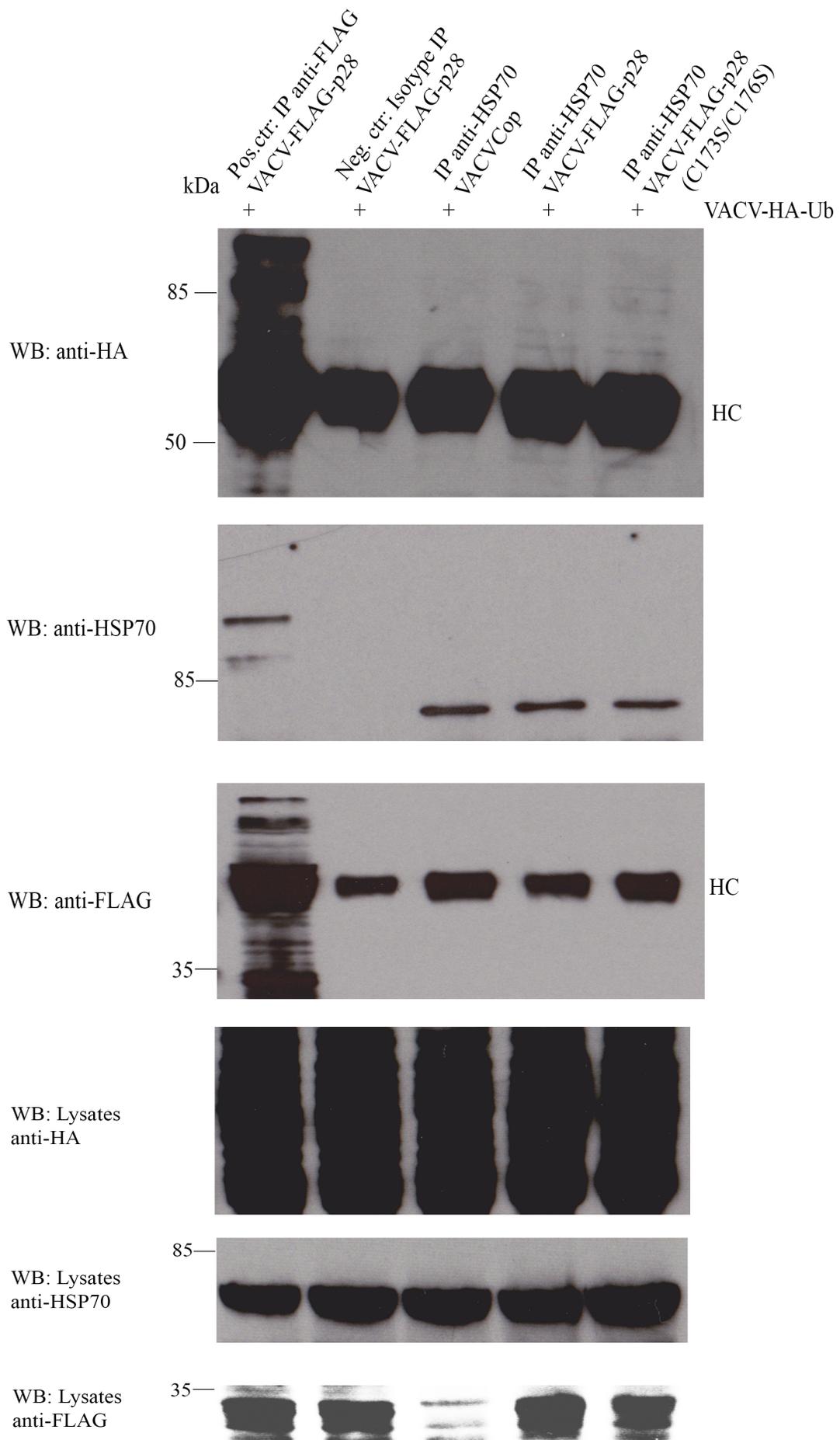
Figure 5.5 p28 does not lead to the ubiquitination of HSP70.

HeLa cells were infected with VACVCop, VACV-FLAG-p28, or VACV-FLAG-p28(C173S/C176S) and co-infected with VACV-HA-Ubiquitin at an MOI of 5 and cells were treated with MG132 for the last 4 hours of 16 hour infection. Cells were lysed in NP-40 lysis buffer containing 5mM NEM, an inhibitor of deubiquitinating enzymes. We performed immunoprecipition for endogenous HSP70. As a positive control for laddering, we examined the ubiquitination associated laddering of FLAG-p28 and as a negative control we used isotype controls (anti-GFP). We stained western blots as stated above. Heavy chain (HC), light chain (LC), Whole cell lysates (WCL)

immunoprecipitated with an isotype control, where little to no ubiquitination smear or only background was expected. We saw a strong ubiquitination smear in our positive control staining anti-HA and little to none in both our negative control and HSP70 immunoprecipitated samples. Western blotting of WCL showed that HA-ubiquitin was expressed in all the samples. Furthermore, endogenous HSP70 was expressed in all the samples in a similar amount. Western blotting anti-FLAG in WCL also demonstrated the presence of FLAG-p28 and FLAG-p28(C173S/C176S) as indicated. We conducted these experiments three times with similar results. Taken together, we were not able to demonstrate that HSP70 is ubiquitinated in presence of p28 and that the enrichment of HSP70 is independent of the ubiquitin ligase activity of p28.

5.2.6 p28 homologues in MYXV and FWPV show a trend to trigger enrichment of HSP70 at the virus factory

In previous chapters, it was demonstrated that p28 homologues in MYXV (M143R) and in FWPV (FWPV150 and FWPV157) act as ubiquitin ligases and are localized to the virus factory. These homologues demonstrate a sequence identity to p28(IHDW) of 20% in MYXV homologues M143R, and 24% and 43% in the FWPV homologues, FWPV150 and FWPV157, respectively. This experiment was designed to investigate whether the effect of HSP70 enrichment at the virus factory is conserved in these homologues. We infected HeLa cells with VACVCop, VACV-FLAG-p28, VACV-FLAG-M143R, or transfected VACVCop infected cells with pSC66-FLAG-FWPV150, or pSC66-FLAG-FWPV157 at an MOI of 5 for 14 hours. Cells were fixed in 4% PFA and stained for anti-FLAG, anti-HSP70, and DAPI and were evaluated using confocal analysis **(Figure 5.6)**.
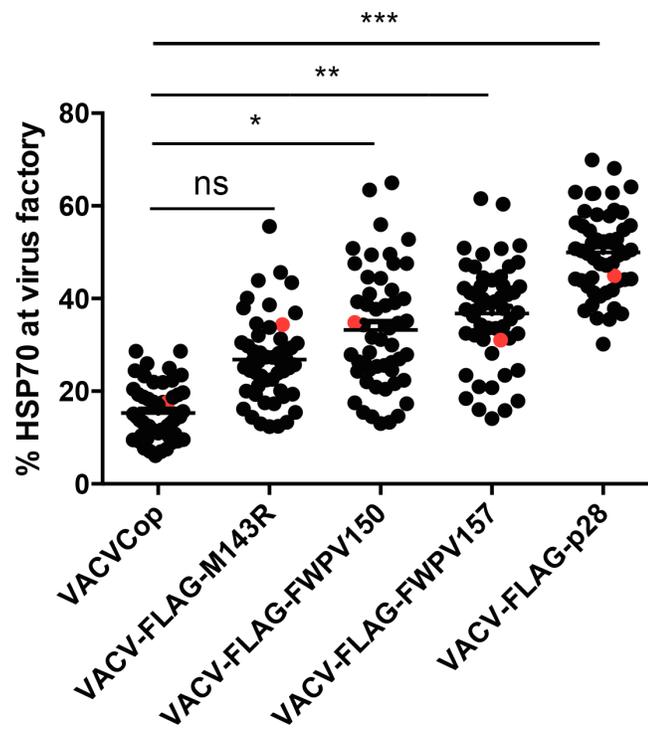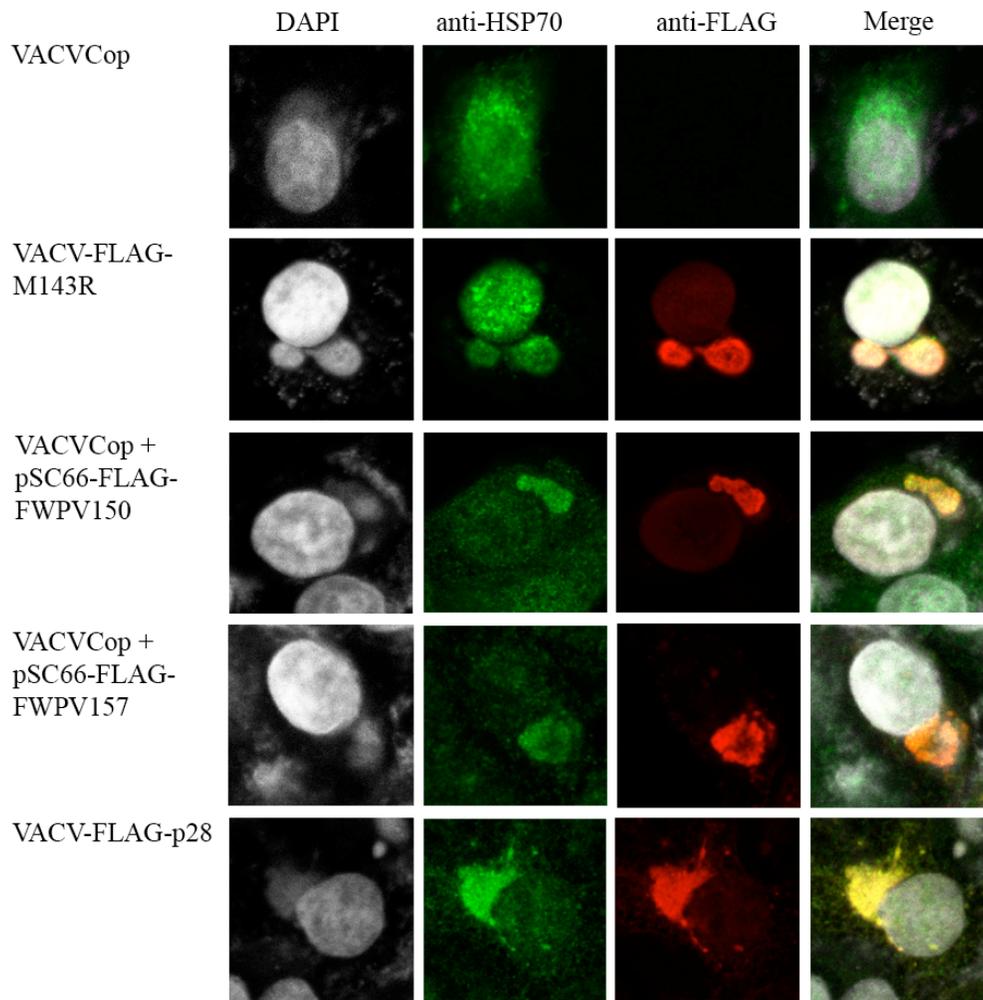
Figure 5.6 p28 homologues in MYXV and FWPV show a trend to trigger enrichment of HSP70 at the virus factory.

HeLa cells were infected with VACVCop, VACV-FLAG-M143R, VACV-FLAG-p28, or VACVCop infected cells were transfected with pSC66-FLAG-FWPV150, or pSC66-FLAG-FWPV157, at an MOI of 5 for 14 hours. Cells were fixed in 4% PFA stained for mouse anti-FLAG, DAPI, and anti-HSP70 and analyzed with confocal analysis and Fiji/Prism. One-way Analysis of variance (ANOVA) tests with Tukey post-hoc column comparison were used to compare calculate statistical significance. $p < 0.05$ was considered significant and statistically significant results were reported as: * $p < 0.05$, **$p < 0.01$, and *** $p < 0.001$. Actual p-values were: VACV vs M143: $p < 0.1511$ (ns), VACV vs FWPV150: $p < 0.0163$ (*), VACV vs FWPV157: $p < 0.0040$ (**), and VACV vs p28: $p < 0.0001$ (***).The dots, which correspond to the cells in the panels, are marked in red. The experiments in Figure 5.6 and Figure 5.7 were conducted at the same time and the results were split into two Figures; therefore the same controls of VACVCop and VACV-FLAG-p28 appear in both Figures.

We observed a trend of HSP70 enrichment at the virus factory in all the homologues. Interestingly, the MYXV homologue seemed to trigger only enrichment of cytoplasmic HSP70 to the virus factory, while a strong nuclear staining of HSP70 was observed. In contrast, both FWPV homologues recruited both, nuclear and cytoplasmic HSP70 to the virus factory. From all tested homologues, FWPV157 demonstrated the highest HSP70 enrichment, though not to the same degree as p28. Overall, the effect of HSP70 enrichment at the virus factory was conserved across p28 homologues in both MYXV and FWPV. The experiments in Figure 5.6 and Figure 5.7 were conducted at the same time and the results were split into two Figures; therefore the same controls of VACVCop and VACV-FLAG-p28 appear in both Figures. In the next subsection, we will investigate, which part of p28 is important for the enrichment of HSP70 to the virus factories.

5.2.7 Residues 1-204 within p28 are necessary for recruiting HSP70 to the virus factory

The previous subsection showed that the presence of p28 leads to the enrichment of HSP70 to the virus factory. Here, we investigated which part of p28 is crucial for the enrichment of HSP70 at the virus factories by utilizing p28 truncation mutants described in the previous chapter (318) including the KilA-N only p28(1-152), and truncations within the RING domain p28(1-184) and p28(1-204) **(Figure 5.7)**. HeLa cells were infected with VACVCop, VACV-FLAG-p28(1-152), VACV-FLAG-p28(1-184), VACV-FLAG-p28(1-204), or VACV-FLAG-p28 **(Figure 5.7)** and the HSP70 localization was investigated as described above. p28(1-152) and p28(1-184) demonstrated only
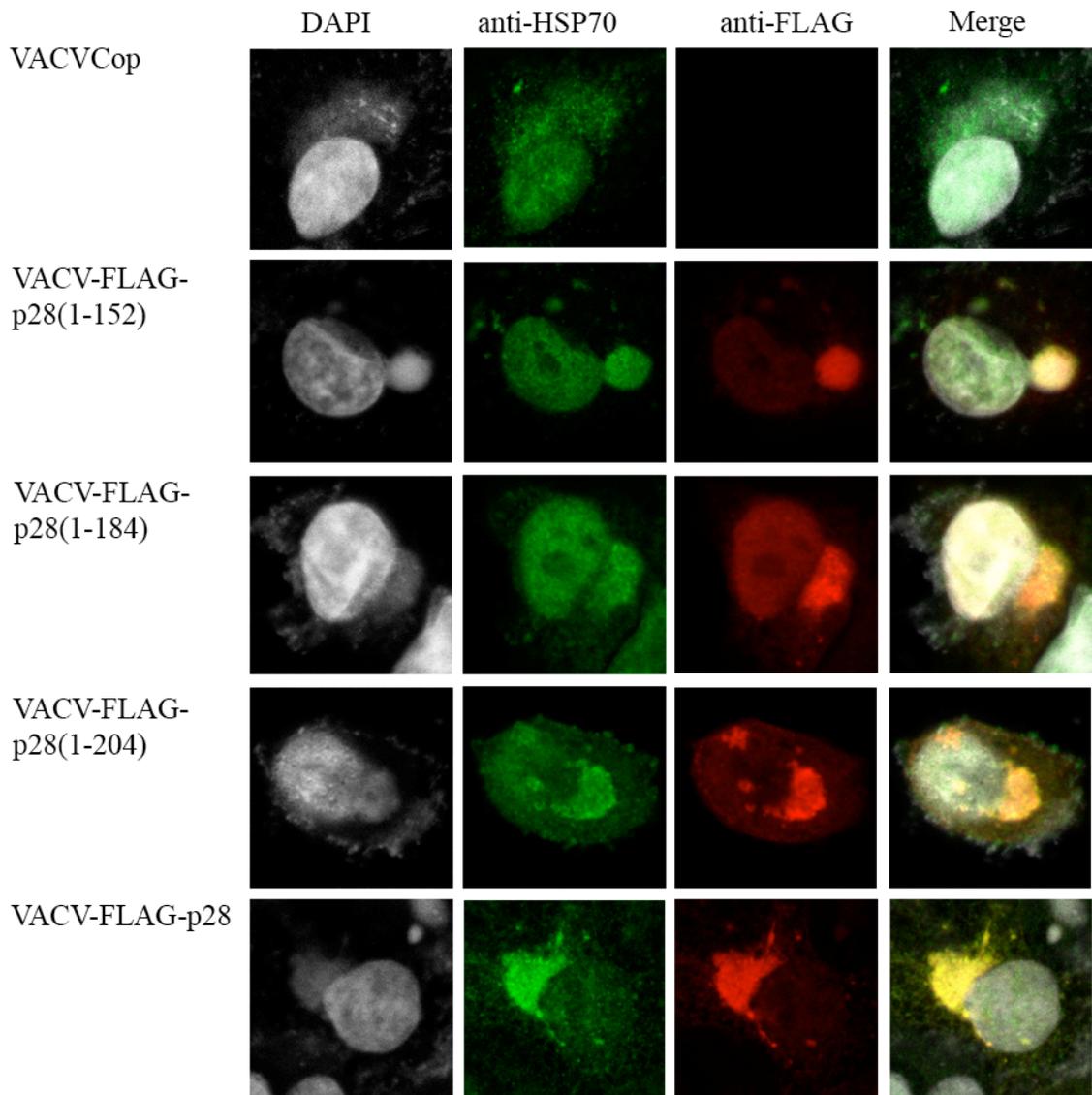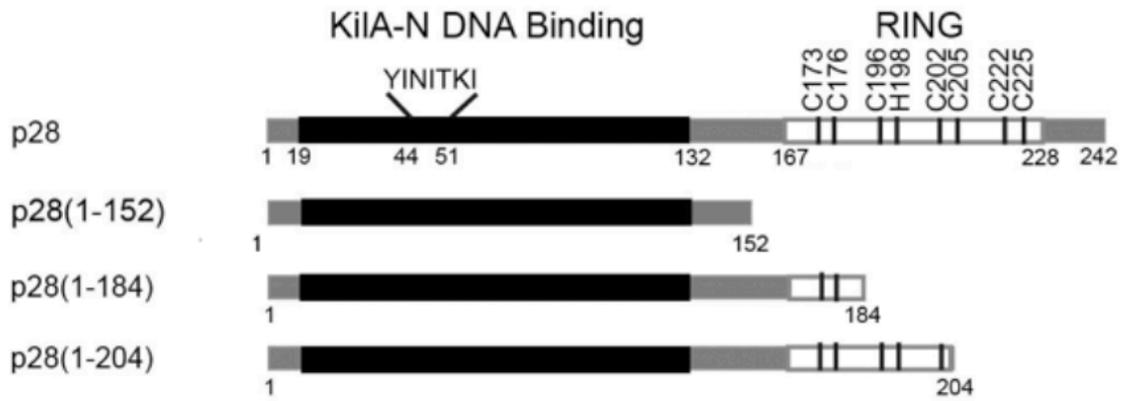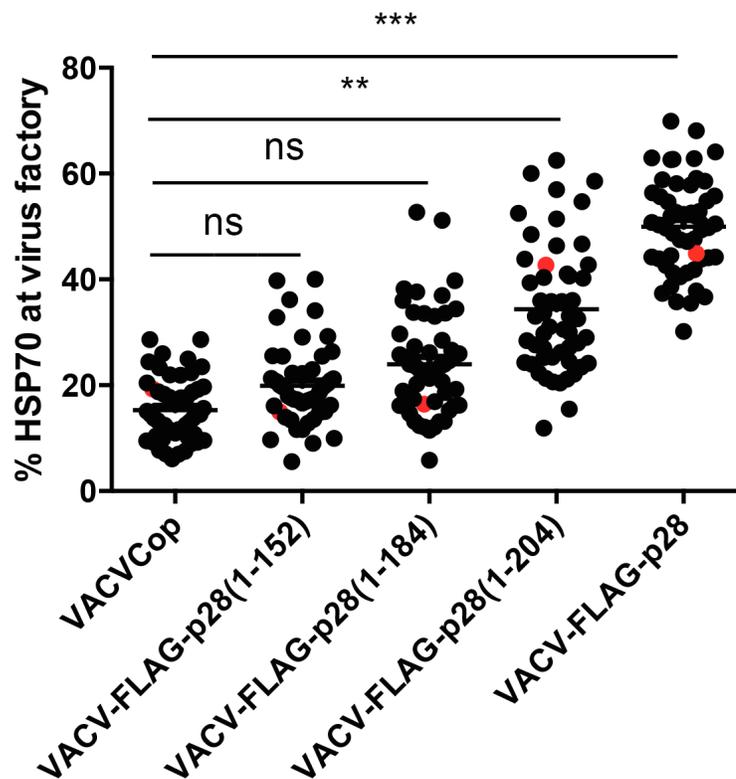
Figure 5.7 Residues 1-204 within p28 are necessary for recruiting HSP70 to the virus factory.

HeLa cells were infected with VACVCop, VACV-FLAG-p28(1-152), VACV-FLAG-p28(1-184), VACV-FLAG-p28(1-204), or VACV-FLAG-p28 at an MOI of 5 for 14 hours. Cells were fixed in 4% PFA stained for mouse anti-FLAG, DAPI, and anti-HSP70 and analyzed with confocal analysis and Fiji/Prism. One-way Analysis of variance (ANOVA) tests with Tukey post-hoc column comparison were used to compare calculate statistical significance. $p < 0.05$ was considered significant and statistically significant results were reported as: * $p < 0.05$, **$p < 0.01$, and *** $p < 0.001$. Actual p-values were: VACV vs p28(1-152): $p < 0.9986$ (ns); VACV vs p28(1-184): $p < 0.8622$ (ns); VACV vs p28(1-204): $p < 0.0052$ (**); VACV vs p28: $p < 0.0001$ (***).The dots, which correspond to the cells in the panels, are marked in red. The experiments in Figure 5.6 and Figure 5.7 were conducted at the same time and the results were split into two Figures; therefore the same controls of VACVCop and VACV-FLAG-p28 appear in both Figures.

enrichment of cytoplasmic HSP70 to the virus factory, while there was still a strong

nuclear HSP70 staining detected. In contrast, p28(1-204) showed a drastic enrichment of

HSP70 at the virus factory, though not to the same degree as full length p28. Only p28(1-

204) and full length p28 seem to have an effect of recruiting nuclear HSP70 to the virus

factory. In summary, the residues 1-204 within p28 are necessary for recruiting HSP70

from the entire cellular pool to the virus factory.

5.3 Summary and Brief Discussion

The goal of this study was to identify interaction partners or substrates of the poxviral

encoded ubiquitin ligase p28. It was hypothesized that the host or viral proteins involved

in VACV replication, viral morphogenesis, or viral sensing may be identified as potential

interacting partners for p28. It is known that p28 is a virulence factor during ECTV

infection, since ECTV(Δp28) cannot replicate in macrophages, which are important for

virus dissemination from skin lesions to lymph nodes and the liver (245). However, the

substrates of p28, or how p28 is regulated is unknown. To this end, we applied FLAG-

immunoprecipitation of FLAG-p28, and the FLAG-p28(C173S/C176S) mutant during

VACV infection followed by Coomassie stain of SDS-PAGE gel and mass spectrometry

analysis to identify potential substrates of p28 and attempt to shed light on how this viral

protein may function as a virulence factor. As validation, we employed

immunoprecipitation and western blotting with specific antibodies, which confirmed

protein-protein interaction of potential hits with p28. Furthermore, we expected that

potential interacting proteins would co-localize with p28 at the viral factories. We

demonstrated that co-immunoprecipitated proteins also accumulate within viral factories. However, only one hit, HSP70, demonstrated enrichment in the presence of p28 when compared with VACVCop infected cells alone. We followed up with ubiquitination studies and demonstrated that HSP70 is not a substrate of p28, but rather an interaction partner. The enrichment of HSP70 was conserved within p28 homologues and we pinpointed residues 184-204 within p28 as being important for recruiting HSP70 from the cellular nuclear and cytoplasmic pool to the virus factory.

There is an existing body of research, which highlights the important role of HSPs, including HSP70 and HSP90, during virus replication. For almost every step in viral replication, there is an example of how viruses hijack HSPs for their own purposes. HSP induction has been reported to propagate viral cell entry, uncoating, transcription and genome replication, virion morphogenesis, encapsidation, and virion release in numerous cases of viral infections (313-316, 319-331). In fact, it was recently reported that upregulation of HSPs enhances VACV replication and provides a mechanism for how VACV ensures sufficient levels of multiple chaperones (332). Further evidence that HSPs are beneficial for poxvirus infections is the fact that Crocodilepox virus and MCV encode their own version of HSP40 (333, 334). In addition, HSP70, HSP72 and HSP90 are packed into mature VACV virions to be readily available in the next round of infection (227, 335, 336). Interactions between HSPs and poxviral proteins were previously described: HSP90 interacts with A10L, while HSP27 binds to C2L and I4L (337, 338). However, the function of these interactions remains elusive. Our study contributes to this existing body of research by demonstrating that HSP70 is associated with p28 in co-immunoprecipitation studies. Furthermore, the presence of p28 enriches

HSP70 to the virus factory. This study demonstrates that HSP70 is not a substrate of p28, but rather an interacting partner. Future experiments will investigate if this p28 mediated HSP70 recruitment to the virus factory is crucial for VACV replication and if this is important for p28 to function as a virulence factor.

CHAPTER 6: DISCUSSION AND FUTURE DIRECTIONS

6.1 Poxviruses modulate the UPS

Members of the poxvirus family encode a variety of proteins aimed at modulating the cellular UPS for their own advantage (233). These include p28, the membrane-bound ubiquitin ligase M153R, and adapter proteins that function in complexes with cellular ubiquitin ligases, such as BTB/kelch and F-box/ankyrin proteins (73, 74, 208, 235, 237). In addition, a functional UPS is essential for poxvirus infection at the level of uncoating and DNA replication (116, 117). The general shutdown of the UPS prevents VACV infection during early stages (116, 117). To date, the functions of most ubiquitin modulators remain unexplored. To this end, the present study sought to characterize the poxviral encoded ubiquitin ligase p28.

6.2 p28 homologues in FWPV

Many Chordopoxviruses described to date encode, or are predicted to encode, a p28 ubiquitin ligase homologue **(Table 1.2)**. This is especially the case for "wildtype" strains, such as ECTV, CWPV, VARV, and MPXV. These strains cause severe disease for natural hosts (242). Within these strains, p28 homologues demonstrate a sequence conservation of up to 90%, with about 97% identity in the RING domain (242). In contrast, strains of VACV that have been repeatedly passaged in tissue culture (e.g. WR, Cop, Ankara, or MVA), have either inactivating mutations in, or lost their p28 gene (29, 238, 242). This suggests that while p28 may be important as a virulence factor *in vivo*, it is not necessary for efficient replication in tissue culture settings.

Only members of the *Avipoxvirus* family, encode two homologues to p28. In FWPV these are FWPV150 and FWPV157 (22). While these proteins show sequence divergence from other p28 homologs, they encode the critical KilA-N (YINITKI) and RING (C_3HC_4) domains found in functional ubiquitin ligases (Figure 3.2). These observations suggested that FWPV150 and FWPV157 may be functional ubiquitin ligases.

This study began by investigating whether both p28 homologues, FWPV150 and FWPV157, were produced during FWPV infection, if they were functional ubiquitin ligases, and how they interacted with ubiquitination. In Chapter 3 I demonstrated that FWPV150 and FWPV157 are indeed functional ubiquitin ligases, which are regulated by ubiquitination during infection. Interestingly, FWPV150 is expressed early on during infection and persists throughout infection, while FWPV157 is a late gene, as evident by AraC treatment and time course RT-PCR evaluation **(Figure 3.8)**. The p28 homologue in ECTV is an early/intermediate gene, whereas the p28 protein is expressed as early as 2 hours post-infection and further accumulates throughout the infection cycle (245).

The biological relevance of FWPV expressing two p28 homologs, with different expression kinetics, is unknown. It would be interesting to examine if these proteins have different functions during infection or if a temporal switch occurs whereby FWPV157 takes over the function of FWPV150, or if both proteins can function late during infection. Unfortunately, no antibodies against FWPV150 or FWPV157 are available to examine their protein levels during infection. It is also possible that the single p28 version in the Chordopoxviruses have gathered and combined the substrate binding domains, which are present in FWPV150 and FWPV157, to act as a multifunctional

ubiquitin ligase. The evolutionary more divergent FWPV might still have two distinct proteins, FWPV150 and FWPV157, whose functions combine in the *Orthopoxvirus* versions of p28. Future experiments could include gene knockouts of p28 homologues in FWPV and will address if this affects FWPV replication *in vitro*. We should further investigate what happens when we switch the FWPV150 and FWPV157 promoters from early to late and vice versa in terms of virus replication and spread. This will show whether the temporal expression pattern is important, or if it is instead the case that FWPV150 and FWPV157 are interchangeable. Based on the effect of the p28 homologues in ECTV, it is possible that the absence of p28 homologues in FWPV will not affect viral growth *in vitro*. We will have to establish an animal model to evaluate the role of FWPV150 and FWPV157 *in vivo*. Infection models for FWPV have been rare and mostly focused on the evaluation of FWPV used as a vaccination vector (27, 260, 339). Less work has been published on models to evaluate virulence factors in FWPV during infection in a natural host, such as chicken and chicken embryos (chorioallantoic membrane (CAM)) (260, 340, 341).

An animal model has been established by the Buller group to evaluate ECTV infection lacking p28 (ECTVΔp28), which produces an attenuated infection in mice compared to wildtype ECTV (244). Alternatively we could attempt to complement a p28-deficient strain of ECTV with combinations of FWPV150 and/or FWPV157. Recombinant ECTVs expressing these FPWV genes could then be tested for virulence in mice. This will indicate whether p28 in ECTV has combined the functions of FWPV150 and FWPV157. This is given, that the substrates of FWPV150 and FWPV157 in birds are similar enough to the homologues in mice to allow them to be recognized. Furthermore, it

is not to rule out, that FWPV150 and FWPV157 have functions, which dictate host tropism in birds. In this case, no rescue effect would be seen for ECTVΔp28-FWPV150-FWPV157 infection in mice compared to ECTV infection. Another pitfall in this experimental setup is that the FWPV homologues might need another FWPV protein to function, which might be lacking in the ECTV infection in mice.

6.3 Speculations on the origin of the p28 gene

It has been suggested that poxviruses may have gained some viral genes by incorporating cellular genes via recombination events (342-347). Nicholls and Gray found that p28 shares some homology to members of the cellular Makorin proteins (348). Makorin has been reported to induce degradation of West Nile Virus capsid proteins (349) and has been thought to regulate the functions of cellular RNA polymerase II-dependent transcription (350). Makorin and p28 show homology within the RING domain (351). However, the rest of the sequence varies considerably in amino acid composition. It is possible that p28 in poxviruses was derived by virus incorporation of Makorin cDNA during infection and recombination events.

Unlike other poxvirus p28 homologs, which show only similarity to Makorin RING domains, *Avipoxvirus* p28 homologues in FWPV and CNPV contain additional regions which show similarity to extra Makorin motifs, including a third zinc finger domain (348). This further suggests a connection between cellular Makorin and p28. Furthermore, Avipoxviruses FWPV and CNPV contain multiple copies of the KilA-N domain, but only two of these ORFs in each virus combine the KilA-N with a C-terminal RING domain. This could mean that p28 was acquired in the *Avipoxvirus* clade as a

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fusion event between a KilA-N domain and a RING domain, potentially from a Makorin-like protein.

6.4 Regulation of p28

While much is known about how ubiquitin ligases ubiquitinate other proteins leading to their degradation by the 26S proteasome (142, 144, 145), less is known about how ubiquitin ligases themselves are regulated. It is known that some ubiquitin ligases can be ubiquinated by other ligases or can catalyze self-ubiquitation in a process known as autoubiquitation. (171). In Chapter 4, evidence was presented in form of flow cytometry data, where p28 and the p28 double cysteine mutant were both stabilized in presence of MG132 proteasomal inhibitor **(Figure 4.8)**. These results indicated that mutation within the RING domain results in increased stabilization of the p28 mutant proteins, suggesting that wild type p28 uses its RING domain to regulate itself by autoubiquitination. However, since the RING mutants demonstrated an increase in stabilization that suggests that p28 may be potentially regulated by a combination of autoubiquitination via another ubiquitin ligase **(Figure 4.8)**. The identity of the exogenous ubiquitin ligase is yet to be explored. Furthermore, it is not clear whether the autoubiquitination reaction is intermolecular or intramolecular.

Intermolecular self-ubiquitination requires the formation of dimers, which has been described for a number of ubiquitin ligases including E6-AP, seven in absentia 1 (SIAH1), and TRAF6 (352-354). Other ubiquitin ligases, including F-box protein Grr1p and HECT ligases Rsp5, WWP1, ITCH, SMURF2, NEDD4-1, and NEDD4-2 do not

form dimers and can autoubiquinate themselves (355, 356). This process seems to be regulated by modifications, such as phosphorylation (357-359).

Autoubiquitination typically targets the ubiquitin ligase for degradation as a self-regulatory process within a negative feedback loop, which was demonstrated for CBL ligases (360), E6-AP (361), and Mdm2 (266, 362). Furthermore, autoubiquitination can happen in substrate dependent and independent ways, while the presence of the substrate can protect the ubiquitin ligase from autoubiquitination as part of the feedback loop (131, 171, 360, 363). In some instances, autoubiquitination does not lead to proteasomal degradation, but rather regulates the activity of the ubiquitin ligase or the recruitment of substrates (171).

Future studies should evaluate whether p28 autoubiquitination occurs intermolecularly or intramolecularly. By identifying whether p28 forms dimers one could gain information as to which method occurs. This could be done by a number of different methods including immunoprecipitation studies with two different tagged constructs of p28 (e.g. Myc and FLAG), solving the crystal structure of p28, which could also identify any potential interaction amino acids, or gel filtration analysis to identify any higher order molecular p28 complexes.

A scenario of dimerization would speak in favor of intermolecular autoubiquitination. In the case of monomeric p28, we will further evaluate the phosphorylation status and, in particular, whether conformational changes regulate p28 (357). This new knowledge will further contribute to our understanding of how the poxvirus encoded ubiquitin ligase and virulence factor is regulated.

6.5 Substrate identification of p28

6.5.1 Limitations of mass spectrometry approach in this thesis

Most substrates for poxviral proteins that manipulate the ubiquitin-proteasome system are unknown, with the exception of myxoma virus protein M153R (73, 74, 231). A preliminary 2D gel and mass spectrometry screen by a former graduate student in the Dr. Barry group identified a potential interaction of p28 with viral A6 protein, a virion core protein required for the formation of mature virion, but the group could not verify this interaction (253).

In Chapter 5, I report our efforts to identify interaction partners and substrates of p28. Following immunoprecipitation of p28 in virally-infected cells we performed mass-spectrometry on proteins that were co-immunoprecipitated. I am unable to comment on the false-negative rate of hits in this screen, since no previously known hits of p28 had been identified. It is noteworthy that A6 was also a hit in our screen, but we refrained from following up on this interaction due to a lack of easily obtainable reagents for A6. Our attempts to validate many of these putative hits were accompanied by a high false-positive rate.

We conducted our search for p28 substrates by identifying both viral and cellular proteins that co-immunoprecipated with p28 using LC-MS/MS **(Figure 5.1)**. This approach resulted in a high level of false positives. Given that p28 contains a DNA-binding domain, which localizes it to the virus factory, and many of these false hits also appear to localize to viral factories, it is possible that these false-positives were detected due to a DNA-mediated interaction. We tried a benzonase treatment step in the lysis buffer to remove the DNA in the virus factory. But despite this DNAse treatment step, we

still co-immunoprecipitated the positive target proteins. Furthermore, we utilized the proteasomal inhibitor (MG132) for our experiments, since ubiquitinated proteins, including p28, are rapidly turned over by the 26S proteasome and this treatment stabilized p28 and the target proteins **(Chapter 4)**. One downside of the MG132 treatment is the increase in the overall amount of ubiquitinated proteins in the cell. This could have increased the background of false positive hits.

A high level of 26S proteasomal subunits were found in the list of potential p28-interacting partners in our screen. Previous work by the Barry laboratory found that p28 expression results in the redistribution of the 26S proteasome to virus factories (364). It is possible that immunoprecipitation of p28 could pull down these proteasome subunits, which not only contain ubiquitinated p28, but also other cellular and viral proteins, contributing to the high false positive hits on our mass spectrometry screen. While leading to a high level of false positives this may have had the unintentional consequences of potentially identifying a list of viral proteins that are degraded by the 26S proteasome before the virion assembly occurs.

6.5.2 Alternative mass spectrometry approaches

An alternative approach that could have been used to identify ubiquitinated substrates of p28 is stable isotope labeling of amino acids in cell culture (SILAC). SILAC was previously used to successfully identify substrates for cellular E3 ligases (365-367). In this approach two samples are prepared. In the first sample cells are grown in the presence of heavy amino acids (^{13}C labeling), while the second set is grown under normal conditions (unlabeled ^{12}C) (365-367). Samples are then mixed and compared using

quantitative mass spectrometry. In our future studies, heavily labelled cells could be infected with VACV-FLAG-p28(C173S/C176S) expressing only a non-functional p28, while the cells in normal media would be infected with VACV-FLAG-p28, expressing functional p28 ubiquitin ligase. Protein samples will be collected and compared using quantitative mass spectrometry, where the readout is expected contain higher amounts of ^{13}C labeled target proteins from the mutant sample and less of the unlabeled target protein from the wildtype p28 containing sample. This approach is well-suited for K48 ubiquitination, where substrates are degraded by the 26S proteasome.

Another method that could be used to identify p28 substrates makes use of a recent advance in the mass spectrometry field whereby antibodies specific for the remnants of ubiquinated proteins (Lys-e-Gly-Gly (diGly)) are used (140, 292, 296, 368-371). In this approach cell lysates are trypsin digested and incubated with diGly-specific antibodies as affinity purification followed by mass spectrometry.

We could also combine SILAC and diGy affinity capture, which will further allow the comparison of enriched ubiquitinated proteins in the presence or absence of functional p28. This will not only allow identification of K48 ubiquitinated targets, which were degraded by the 26S proteasome, but also other non-proteolytic ubiquitination via K63 chains.

Another promising approach is to clone only the substrate-binding domain of a ubiquitin ligase to use as bait for immunoprecipitation and LC-MS/MS studies. This construct will still bind to the substrates, but without a ubiquitination reaction. This is a more stable interaction. Zhuang *et al.* developed an elegant method, the so-called Neddylator, where the substrate-binding domain is linked with a Neddylation reaction

(372). Since neddylation is more rare in cells compared to the overwhelming ubiquitination found throughout the cell, there would be less background when looking for neddylated substrates. Unfortunately, the substrate-binding domain of p28 is unknown to date and would be required for this approach to be successful defined.

6.5.3 Choice of cell lines and virus

A potential limitation of this study concerns the choice of cell line. This study mainly used HeLa cells. This was the cell line of choice at the time this project was initiated. For future experiments, we propose to investigate a cell type that is known to be more physiologically relevant. As mentioned above, the only recorded cell type, which demonstrated a different phenotype in the absence of p28 was murine resident peritoneal macrophages in the susceptible mouse strain A (though this was not the case for the resistant B6 mouse strain) (244, 245). In the future we could amend an animal protocol, and isolate primary peritoneal macrophages from susceptible (strain A), or resistant (strain B6) mouse strains, following the protocol established by the Buller group (245). Infection studies of these macrophages could allow us to draw a more relevant comparison between ECTV infections in the presence or absence of p28, for SILAC based mass spectrometry experiments. Follow up validation experiments would be more streamlined, since we would be able to evaluate which cellular factors and p28 interaction partners allow ECTV replication in susceptible macrophages.

6.5.4 Validation of poxvirus proteins as substrates of p28

In our mass spectrometry validation experiments, we mostly focused on cellular substrates. For follow-up experiments on poxviral hits, we limited our validation to hits where antibodies were readily available (A55, A34, G1, I3, and E9). We initially speculated that p28 might be involved in poxvirus uncoating steps mediated by degradation of core proteins, transcription, replication or the production of early immunomodulatory proteins. This is based on the data from ECTV p28 knockout viruses and the sequence homology to Makorin (244, 245, 348). Senkevich *et al.* demonstrated the importance of the p28 homologue in ECTV for replication in murine resident peritoneal macrophages and dissemination to distant organs, such as the liver and the spleen (245). ECTV lacking a functional p28 gene (ECTVΔp28) could not replicate in the aforementioned cells to the point where no virus factories were formed and no late genes were expressed (245). This phenotype was only observed in macrophages from the susceptible mouse A strain, but not in the resistant B6 mouse strain (244, 245). Furthermore, ECTVΔp28 could successfully infect other cell lines isolated from the A strain. It is likely that in murine-resident peritoneal macrophages from strain A (which are a highly specialized non-dividing type of cell), p28 substitutes or activates an unknown cellular factor, which is required for virus replication. It is plausible that this factor is not readily available to the same levels in the susceptible A strain and the resistant B6 mouse strain.

Given the almost identical sequence identity to the p28 homologue in ECTV, we speculate that p28 in VACV has a similar function. VACV infection experiments in mouse resident peritoneal macrophages had different outcomes (373). It was mainly

reported that VACV-WR infection in mouse resident peritoneal macrophages led to the expression of early genes, partial uncoating and the activation of the virion-associated DNA-dependent RNA Polymerase (373). In the end, the virus did not fully uncoat, there was no virus replication and VACV-WR resulted in an aborted infection (373). VACV-WR infection in mouse macrophage J774 cells demonstrated virus replication, but not to the same degree as in other tested cell lines (373). We tested J774 and Raw264 mouse macrophage cell lines for VACVCop infection (Chapter 4), in the presence and absence of functional p28. In all cases, virus factories for virus replication were formed and p28 triggered the accumulation of conjugated ubiquitin to the virus factory. It is possible, that the function of p28 is required for the cell-type specific poxviral replication in primary peritoneal macrophages.

It would be interesting to investigate whether similar virus functions are impaired in VACVCop (which does not have p28) and ECTV(Δp28) and whether the inability of these viruses to replicate in mouse resident peritoneal macrophages correlates with the absence of p28. Future studies will investigate the effect of VACV infection in the presence and absence of p28 in resident peritoneal mouse macrophages. We will investigate whether the presence of functional p28 can rescue the VACVCop replication. We will conduct growth curves in these macrophages to test for productive replication, investigate the formation of virus factories via microscopy, and test the expression levels of early and late genes.

Ubiquitin is incorporated into vaccinia virus virions and is readily accessible for the next round of infection (227). These data suggest that ubiquitination of specific proteins is important early during infection at a point prior to virion uncoating (227).

Furthermore, the expression of early genes is required for this uncoating step and a functional proteasome is required for a full poxvirus replication cycle (117, 374). We speculate that p28, which is expressed early during infection, might target viral core proteins for ubiquitination and degradation to promote viral uncoating. This is strengthened by observations that the cellular homologue of p28, Makorin-1, has been reported to ubiquitinate the West Nile virus capsid protein and target it for proteasome-mediated degradation (349). While Makorin-1 plays a protective role for the host cell in doing so, the function of p28 might be to ubiquitinate viral core proteins, uncoating and lead to productive virus infection.

Some of the major virion components are A10, A3, A4, L4, and F17, which collectively function to protect the dsDNA viral genome. (375, 376). Intriguingly, one major hit in our p28 mass spectrometry screen was the core protein A10. In future studies, we should evaluate the role of p28 on the ubiquitination and subsequent proteasomal degradation of A10. We will also explore whether p28 is involved in enhancing viral uncoating.

6.5.5 p28 interaction with HSP70

Despite the above discussed high false-positive rate and limitation of our mass spectrometry approach, we verified HSP70 as an interaction partner of p28 during VACV infection. We could demonstrate that HSP70 and HSP90 are associated with p28 in co-immunoprecipitation studies (Chapter 5). Furthermore, the presence of p28 enriched HSP70 at the virus factory **(Figure 5.4.1 E)**. In fact, HSP70 was the sole hit in our mass spectrometry validation, which demonstrated enrichment at the virus factory in the

presence of p28. While HSP70 interacted with p28 we found no evidence that this interaction resulted in the ubiquitination of HSP70, hence we could not verify HSP70 as a substrate of p28 **(Figure 5.5)**. The p28-mediated accumulation of HSP70 at the virus factory was also seen in homologues of p28 **(Figure 5.6)**. In the end, HSP70 proteins were validated as interaction partners of p28, but the biological significance of HSP70 enrichment at the virus factory remains elusive.

HSP families are named after their molecular mass, HSP40, HSP60, HSP70, HSP90, and HSP110 (377). The different families of HSP can facilitate protein trafficking, formation of complexes, and maintenance of protein conformation (377). Furthermore, some HSP families have the so-called HSP-mediated activity control, where HSPs can bind native proteins and alter their conformation and function (378). In general, HSPs are mostly known as stress response proteins, which include physiological stress, environmental stress, and infections by pathogens, including viruses (308, 379).

A number of DNA and RNA viruses have been reported to induce a HSP response. (308-312). Viruses hijack HSPs for their own purposes, including the propagation of viral cell entry, uncoating, transcription and genome replication, virion morphogenesis, encapsidation, and virion release in numerous cases of viral infections (313-316, 319, 320, 322-324, 326-328, 330, 331). HSP70 has previously been reported to associate with distinct viral proteins in the case of hepatitis C virus, porcine reproductive and respiratory syndrome virus, adenovirus and poliovirus (312, 331, 380-382).

It was recently shown that VACV-WR infection triggers heat shock factor 1 (HSF1) activation, the master transcriptional regulator of the HSP response. This may explain how VACV can ensure sufficient levels of multiple chaperones throughout

infection as the activation of HSF1 can lead to the induction of many HSP proteins (332). HSF1 appears to be important for VACV replication as siRNA-mediated silencing of HSF1 was reported to lower the rates of VACV-WR gene expression. In this context, VACV induces an increase in expression levels of HSP70 by 20-fold, while HSP90 and HSP60 levels were only increased by 3-fold (309, 383).

We observed an increase of HSP70 levels during VACVCop infection compared to uninfected cells; but the HSP70 levels did not differ between VACVCop, VACV-FLAG-p28, or VACV-FLAG-p28(C173S/C176S) infected cells. Interestingly, HSP70, HSP72 and HSP90 are also packed into mature poxvirus virions, which suggest the importance for chaperones in early stages of virus infection (227, 335, 336). Several poxviral proteins have been reported to interact or bind to HSPs during infection. Jindal *et al.* showed that HSP70 co-immunoprecipitated intracellular VACV proteins, which identities are still unknown (383). HSP90 interacts with A10, also known as VACV-WR core protein 4a, and co-localizes at the virus factory (337). Furthermore, several poxviral proteins are bound to HSP70/72 (383). HSP27 has been reported to bind to VACV proteins VACV-WR002, C2, and I4 in a yeast-two hybrid screen, but awaits confirmation (338). However, the function of these interactions remains elusive.

Our study contributes to this existing body of research by demonstrating that HSP70 and HSP90 are associated with p28 in co-immunoprecipitation studies and the presence of p28 homologues enrich HSP70 at the virus factory **(Figure 5.4.1 E, and 5.2.6)**. It appeared that p28 and FWPV homologues were capable of triggering HSP70 from both the cytoplasm and the nucleus to the virus factory, whereas VACVCop infection alone and the M143R homologue in MYXV only seemed to have an effect on

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cytoplasmic HSP70 enrichment at the virus factory **(Figure 5.6)**. The effect of recruiting cytoplasmic and/or nuclear HSP70 remains elusive. Furthermore, the HSP70 family contains multiple homologues and isoforms. The most studied HSP70 members include the constitutively expressed cytosolic Hsc70 and the stress-induced HSP70 homologues (384-386). It is possible that we detected both Hsc70 and HSP70 due to their high sequence identity (>90%) and since many HSP70 antibodies are cross-reactive. The HSP70 isoform Hsc70 is constitutively expressed at elevated levels in the cytoplasm and is reported to be packed into mature virions during VACV-WR infection (227). We speculate that Hsc70 was also recruited to the virus factories during VACVCop infection. Interestingly, p28 mass spectrometry hits included both HSP70 members Hsc70 and HSPA1A. In contrast to Hsc70, which is readily available in the cytoplasm, HSPA1A is induced by HSF1 activation (387). Future studies should evaluate which HSP70 family members are packed into mature virions in the presence and absence of p28 and if there is a biological relevance to this.

While poxviruses appear to utilize different cellular chaperones at different stages throughout their lifecycle, some poxviruses encode their own HSF1-regulated genes which may lower their dependence on these host HSPs. MCV and Crocodilepox encode viral homologs of HSP40, which is lacking in all the other poxviruses (333, 334). Members of the HSP40 family typically regulate protein folding, assembly, and protein transport. HSP40 is a co-factor for HSP70, recognizing unfolded-proteins as well as chaperone proteins (388). It is possible that HSP70 works with HSP40 to help chaperone poxviral proteins at the virus factories. HSP40 was another hit in our p28 mass spectrometry screen, but we had no available antibody to follow up with HSP40. Since

VACVCop is lacking a HSP40 homologue, p28 might be also responsible for recruiting HSP40, together with HSP70 to the virus factories. Future studies will investigate the employment of HSP40 during VACV infection and interaction with p28. Other large DNA viruses also encode their own HSPs, including mimiviruses, which express HSP70 and HSP40 homologues (332, 389, 390). There seems to be a trend towards the large cytosolic DNA viruses utilizing large amounts of host cellular HSPs for chaperoning purposes.

It is unknown how poxviruses activate HSF1, resulting in the up-regulation of HSPs. HSF1 in its inactive form is kept in the cytoplasm by binding to HSP70 or HSP90 (391-393). VACV may activate HSF1 by recruiting HSPs to the virus factory, resulting in post-translational modification and nuclear translocation of free HSF1. So far, only HSP90 accumulation at the virus factories has been described (337). Here, we further showed HSP70 enrichment at the virus factory, which is significantly enhanced by the presence of p28. In future experiments we will investigate whether p28 affects HSP70 localization to the virus factory in order to permit HSF1 nuclear translocation and the subsequent upregulation of HSP gene transcription. Future experiments should also investigate if this p28 mediated HSP70 recruitment to the virus factory is crucial for VACV replication and if this is important for p28 to function as a virulence factor.

Interestingly, HSP70 can associate with the E3-ubiquitin ligase C-terminus of HSP70 Interacting Protein (CHIP) (394). CHIP assists in the decision-making, whether HSP70 is chaperoning the substrate proteins, or if it promotes the ubiquitination of HSP70 substrates leading to their proteasomal degradation (394, 395). In fact, CHIP interacts with the substrate-binding domain of HSP70 and negatively influences the

chaperone activity of HSP70. Furthermore, the ubiquitin ligase activity of CHIP can promote the degradation of proteins that were interaction partners of HSP70 (395-397). Future ubiquitination experiments could also explore whether CHIP is the unknown ubiquitin ligase, which mediates the ubiquitination of p28, and if this interaction is mediated through HSP70.

Conclusions

This thesis characterized the poxviral encoded ubiquitin ligase p28 and homologues in FWPV. The large genomes of the prototypical members of *Avipoxvirus*, FWPV and CNPV, encode a number of yet uncharacterized proteins. We demonstrated that FWPV encodes two functional p28 homologues, which act as ubiquitin ligases. Interestingly, one is expressed early, while the other homologue is expressed only late during infection. An additional observation was that all KilA-N only and KilA-N-DUF genes were actively transcribed during FWPV infection. The function of the poxviral encoded KilA-N DNA binding domains seems to aid in the localization of the protein to the virus factories. The biological relevance of expressing two p28 homologs, which contain KilA-N a domain paired with a RING domain remains unknown. In contrast, non-Avipoxviruses encode only one p28-like ubiquitin ligase. FWPV further encodes two KilA-N domains paired with DUF (FWPV124 and FWPV155). We used BLAST analysis to speculate on the role of these DUFs, but we were unable to find similar proteins. It would be interesting to investigate what functions the DUF in FWPV124 and FWPV155 hold, in addition to the p28 homologues in FWPV.

The new knowledge gained from this thesis has increased our understanding into the regulation of p28 via ubiquitination and the UPS. Our data confirmed a role of the RING domain for the p28 ubiquitin ligase activity. The investigation of non-functional p28 RING mutants further revealed that p28 is regulated by both self-ubiquitination and ubiquitination by another unknown ubiquitin ligase(s). Continued work on the ubiquitination of p28 will allow us to better understand this regulatory mechanism. It will help us to clarify whether the ubiquitination events are a simple consequence of the E3 activity with no functional impact, or if ubiquitination is a feedback mechanism, where the presence of substrate shields the ubiquitin ligase from ubiquitination. This homeostatic feedback mechanism would result in accumulation of the ubiquitin ligase only in presence of abundant substrate (139, 155, 362, 398). In some cases, ubiquitination can have the opposite effect exemplified by the ubiquitin ligases Bmi1-Ring1b and Bard1-Brca1, where ubiquitination leads to an increase in their E3 ligase activity (399, 400). We could also distinguish, if the ubiquitination of p28 is solely required for the proteasomal degradation of p28 at a certain stage during infection, or if the ubiquitination is nonproteolytic and activate signaling, as in the case of TRAF6 (139).

Finally, we conducted a mass spectrometry screen for p28 interaction partners in an attempt to understand the function of this ubiquitin ligase. We identified HSP70 as an interaction partner of p28 during VACV infection. HSP70 was enriched at the virus factory in the presence of p28 compared with VACVCop infection alone. Many viruses use HSP70 for their own purposes, including viral cell entry, uncoating, transcription and genome replication, virion morphogenesis, encapsidation, virion release, and co-chaperoning purposes in general (313-316, 319, 320, 322-324, 326-328, 330, 331). It

remains elusive which purpose the p28 mediated HSP70 recruitment to the virus factory holds. This study of how viruses exploit cellular signaling pathways provides a stepping-stone to further understand virus host interactions with the ultimate goal of developing therapeutic anti-viral drugs for the treatment of virus infection.

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Appendix: Supporting methodology and data

A.1 Generation of recombinant VACVCop expressing FLAG-p28 mutants

Many experiments presented in this thesis were conducted with poxviral infection and simultaneously transfection of pSC66 plasmids expressing FLAG-tagged p28 mutant proteins, following the general infection/transfection protocol **(Section 2.3.2)**. We list the generation of recombinant viruses in the Appendix, since we did not use any of these viruses in the main thesis. However, the recombinant viruses could be used in future experiments to avoid a low transfection rate and allow protein expression in macrophages during VACV infection.

Recombinant VACVCop viruses that express FLAG-tagged versions of p28 mutants were generated by homologous recombination into the TK locus. CV-1 cells were infected with VACVCop and simultaneously transfected with pSC66-FLAG-p28(1-152), pSC66-FLAG-p28(153-242), pSC66-FLAG-p28(Δ44-51), pSC66-FLAG-p28(1-184), or pSC66-FLAG-p28(1-204). The pSC66 plasmid contains both a *LacZ* gene and FLAG tagged p28 mutant under control of poxviral promoters flanked by regions of homology to the VACV TK locus **(Figure A.1.A)**. Cells that become both infected and transfected may undergo a double-crossover event between the VACV TK locus and flanking regions of homology in pSC66, resulting in a TK negative virus that expresses both FLAG-tagged protein and β-galactosidase. Briefly, CV-1 cells (ATCC) (8×10^5) were infected with VACV at an MOI of 0.05 in 500 µl of OptiMEM for 1 hour. During this time, 10 µg of pSC66-FLAG-p28(1-152), pSC66-FLAG-p28(153-242), pSC66-FLAG-p28(Δ44-51), pSC66-FLAG-p28(1-184), or pSC66-FLAG-p28(1-204) **(Figure A.1.A)** was mixed with 10 µl of Lipofectamine 2000 in 200 µl of OptiMEM as described

in 2.3.1. After one hour, the infection media was removed and replaced with 800 µl of OptiMEM along with the 200 µl DNA-Lipid mixture, then allowed to incubate at 37°C and 5% CO_2 for 5 hours. The cells were then supplemented with 1 mL of recovery media and incubated for another 24 hours. Cells were harvested in SSC, centrifuged at 1000 x g, and re-suspended in 100 µl of ice-cold swelling buffer. For selection of recombinant VACV-FLAG-p28(1-152), VACV-FLAG-p28(153-242), VACV-FLAG-p28(Δ44-51), VACV-FLAG-p28(1-184), or VACV-FLAG-p28(1-204), cell monolayers (1 x 10^6) infected with serial dilutions of virus were overlaid with 1.5 mL of a low melting point (LMP) agarose (Invitrogen) mixture containing 2.5 mL of 3% LMP agarose in water (w/v), 2.5 mL DMEM, 1 mL NCS, and 100 µl of 100 mg/mL X-gal. The overlay was allowed to solidify at room temperature for 15 minutes, followed by incubation at 37°C and 5% CO_2. Recombinant viruses will express β-galactosidase, the product of the *LacZ* gene, which will hydrolyze X-gal in the overlay and result in a bright blue product. Blue plaques were picked the following day using a pasteur pipette and suspended in 100 µl of ice cold swelling buffer. Plaque picks containing virus-infected cells underwent three freeze-thaw cycles at -80°C and 37°C followed by sonication to lyse cells and release infectious virus to be used for subsequent purification rounds or amplification. After 4 to 5 purifying rounds in CV-1, the same screening process was executed in HuTK$^{-/-}$-143B in the presence of 25 µg/mL BrdU. Any virus with an intact TK gene will be unable to grow in the presence of BrdU. To check purity of the recombinant viruses, virus genomic DNA was isolated (see 2.4.6) and subjected to PCR analysis with TK- primer **(Figure A.1.C)**.

A

B

KilA-N DNA Binding   RING

C
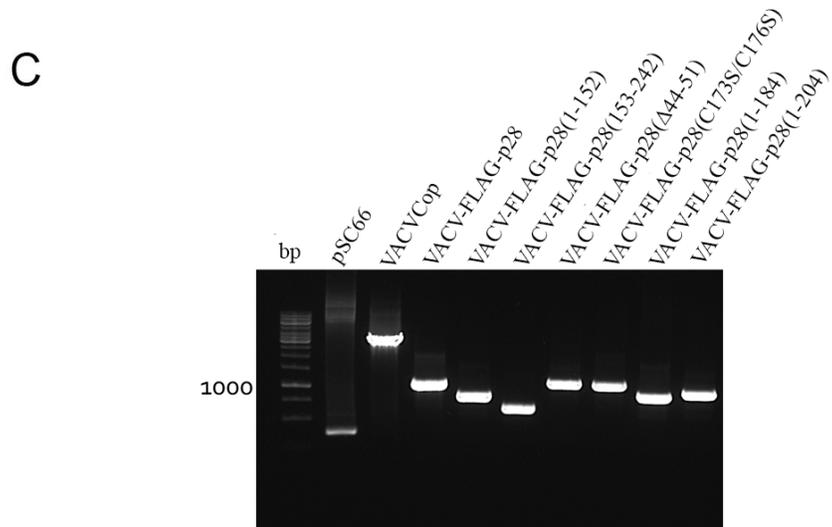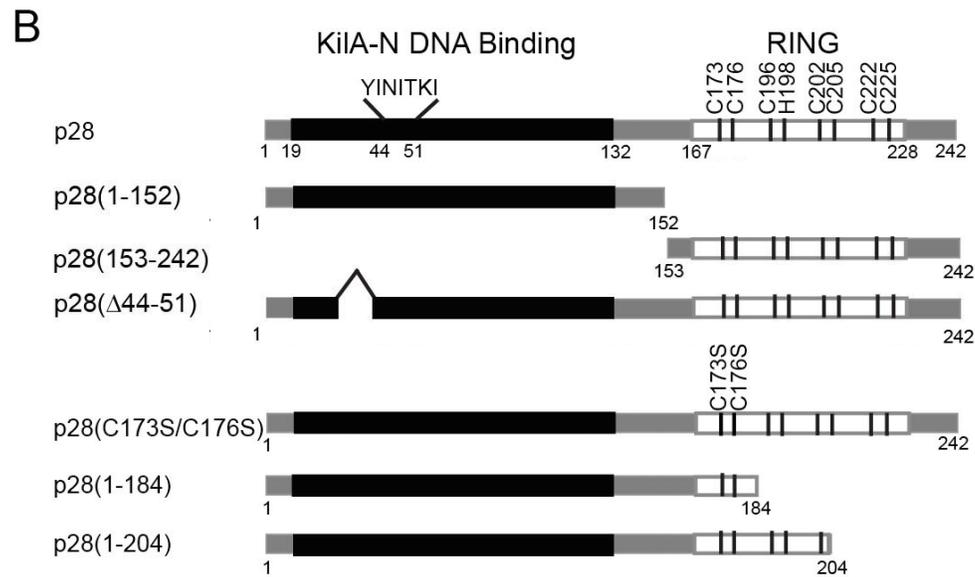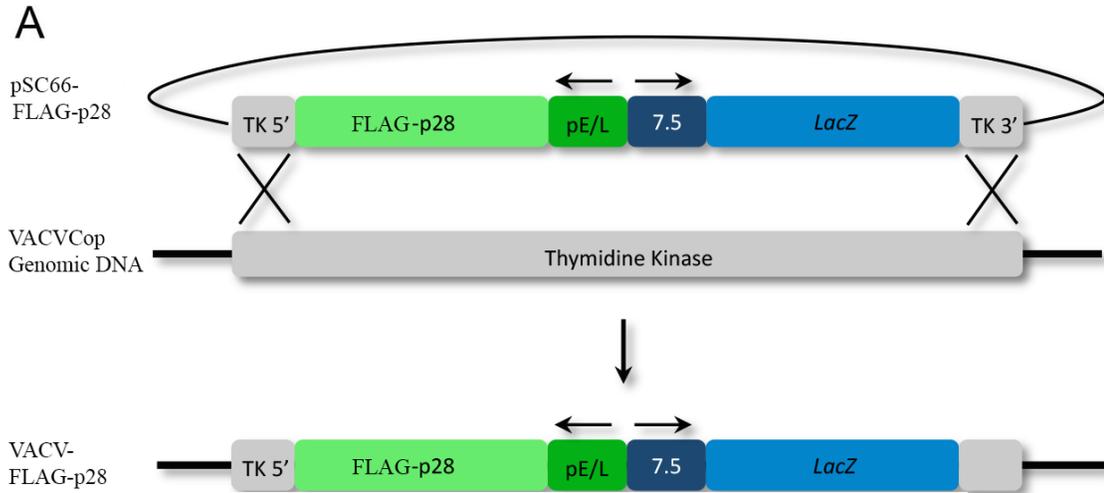
Figure A.1 Generation of recombinant VACV strains. To generate the VACV-FLAG-p28 mutant viruses, CV-1 cells were infected with VACVCopWhite and simultaneously transfected with pSC66-FLAG-p28(1-152), pSC66-FLAG-p28(153-242), pSC66-FLAG-p28(Δ44-51), pSC66-FLAG-p28(1-184), and pSC66-FLAG-p28(1-204), here referred as to FLAG-p28 mutants. **(A)** The pSC66 plasmid contains the *LacZ* gene under a 7.5 poxviral promoter and the gene of interest (FLAG-p28 mutant) under control of a synthetic poxviral early/late promoter, both flanked by regions of homology to the VACVCopWhite thymidine kinase locus (289). CV-1 cells, which are infected and transfected can undergo homologous recombination, resulting in a recombinant virus expressing FLAG-p28 mutants, β-galactosidase, and are lacking a functional thymidine kinase**. (B)** List of p28 mutants, which were used to generate recombinant viruses. **(C)** Genomic DNA from recombinant viruses was isolated and PCR was performed with specific primers for the TK-site. Shown is a SYBR safe stained agarose gel, which shows the presence of p28 mutant genes in the recombinant virus TK-sites. pSC66 control shows TK-site without inserted gene.

A.2 Validation of p28 mass spectrometry hits revealed a high false positive rate.

Chapter 5 described the main approach of mass spectrometry validation for p28 co-immunoprecipitated proteins, belonging to the major functional clusters of annotation. Here, we present further validation of p28 hits, where antibodies were readily available. These include valosin-containing protein (VCP), proliferating cell nuclear antigen (PCNA), and proteasomal subunits Rpt6 and PSMB3. We further tested if the FLAG-tagged Karyopherin (KPNA) subtypes 1-6 interacted with EGFP-tagged p28. Methodology for co-immunoprecipitation and confocal analysis was similar to Chapter 5. In brief, HeLa cells were infected with VACVCop, VACV expressing FLAG-p28, or FLAG-p28(C173S/C176S) at an MOI of 5 and cells were treated with MG132 (20μM) for the last 4 hours of 16 hour infection. Immunoprecipitations were performed using the anti-FLAG M2 mAB. Protein G beads were washed with 1% NP-40 lysis buffer. Immunoprecipitated proteins were then eluted from the beads by boiling in SDS-PAGE sample loading buffer. For mass spectrometry follow-up immunoprecipitations and western blotting, primary antibody staining were anti-VCP, anti-PCNA, anti-Rpt6, and anti-PSMB3 **(Figure A.2.1)**. WCL of HeLa cells infected with VACVCop expressing FLAG-p28 were also included to confirm that the interacting proteins were expressed in cells. Anti-FLAG western blot shown is a representative from one immunoprecipitation and demonstrates that the anti-FLAG immunoprecipitations worked. This representative blot is also shown in Figure 5.3.2 and 5.3.1. We detected VCP, PCNA and Rpt6 in WCL, but we were unable to detect PSMB3. We could only detect weak bands for co-immunoprecipitated Rpt6 with FLAG-p28 and FLAG-p28(C173S/C176S); none of the other tested proteins co-immunoprecipitated with FLAG-p28 in lysates of VACV

IP: anti-FLAG
WB: anti-FLAG

IP: anti-FLAG
WB: anti-VCP

IP: anti-FLAG
WB: anti-PCNA

IP: anti-FLAG
WB: anti-Rpt6

IP: anti-FLAG
WB: anti-PSMB3
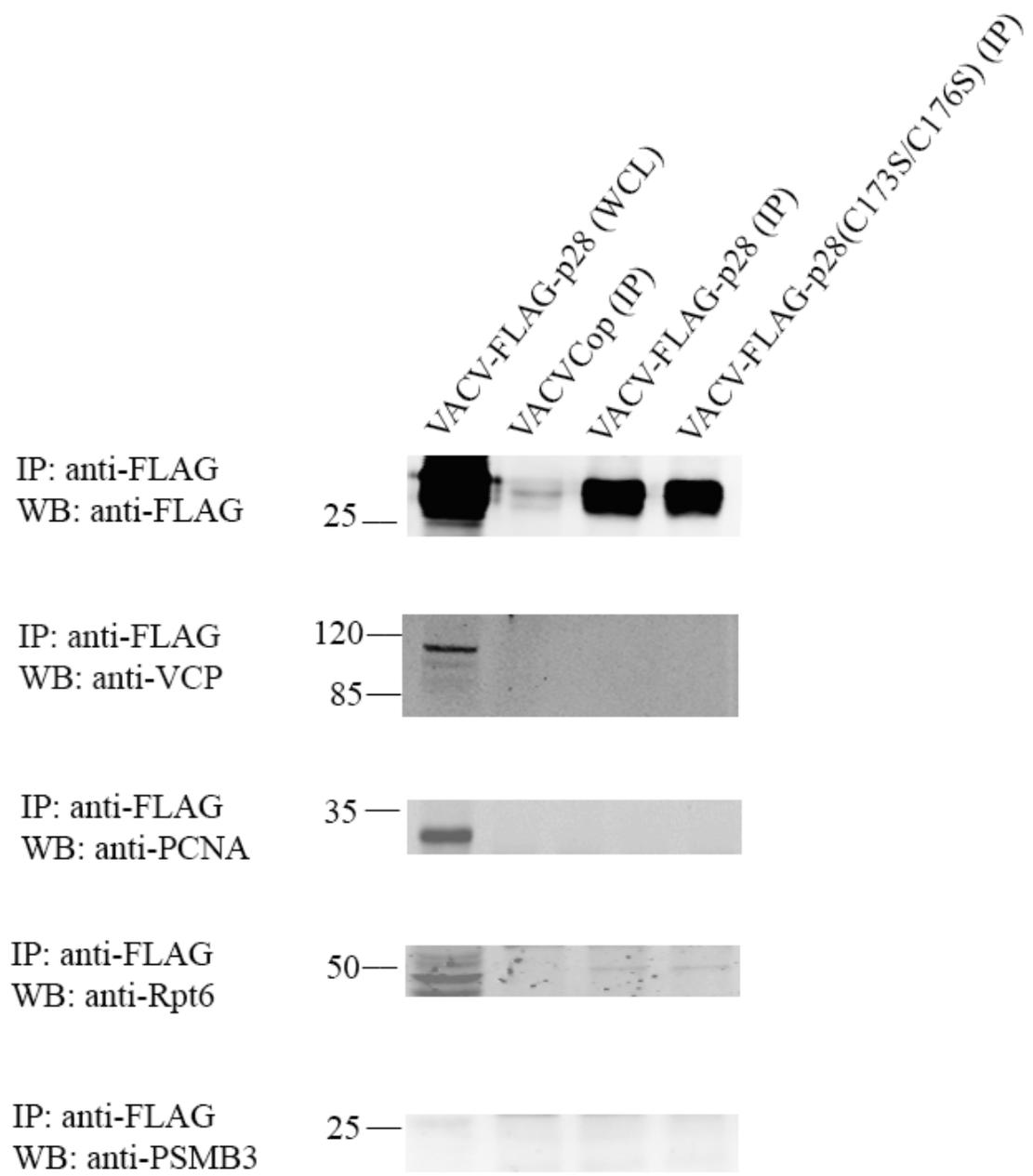
Figure A.2.1 Validation of p28 mass spectrometry hits VCP, PCNA, Rpt6 and PSMB3 revealed no interaction in co-immunoprecipitation studies. HeLa cells were infected with VACVCop, VACV-FLAG-p28, or VACV-FLAG-p28(C173S/C176S) at an MOI of 5 and cells were treated with MG132 (20μM) for the last 4 hours of 16 hour infection. Immunoprecipitations were performed using the anti-FLAG M2 mAB in with 1% NP-40 lysis buffer. For mass spectrometry follow-up immunoprecipitations and western blotting were performed with primary antibody staining for anti-VCP, anti-PCNA, anti-Rpt6, and anti-PSMB3. WCL of HeLa cells infected with VACVCop expressing FLAG-p28 were also included to confirm that the interacting proteins were expressed in cells, which was the case for VCP, PCNA and Rpt6; but we were unable to detect PSMB3. Note: All though some of these blots are from separate anti-FLAG IPs, we verified that the FLAG proteins were precipitated in all IPs. The anti-FLAG blot shown is representative from one IP. This representative blot is also shown in Figure 5.3.1 and 5.3.2.

infected cells expressing FLAG-p28. Since the KPNA subtypes 1-6 were readily available in FLAG-tagged pcDNA3 vectors (generous gift from D. Evans), we tested, if any of the subtypes co-immunoprecipitated with EGFP-p28. HeLa cells were transfected with either of the pcDNA-FLAG-tagged KPNA subtypes 1-6 and cells were co-transfected with pEGFP-p28. Sixteen hours post-transfection, cells were lysed in NP-40 lysis buffer and immunoprecipitations were performed using the anti-EGFP antibody. Protein G beads were washed with 1% NP-40 lysis buffer. Immunoprecipitated proteins were then eluted from the beads by boiling in SDS-PAGE sample loading buffer. The immunoprecipitates were analysed by western blotting using antibodies specific for EGFP to detect the pulldown EGFP-p28, and FLAG to investigate, if any KPNA subtypes co-immunoprecipitated with EGFP-p28. WCL of transfected HeLa cells with KPNA subtypes were also included to confirm their expression in cells. We were unable to detect any of the tested proteins in anti-EGFP-immunoprecipitates of cells expressing FLAG-KPNA-1, FLAG-KPNA-2, FLAG-KPNA-3, FLAG-KPNA-4, FLAG-KPNA-5, or FLAG-KPNA-6, even though all proteins were expressed and were present in the WCL **(Figure A.2.2)**. We were unable to validate the interaction of KPNA subtypes with p28.

We further follow up with confocal analysis, to investigate, if VACVCop, VACVCop expressing FLAG-p28, or FLAG-p28(C173S/C176S) had an effect on the cellular distribution of PCNA, Rpt6, PSMB3, and MSH6. Unfortunately, the VCP antibody was unsuitable for immunofluorescence analysis. In brief, HeLa cells were mock-infected, or infected with VACVCop, VACVCop expressing FLAG-p28, or VACVCop expressing FLAG-p28(C17S/C176S) at an MOI of 5 for 14 hours.
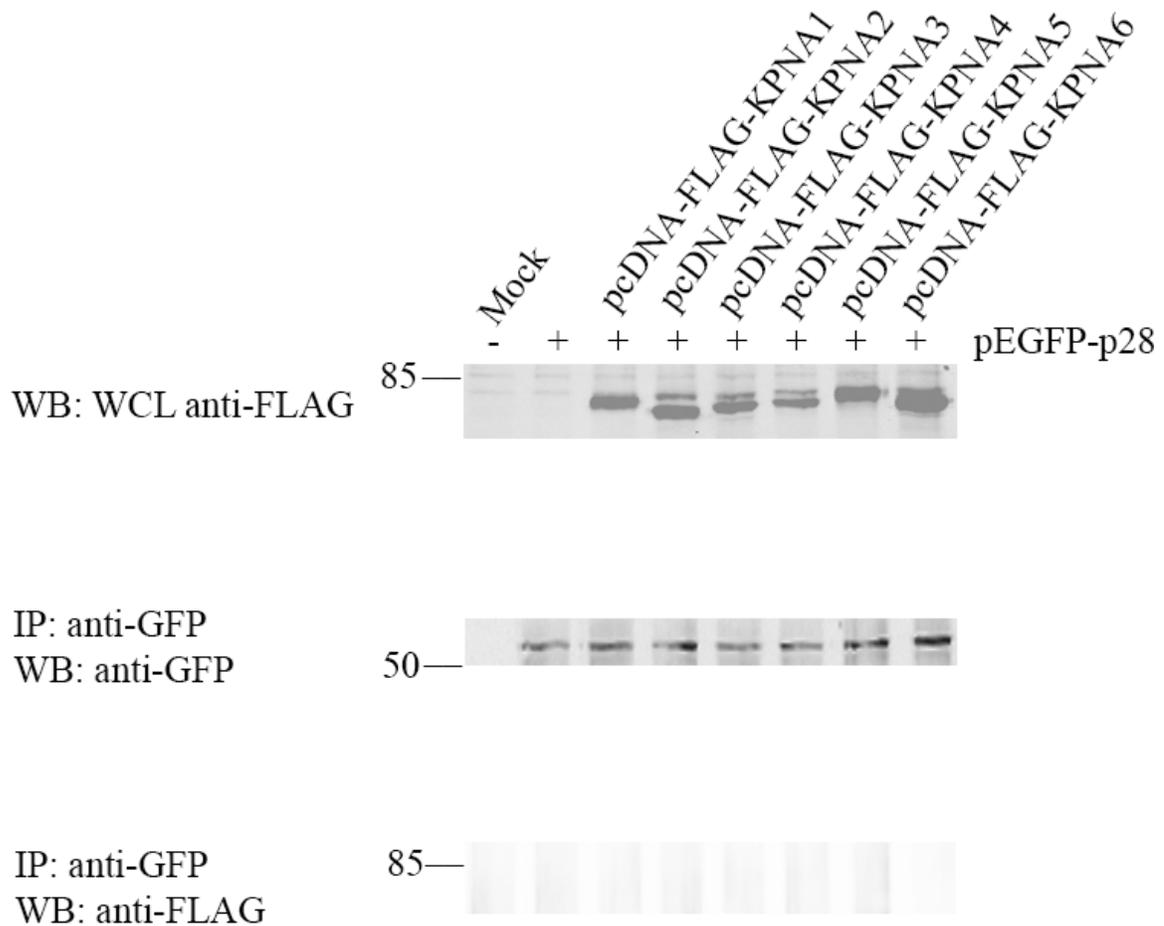
Figure A.2.2 Karyopherin subtypes 1-6 did not co-immunoprecipitate with EGFP-p28. HeLa cells were transfected with pcDNA plasmids expressing FLAG-tagged KPNA subtypes 1-6 and were co-transfected with pEGFP-p28 for 16 hours. Cells were lysed in NP-40 lysis buffer and immunoprecipitated with an antibody against EGFP. The immunoprecipitates were analysed by western blotting using antibodies specific for EGFP to detect the pulldown EGFP-p28, and FLAG-taggedy KPNA subtypes. WCL of HeLas transfected with KPNA subtypes were included to confirm their expression. We were unable to detect any of the tested proteins in anti-EGFP-immunoprecipitates of cells expressing FLAG-KPNA-1, FLAG-KPNA-2, FLAG-KPNA-3, FLAG-KPNA-4, FLAG-KPNA-5, or FLAG-KPNA-6, even though all proteins were expressed and were present in the WCL.

Cells were stained with anti-PCNA, anti-MSH6, anti-Rpt6, or anti-PSMB3. We investigated whether p28 affected the cellular localization of the mass spectrometry hits. Both, PCNA and MSH6 localized mostly in the nucleus in mock-infected cells **(Figure A.2.3 and A.2.4)**. Some of the cellular PCNA and MSH6 were re-localized from the nucleus to the virus factory during VACVCop infection. However, the presence of p28 or p28(C173S/C176S) did not change this phenotype. We were unable to demonstrate that MSH6 co-immunoprecipitates with p28 **(Figure 5.3.2)**. Here we confirm with immunofluorescence that p28 has no effect of MSH6 localization to the virus factory.

Since a significant functional cluster of p28 mass spectrometry hits were parts of the 26S proteasome, we investigated the effect of the presence of FLAG-p28 on their subcellular localization, similar as seen in the sections above. We demonstrate in **Figure A.2.5** that the presence of FLAG-p28 leads to the enrichment of proteasomal subunit PSMB3 at the virus factory, but has no effect on the subunit Rpt6. This effect is similar to what was seen in previous work by the Barry group (362).
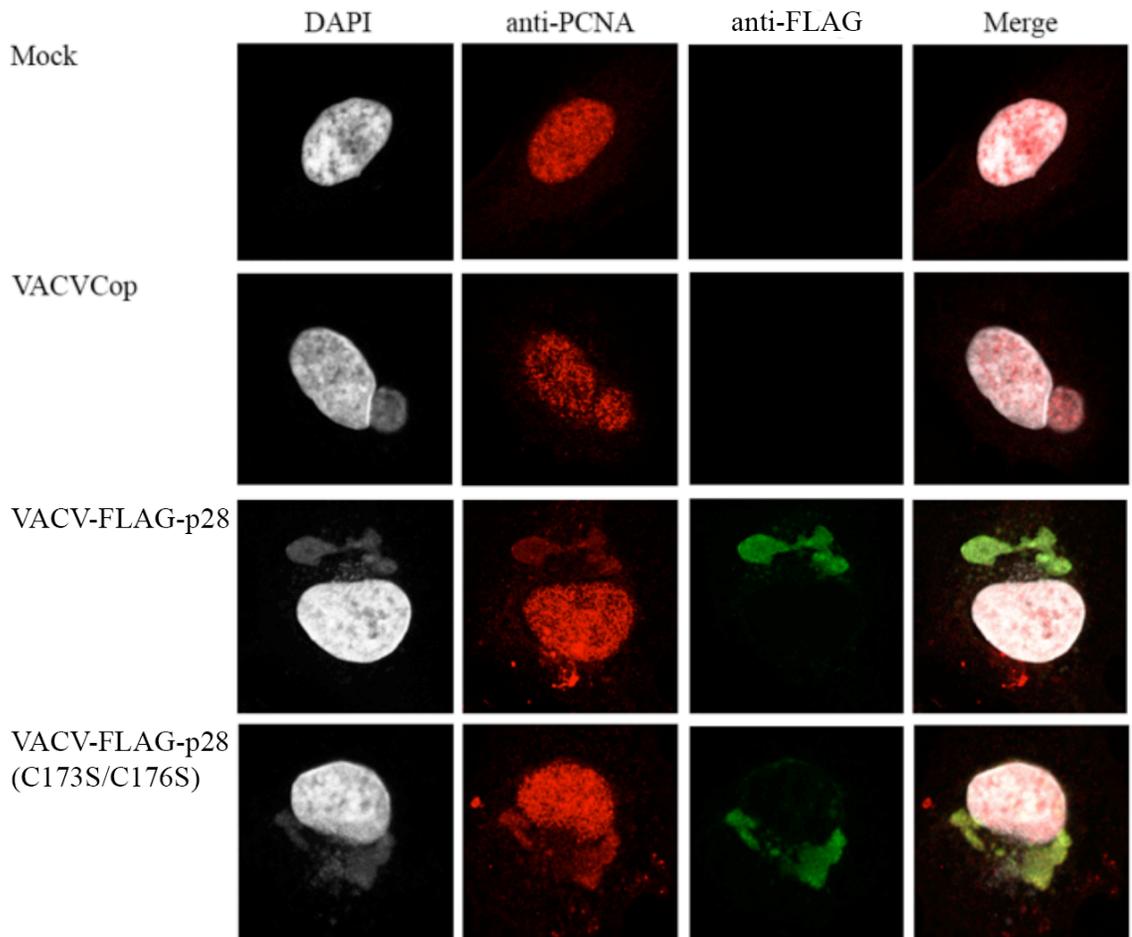
Figure A.2.3 VACVCop infection leads to PCNA localization to the virus factories, independent on the presence of functional p28. HeLa cells were mock-infected, or infected with VACVCop, VACV-FLAG-p28, or VACV-FLAG-p28(C17S/C176S) at an MOI of 5 for 14 hours. Cells were fixed with 4% PFA and permeabilized with 1% NP-40. Cells were stained with anti-PCNA, anti-FLAG and DAPI to visualize nuclei and cytoplasmic viral factories. Cells were examined using a Leica SP5 confocal microscope. We investigated whether p28 affected the cellular localization of the potential mass spectrometry hit PCNA. PCNA localized mostly in the nucleus in mock infected cells. Some of the cellular PCNA was re-localized from the nucleus to the virus factory during VACVCop infection. The presence of p28 or p28(C173S/C176S) did not change this phenotype.
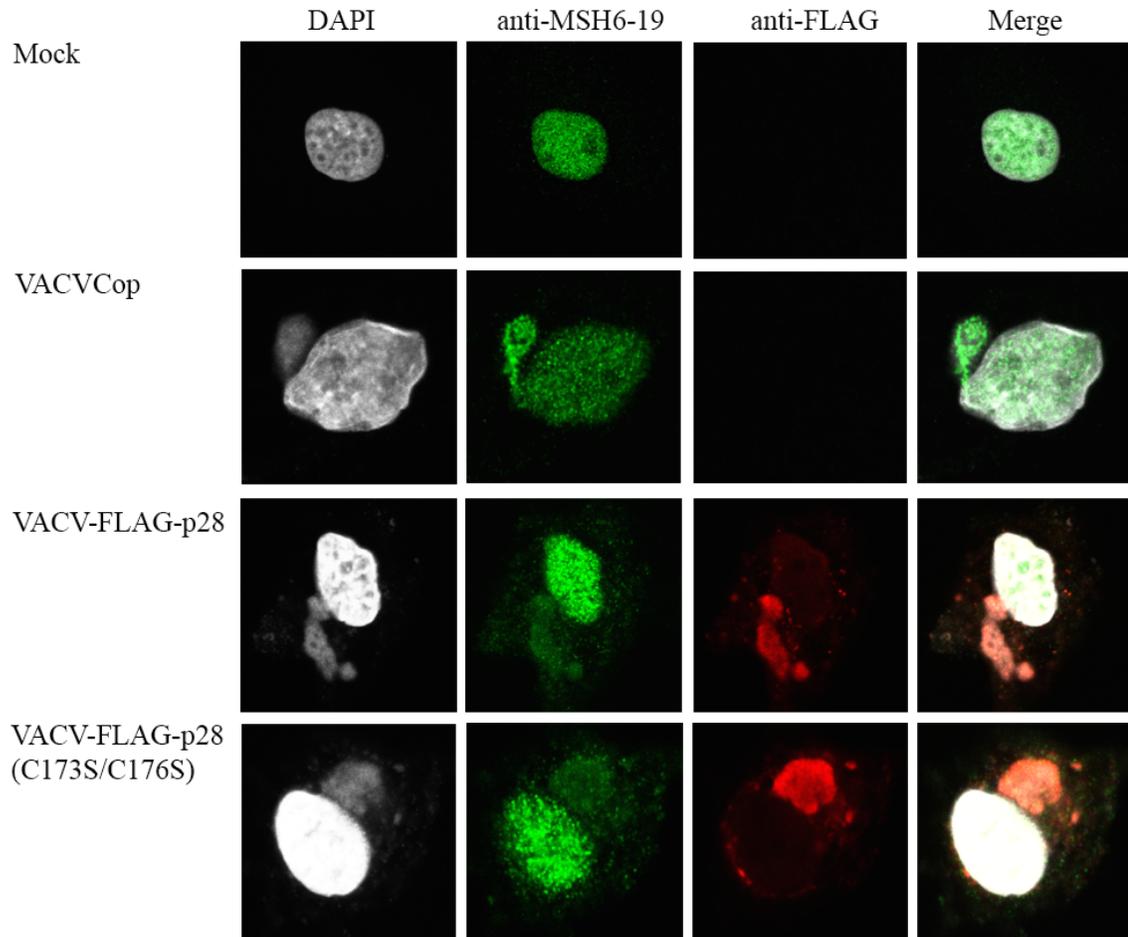
Figure A.2.4 VACVCop infection leads to MSH6 localization to the virus factories, independent on the presence of functional p28. HeLa cells were mock-infected or infected with VACVCop, VACV-FLAG-p28, or VACV-FLAG-p28(C17S/C176S) at an MOI of 5 for 14 hours. Cells were fixed in 4% PFA and stained for anti-FLAG, anti-MSH6, DAPI and were analyzed with confocal analysis. We compared the localization of MSH6 at the virus factory where p28 resides, which was evaluated with confocal analysis. In VACVCop infected cells, MSH6 localized to the virus factory. A similar effect was seen for VACVCop infected cells expressing FLAG-p28 or FLAG-p28(C173S/C176S). We did not further follow up on MSH6, since VACVCop infection led to MSH6 localization to the virus factories, independent on the presence of functional p28.

A

	DAPI	anti-PSMB	anti-FLAG	Merge
Mock				
VACVCop				
VACV-FLAG-p28				
VACVCop-FLAG-p28 (C173/176S)				
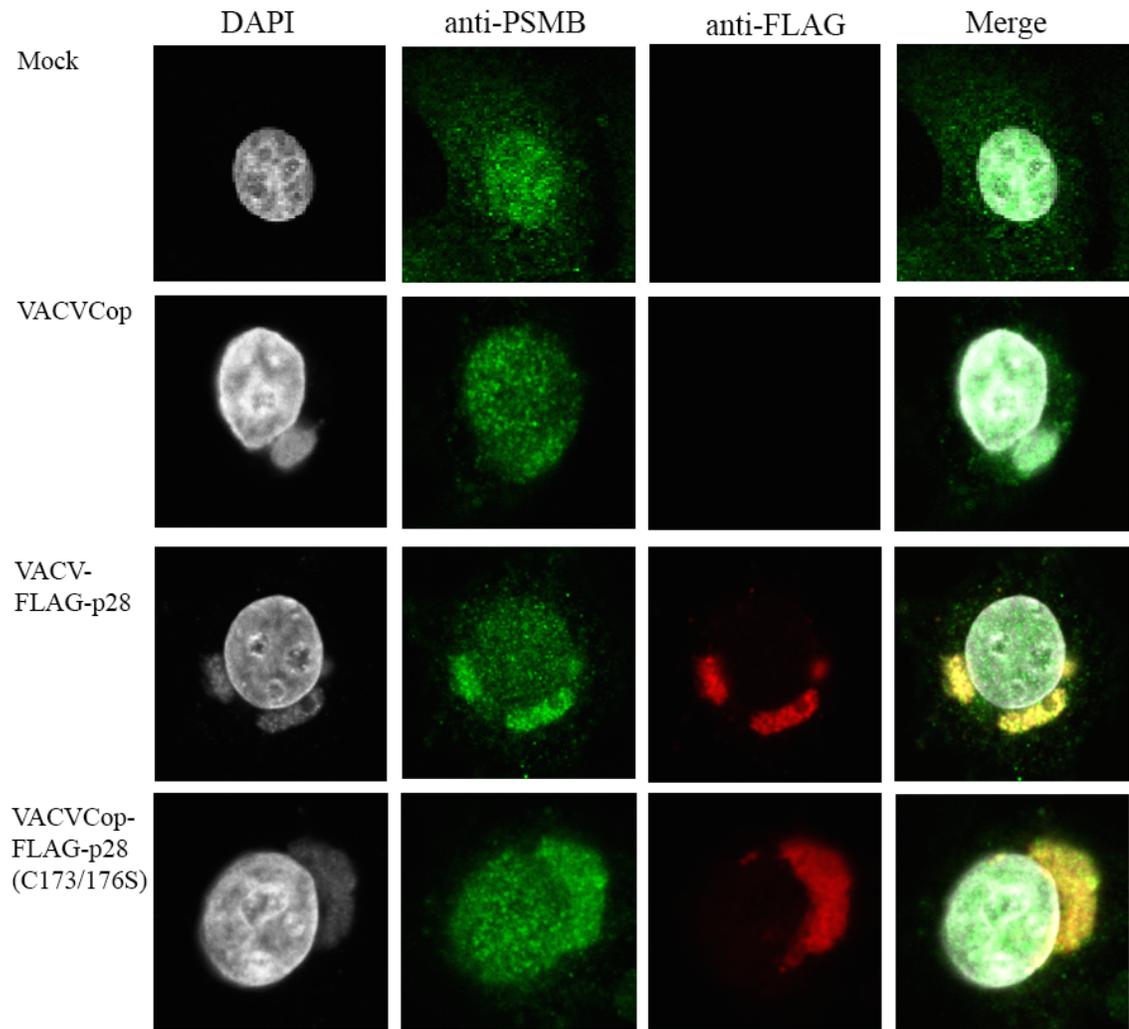
B
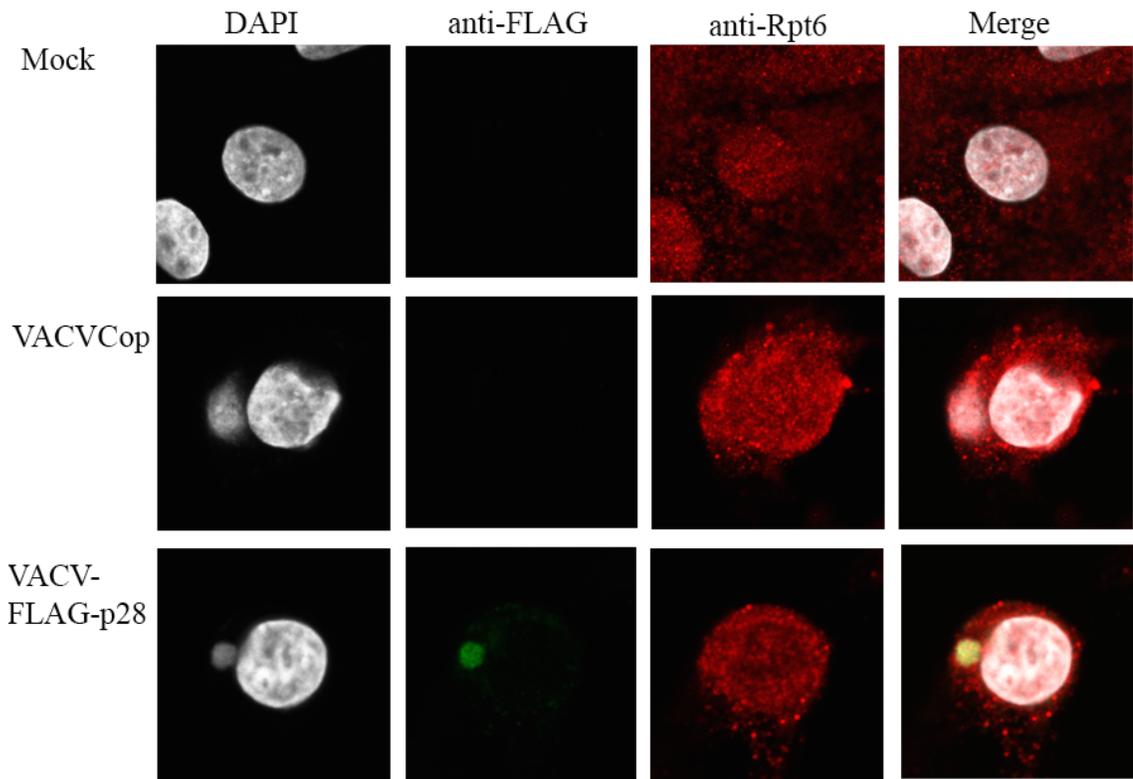
Figure A.2.5 p28 leads to the enrichment of proteasomal subunit PSMB3 to the virus factory, but has no effect on the subunit Rpt6. HeLa cells were infected with VACVCop, VACV-FLAG-p28, or VACV-FLAG-p28(C17S/C176S) at an MOI of 5 for 14 hours. Cells were fixed in 4% PFA and stained for mouse anti-FLAG, DAPI, and **(A)** rabbit anti-PSMB, or **(B)** anti-Rpt6 and were analyzed with confocal analysis. The presence of FLAG-p28 led to the enrichment of PSMB to the virus factory, but had no effect on Rpt6.

Table A.1 Cellular mass spectrometry results of FLAG-p28 and FLAG-p28(C173S/C176S) pull-downs. Score: Abundance of peptides in the sample. Coverage: percentage of protein recovered.

Accession	Description	Score VACV-FLAG p28 (C173S/ C176S)	Coverage VACV-FLAG p28 (C173S/ C176S)	Score VACV-FLAG-p28	Coverage VACV-FLAG-p28
P0CG48	Polyubiquitin-C OS=Homo sapiens GN=UBC PE=1 SV=3 - [UBC_HUMAN]	62.24	61.75	90.88	49.93
P87607	E3 ubiquitin-protein ligase p28-like OS=Cowpox virus (strain GRI-90 / Grishak) GN=p28 PE=3 SV=1 - [P28_CWPXG]	864.99	38.84	799.26	48.76
P11142	Heat shock cognate 71 kDa protein OS=Homo sapiens GN=HSPA8 PE=1 SV=1 - [HSP7C_HUM AN]	688.63	49.85	430.05	47.83
O57224	25 kDa core protein A12L OS=Vaccinia virus (strain Ankara) GN=MVA123L PE=2 SV=1 - [A12_VACCA]	16.85	34.76	48.57	47.59

P23396	40S ribosomal protein S3 OS=Homo sapiens GN=RPS3 PE=1 SV=2 - [RS3_HUMAN]	52.46	44.86	20.50	31.28
O60884	DnaJ homolog subfamily A member 2 OS=Homo sapiens GN=DNAJA2 PE=1 SV=1 - [DNJA2_HUM AN]	39.23	25.97	77.98	35.44
Q49PZ0	E3 ubiquitin ligase p28-like OS=Vaccinia virus (strain LC16m0) GN=p28 PE=3 SV=1 - [P28_VACC0]	904.07	38.49	883.42	37.66
P35998	26S protease regulatory subunit 7 OS=Homo sapiens GN=PSMC2 PE=1 SV=3 - [PRS7_HUMA N]	33.57	30.02	39.31	27.02
P49411	Elongation factor Tu, mitochondrial OS=Homo sapiens GN=TUFM PE=1 SV=2 - [EFTU_HUMA N]	54.80	29.87	105.03	38.05
P38646	Stress-70 protein, mitochondrial OS=Homo	120.85	35.20	139.19	26.66

	sapiens GN=HSPA9 PE=1 SV=2 - [GRP75_HUMAN]				
P25705	ATP synthase subunit alpha, mitochondrial OS=Homo sapiens GN=ATP5A1 PE=1 SV=1 - [ATPA_HUMAN]	40.63	20.07	104.39	34.36
P08107	Heat shock 70 kDa protein 1A/1B OS=Homo sapiens GN=HSPA1A PE=1 SV=5 - [HSP71_HUMAN]	150.07	32.14	179.39	27.61
P60709	Actin, cytoplasmic 1 OS=Homo sapiens GN=ACTB PE=1 SV=1 - [ACTB_HUMAN]	49.02	25.87	77.88	29.33
P20643	Major core protein 4b OS=Vaccinia virus (strain Copenhagen) GN=A3L PE=3 SV=1 - [P4B_VACCC]	86.88	18.48	287.94	29.50
P55072	Transitional endoplasmic reticulum ATPase OS=Homo sapiens GN=VCP PE=1	87.64	28.04	25.90	13.03

	SV=4 - [TERA_HUMAN]				
P05141	ADP/ATP translocase 2 OS=Homo sapiens GN=SLC25A5 PE=1 SV=7 - [ADT2_HUMAN]	101.17	29.19	118.65	19.80
P04792	Heat shock protein beta-1 OS=Homo sapiens GN=HSPB1 PE=1 SV=2 - [HSPB1_HUMAN]	8.27	18.05	22.69	23.41
Q07021	Complement component 1 Q subcomponent-binding protein, mitochondrial OS=Homo sapiens GN=C1QBP PE=1 SV=1 - [C1QBP_HUMAN]	27.01	26.95		0.00
P20638	Envelope protein F13 OS=Vaccinia virus (strain Copenhagen) GN=F13L PE=3 SV=1 - [F13_VACCC]	55.66	26.34	40.42	23.92
P31689	DnaJ homolog subfamily A member 1 OS=Homo sapiens GN=DNAJA1 PE=1 SV=2 - [DNJA1_HUM	32.93	23.17	68.39	26.20

	AN]				
Q9NS69	Mitochondrial import receptor subunit TOM22 homolog OS=Homo sapiens GN=TOMM22 PE=1 SV=3 - [TOM22_HUM AN]		0.00	10.90	26.06
P12236	ADP/ATP translocase 3 OS=Homo sapiens GN=SLC25A6 PE=1 SV=4 - [ADT3_HUMA N]	43.17	25.84	25.98	16.11
Q9UJS0	Calcium-binding mitochondrial carrier protein Aralar2 OS=Homo sapiens GN=SLC25A13 PE=1 SV=2 - [CMC2_HUMA N]	54.27	25.33	18.72	8.44
P62195	26S protease regulatory subunit 8 OS=Homo sapiens GN=PSMC5 PE=1 SV=1 - [PRS8_HUMA N]	37.89	22.66	40.08	22.17
O95816	BAG family molecular chaperone regulator 2 OS=Homo sapiens GN=BAG2 PE=1 SV=1 -	15.08	23.22	17.59	15.64

	[BAG2_HUMA N]				
P62191	26S protease regulatory subunit 4 OS=Homo sapiens GN=PSMC1 PE=1 SV=1 - [PRS4_HUMA N]	28.01	18.18	55.84	22.73
P43686	26S protease regulatory subunit 6B OS=Homo sapiens GN=PSMC4 PE=1 SV=2 - [PRS6B_HUM AN]	21.03	9.09	43.59	22.49
P12004	Proliferating cell nuclear antigen OS=Homo sapiens GN=PCNA PE=1 SV=1 - [PCNA_HUMA N]	30.71	22.22	14.66	16.48
Q92616	Translational activator GCN1 OS=Homo sapiens GN=GCN1L1 PE=1 SV=6 - [GCN1L_HUM AN]	211.61	21.19	69.59	8.91
P68608	DNA-directed RNA polymerase 22 kDa subunit OS=Vaccinia virus (strain Copenhagen) GN=RPO22 PE=3 SV=1 - [RP22_VACCC	7.73	10.81	13.71	21.08

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Q01813	6-phosphofructoki nase type C OS=Homo sapiens GN=PFKP PE=1 SV=2 - [K6PP_HUMA N]	45.74	19.13	43.84	18.49
P62269	40S ribosomal protein S18 OS=Homo sapiens GN=RPS18 PE=1 SV=3 - [RS18_HUMA N]	16.92	21.05	22.96	21.05
P48047	ATP synthase subunit O, mitochondrial OS=Homo sapiens GN=ATP5O PE=1 SV=1 - [ATPO_HUMA N]	10.60	20.66		0.00
P21057	Protein A34 OS=Vaccinia virus (strain Copenhagen) GN=A34R PE=3 SV=1 - [A34_VACCC]	8.32	13.69	8.84	14.88
P18085	ADP-ribosylation factor 4 OS=Homo sapiens GN=ARF4 PE=1 SV=3 - [ARF4_HUMA N]		0.00	7.92	20.00
Q9BSD7	Cancer-related nucleoside-triphosphatase		0.00	7.88	20.00

	OS=Homo sapiens GN=NTPCR PE=1 SV=1 - [NTPCR_HUMAN]				
P61204	ADP-ribosylation factor 3 OS=Homo sapiens GN=ARF3 PE=1 SV=2 - [ARF3_HUMAN]		0.00	12.43	19.89
Q9UNM6	26S proteasome non-ATPase regulatory subunit 13 OS=Homo sapiens GN=PSMD13 PE=1 SV=2 - [PSD13_HUMAN]	9.68	10.64	15.18	17.02
P51571	Translocon-associated protein subunit delta OS=Homo sapiens GN=SSR4 PE=1 SV=1 - [SSRD_HUMAN]		0.00	8.11	19.65
Q06830	Peroxiredoxin-1 OS=Homo sapiens GN=PRDX1 PE=1 SV=1 - [PRDX1_HUMAN]	10.74	19.60	8.20	14.57
P68457	Late transcription elongation factor G2 OS=Vaccinia	7.76	11.36	12.49	13.64

	virus (strain Copenhagen) GN=G2R PE=3 SV=1 - [G2_VACCC]				
Q92928	Putative Ras-related protein Rab-1C OS=Homo sapiens GN=RAB1C PE=5 SV=2 - [RAB1C_HUM AN]		0.00	5.46	13.43
P30050	60S ribosomal protein L12 OS=Homo sapiens GN=RPL12 PE=1 SV=1 - [RL12_HUMA N]	11.33	18.79	7.96	18.79
P62333	26S protease regulatory subunit 10B OS=Homo sapiens GN=PSMC6 PE=1 SV=1 - [PRS10_HUMA N]	16.01	15.68	15.90	15.68
P17980	26S protease regulatory subunit 6A OS=Homo sapiens GN=PSMC3 PE=1 SV=3 - [PRS6A_HUM AN]	29.34	18.68	23.68	13.21
P36404	ADP-ribosylation factor-like protein 2 OS=Homo sapiens	7.68	18.48		0.00

	GN=ARL2 PE=1 SV=4 - [ARL2_HUMAN]				
P61026	Ras-related protein Rab-10 OS=Homo sapiens GN=RAB10 PE=1 SV=1 - [RAB10_HUMAN]	5.27	11.50		0.00
Q99653	Calcineurin B homologous protein 1 OS=Homo sapiens GN=CHP1 PE=1 SV=3 - [CHP1_HUMAN]		0.00	7.89	16.92
P78527	DNA-dependent protein kinase catalytic subunit OS=Homo sapiens GN=PRKDC PE=1 SV=3 - [PRKDC_HUMAN]	282.16	16.25	94.79	7.10
O95831	Apoptosis-inducing factor 1, mitochondrial OS=Homo sapiens GN=AIFM1 PE=1 SV=1 - [AIFM1_HUMAN]	26.56	14.52	19.32	9.79
O57198	Late transcription factor 1 OS=Vaccinia virus (strain Ankara) GN=VLTF1	22.54	16.54	19.47	16.54

	PE=2 SV=1 - [VLTF1_VACC A]				
Q9BZX2	Uridine-cytidine kinase 2 OS=Homo sapiens GN=UCK2 PE=1 SV=1 - [UCK2_HUMA N]		0.00	18.21	16.48
O57245	3 beta-hydroxysteroid dehydrogenase/ Delta 5-->4-isomerase OS=Vaccinia virus (strain Ankara) GN=MVA157L PE=3 SV=1 - [3BHS_VACCA]	12.19	13.87	11.71	10.12
O43242	26S proteasome non-ATPase regulatory subunit 3 OS=Homo sapiens GN=PSMD3 PE=1 SV=2 - [PSMD3_HUM AN]	18.63	13.67	13.33	9.74
P62263	40S ribosomal protein S14 OS=Homo sapiens GN=RPS14 PE=1 SV=3 - [RS14_HUMA N]			5.18	15.89
Q3ZCQ8	Mitochondrial import inner membrane translocase subunit TIM50	7.70	11.33	9.57	12.46

	OS=Homo sapiens GN=TIMM50 PE=1 SV=2 - [TIM50_HUMAN]				
Q9NVI7	ATPase family AAA domain-containing protein 3A OS=Homo sapiens GN=ATAD3A PE=1 SV=2 - [ATD3A_HUMAN]	19.37	8.20	24.09	11.83
O00231	26S proteasome non-ATPase regulatory subunit 11 OS=Homo sapiens GN=PSMD11 PE=1 SV=3 - [PSD11_HUMAN]	11.60	12.56	8.66	5.92
P16615	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2 OS=Homo sapiens GN=ATP2A2 PE=1 SV=1 - [AT2A2_HUMAN]	70.72	13.63	28.24	8.83
P08238	Heat shock protein HSP 90-beta OS=Homo sapiens GN=HSP90AB1 PE=1 SV=4 - [HS90B_HUMAN]	30.88	13.67	32.09	13.12
P20495	Dual specificity protein		0.00	8.37	14.62

	phosphatase H1 OS=Vaccinia virus (strain Copenhagen) GN=H1L PE=2 SV=1 - [DUSP_VACC C]				
O75832	26S proteasome non-ATPase regulatory subunit 10 OS=Homo sapiens GN=PSMD10 PE=1 SV=1 - [PSD10_HUMA N]	6.07	14.60		0.00
O14980	Exportin-1 OS=Homo sapiens GN=XPO1 PE=1 SV=1 - [XPO1_HUMA N]	41.90	13.54	9.32	1.87
P20642	Major core protein 4a precursor OS=Vaccinia virus (strain Copenhagen) GN=A10L PE=3 SV=1 - [P4A_VACCC]	12.55	5.72	53.40	14.25
Q99460	26S proteasome non-ATPase regulatory subunit 1 OS=Homo sapiens GN=PSMD1 PE=1 SV=2 - [PSMD1_HUM AN]	28.97	13.96	8.35	6.40
P53007	Tricarboxylate transport	15.98	13.50	12.99	9.65

	protein, mitochondrial OS=Homo sapiens GN=SLC25A1 PE=1 SV=2 - [TXTP_HUMAN]				
P11441	Ubiquitin-like protein 4A OS=Homo sapiens GN=UBL4A PE=1 SV=1 - [UBL4A_HUMAN]	5.40	13.38	17.29	13.38
Q13200	26S proteasome non-ATPase regulatory subunit 2 OS=Homo sapiens GN=PSMD2 PE=1 SV=3 - [PSMD2_HUMAN]	30.27	9.36	51.97	13.33
O43175	D-3-phosphoglycerate dehydrogenase OS=Homo sapiens GN=PHGDH PE=1 SV=4 - [SERA_HUMAN]	11.96	9.01	21.65	13.32
P10809	60 kDa heat shock protein, mitochondrial OS=Homo sapiens GN=HSPD1 PE=1 SV=2 - [CH60_HUMAN]	10.82	5.76	20.22	11.17
O14925	Mitochondrial import inner	8.92	12.92		0.00

	membrane translocase subunit Tim23 OS=Homo sapiens GN=TIMM23 PE=1 SV=1 - [TIM23_HUMAN]				
Q9UBS4	DnaJ homolog subfamily B member 11 OS=Homo sapiens GN=DNAJB11 PE=1 SV=1 - [DJB11_HUMAN]	10.43	12.85		0.00
Q76RA8	Thymidylate kinase OS=Vaccinia virus (strain Ankara) GN=TMK PE=3 SV=1 - [KTHY_VACCA]		0.00	12.51	12.75
Q9NP72	Ras-related protein Rab-18 OS=Homo sapiens GN=RAB18 PE=1 SV=1 - [RAB18_HUMAN]		0.00	6.19	12.62
P07477	Trypsin-1 OS=Homo sapiens GN=PRSS1 PE=1 SV=1 - [TRY1_HUMAN]	33.93	12.15	35.88	12.15
P20985	Protein A6 OS=Vaccinia virus (strain Copenhagen)	16.12	11.83	6.54	6.45

	GN=A6L PE=3 SV=1 - [A6_VACCC]				
O75746	Calcium-binding mitochondrial carrier protein Aralar1 OS=Homo sapiens GN=SLC25A12 PE=1 SV=2 - [CMC1_HUMAN]	23.93	11.80		0.00
Q07020	60S ribosomal protein L18 OS=Homo sapiens GN=RPL18 PE=1 SV=2 - [RL18_HUMAN]	5.11	11.70		0.00
Q76RB8	Protein A11 OS=Vaccinia virus (strain Ankara) GN=MVA122R PE=2 SV=1 - [A11_VACCA]	9.09	11.64		0.00
Q9NX63	Coiled-coil-helix-coiled-coil-helix domain-containing protein 3, mitochondrial OS=Homo sapiens GN=CHCHD3 PE=1 SV=1 - [CHCH3_HUMAN]	6.90	11.45	7.18	11.45
Q9BWM7	Sideroflexin-3 OS=Homo sapiens GN=SFXN3 PE=1 SV=2 -	8.90	11.38		0.00

	[SFXN3_HUM AN]			
Q8WWC4	Uncharacterized protein C2orf47, mitochondrial OS=Homo sapiens GN=C2orf47 PE=1 SV=1 - [CB047_HUMA N]	0.00	8.17	11.34
P62826	GTP-binding nuclear protein Ran OS=Homo sapiens GN=RAN PE=1 SV=3 - [RAN_HUMAN]	5.82	11.11	0.00
P17844	Probable ATP-dependent RNA helicase DDX5 OS=Homo sapiens GN=DDX5 PE=1 SV=1 - [DDX5_HUMA N]	19.43	10.91	0.00
O93117	Telomere-binding protein I1 OS=Vaccinia virus (strain Ankara) GN=MVA062L PE=2 SV=1 - [I1_VACCA]	18.38	10.90	0.00
P51148	Ras-related protein Rab-5C OS=Homo sapiens GN=RAB5C PE=1 SV=2 - [RAB5C_HUM AN]	5.31	10.65	0.00
O94905	Erlin-2 OS=Homo	9.57	10.62	0.00

	sapiens GN=ERLIN2 PE=1 SV=1 - [ERLN2_HUM AN]				
P22695	Cytochrome b-c1 complex subunit 2, mitochondrial OS=Homo sapiens GN=UQCRC2 PE=1 SV=3 - [QCR2_HUMA N]	9.80	10.60	6.94	7.73
Q9Y3D8	Adenylate kinase isoenzyme 6 OS=Homo sapiens GN=TAF9 PE=1 SV=1 - [KAD6_HUMA N]	5.28	10.47	5.45	10.47
P43246	DNA mismatch repair protein Msh2 OS=Homo sapiens GN=MSH2 PE=1 SV=1 - [MSH2_HUMA N]	29.37	9.31	18.51	6.21
P52597	Heterogeneous nuclear ribonucleoprotei n F OS=Homo sapiens GN=HNRNPF PE=1 SV=3 - [HNRPF_HUM AN]	6.13	7.95	7.36	6.51
O14966	Ras-related protein Rab-7L1 OS=Homo sapiens		0.00	5.24	10.34

	GN=RAB7L1 PE=1 SV=1 - [RAB7L_HUM AN]				
P52701	DNA mismatch repair protein Msh6 OS=Homo sapiens GN=MSH6 PE=1 SV=2 - [MSH6_HUMA N]	31.63	10.22	12.39	2.72
P40939	Trifunctional enzyme subunit alpha, mitochondrial OS=Homo sapiens GN=HADHA PE=1 SV=2 - [ECHA_HUMA N]	6.14	3.28	12.23	6.42
Q00325	Phosphate carrier protein, mitochondrial OS=Homo sapiens GN=SLC25A3 PE=1 SV=2 - [MPCP_HUMA N]	71.87	9.67	36.17	7.18
P50416	Carnitine O-palmitoyltransfe rase 1, liver isoform OS=Homo sapiens GN=CPT1A PE=1 SV=2 - [CPT1A_HUM AN]	11.71	6.60	22.25	9.57
O43592	Exportin-T OS=Homo sapiens GN=XPOT	25.56	9.46		0.00

	PE=1 SV=2 - [XPOT_HUMA N]			
O57187	DNA-directed RNA polymerase 30 kDa polypeptide OS=Vaccinia virus (strain Ankara) GN=RPO30 PE=4 SV=1 - [RPO5_VACCA]		0.00	6.29	9.27
Q96EY1	DnaJ homolog subfamily A member 3, mitochondrial OS=Homo sapiens GN=DNAJA3 PE=1 SV=2 - [DNJA3_HUM AN]	5.29	5.83	6.35	6.67
Q9UBX3	Mitochondrial dicarboxylate carrier OS=Homo sapiens GN=SLC25A10 PE=1 SV=2 - [DIC_HUMAN]	6.74	9.06		0.00
P57088	Transmembrane protein 33 OS=Homo sapiens GN=TMEM33 PE=1 SV=2 - [TMM33_HUM AN]	5.47	8.91		0.00
P22061	Protein-L-isoaspartate(D-aspartate) O-methyltransferas e OS=Homo sapiens	5.06	8.81		0.00

	GN=PCMT1 PE=1 SV=4 - [PIMT_HUMA N]				
P21049	Protein E8 OS=Vaccinia virus (strain Copenhagen) GN=E8R PE=2 SV=1 - [E8_VACCC]	5.13	8.79		
Q9H936	Mitochondrial glutamate carrier 1 OS=Homo sapiens GN=SLC25A22 PE=1 SV=1 - [GHC1_HUMA N]	6.59	8.67		0.00
Q9Y4W6	AFG3-like protein 2 OS=Homo sapiens GN=AFG3L2 PE=1 SV=2 - [AFG32_HUM AN]	11.28	6.15	14.44	7.53
P11310	Medium-chain specific acyl- CoA dehydrogenase, mitochondrial OS=Homo sapiens GN=ACADM PE=1 SV=1 - [ACADM_HU MAN]	8.69	8.55	9.32	8.55
O60762	Dolichol- phosphate mannosyltransfe rase OS=Homo sapiens GN=DPM1 PE=1 SV=1 -	6.19	8.46	6.02	8.46

	[DPM1_HUMA N]				
P46379	Large proline-rich protein BAG6 OS=Homo sapiens GN=BAG6 PE=1 SV=2 - [BAG6_HUMA N]	10.77	4.42	24.18	8.39
Q9H5Q4	Dimethyladenos ine transferase 2, mitochondrial OS=Homo sapiens GN=TFB2M PE=1 SV=1 - [TFB2M_HUM AN]	5.06	4.29	5.75	6.31
P05023	Sodium/potassiu m-transporting ATPase subunit alpha-1 OS=Homo sapiens GN=ATP1A1 PE=1 SV=1 - [AT1A1_HUM AN]	29.44	8.31	6.59	2.74
P49368	T-complex protein 1 subunit gamma OS=Homo sapiens GN=CCT3 PE=1 SV=4 - [TCPG_HUMA N]	8.58	6.24	5.55	4.04
P46781	40S ribosomal protein S9 OS=Homo sapiens GN=RPS9 PE=1 SV=3 - [RS9_HUMAN]	4.99	8.25		0.00

Q92598	Heat shock protein 105 kDa OS=Homo sapiens GN=HSPH1 PE=1 SV=1 - [HS105_HUMAN]	15.17	8.16		0.00
Q9H3U1	Protein unc-45 homolog A OS=Homo sapiens GN=UNC45A PE=1 SV=1 - [UN45A_HUMAN]	19.28	8.16	6.11	2.75
P21080	Protein E2 OS=Vaccinia virus (strain Copenhagen) GN=E2L PE=3 SV=1 - [E2_VACCC]	15.74	8.14	9.56	5.29
Q16401	26S proteasome non-ATPase regulatory subunit 5 OS=Homo sapiens GN=PSMD5 PE=1 SV=3 - [PSMD5_HUMAN]			14.20	7.94
P02768	Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2 - [ALBU_HUMAN]	11.61	4.43	17.89	5.91
P40938	Replication factor C subunit 3 OS=Homo sapiens GN=RFC3 PE=1 SV=2 -		0.00	5.31	7.87

	[RFC3_HUMAN]			
P21047	Protein E6 OS=Vaccinia virus (strain Copenhagen) GN=E6R PE=2 SV=1 - [E6_VACCC]	12.68	7.76	0.00
P36542	ATP synthase subunit gamma, mitochondrial OS=Homo sapiens GN=ATP5C1 PE=1 SV=1 - [ATPG_HUMAN]	6.72	7.72	0.00
O14732	Inositol monophosphatase 2 OS=Homo sapiens GN=IMPA2 PE=1 SV=1 - [IMPA2_HUMAN]	7.87	7.64	0.00
Q96TA2	ATP-dependent zinc metalloprotease YME1L1 OS=Homo sapiens GN=YME1L1 PE=1 SV=2 - [YMEL1_HUMAN]	13.83	7.63	0.00
P55060	Exportin-2 OS=Homo sapiens GN=CSE1L PE=1 SV=3 - [XPO2_HUMAN]	20.71	7.62	0.00
P09651	Heterogeneous nuclear ribonucleoprotei	8.65	7.53	0.00

	n A1 OS=Homo sapiens GN=HNRNPA1 PE=1 SV=5 - [ROA1_HUMAN]				
P51665	26S proteasome non-ATPase regulatory subunit 7 OS=Homo sapiens GN=PSMD7 PE=1 SV=2 - [PSD7_HUMAN]	8.14	7.41		0.00
P62701	40S ribosomal protein S4, X isoform OS=Homo sapiens GN=RPS4X PE=1 SV=2 - [RS4X_HUMAN]	5.31	7.22		0.00
P21060	Protein A37 OS=Vaccinia virus (strain Copenhagen) GN=A37R PE=3 SV=1 - [A37_VACCC]		0.00	5.21	7.22
O57178	Protein F12 OS=Vaccinia virus (strain Ankara) GN=MVA042L PE=3 SV=1 - [F12_VACCA]	13.87	7.09		0.00
Q9UG63	ATP-binding cassette sub-family F member 2 OS=Homo sapiens GN=ABCF2	17.96	7.06	8.30	3.85

	PE=1 SV=2 - [ABCF2_HUM AN]				
Q92841	Probable ATP-dependent RNA helicase DDX17 OS=Homo sapiens GN=DDX17 PE=1 SV=2 - [DDX17_HUM AN]	14.75	7.00		0.00
P05388	60S acidic ribosomal protein P0 OS=Homo sapiens GN=RPLP0 PE=1 SV=1 - [RLA0_HUMA N]	5.76	6.94		0.00
Q86VP6	Cullin-associated NEDD8-dissociated protein 1 OS=Homo sapiens GN=CAND1 PE=1 SV=2 - [CAND1_HUM AN]	11.21	4.31	15.67	4.88
Q2TB90	Putative hexokinase HKDC1 OS=Homo sapiens GN=HKDC1 PE=1 SV=3 - [HKDC1_HUM AN]	21.12	6.87	12.14	3.49
Q15366	Poly(rC)-binding protein 2 OS=Homo sapiens GN=PCBP2	5.51	6.85		0.00

	PE=1 SV=1 - [PCBP2_HUM AN]				
Q92621	Nuclear pore complex protein Nup205 OS=Homo sapiens GN=NUP205 PE=1 SV=3 - [NU205_HUM AN]	32.11	5.86	25.95	4.22
P33810	Scaffold protein D13 OS=Variola virus (isolate Human/India/In d3/1967) GN=D13L PE=3 SV=1 - [D13_VAR67]	5.47	4.72	5.38	4.17
P20700	Lamin-B1 OS=Homo sapiens GN=LMNB1 PE=1 SV=2 - [LMNB1_HUM AN]	7.89	6.14		0.00
P08195	4F2 cell-surface antigen heavy chain OS=Homo sapiens GN=SLC3A2 PE=1 SV=3 - [4F2_HUMAN]	9.15	6.03		0.00
Q8N1F7	Nuclear pore complex protein Nup93 OS=Homo sapiens GN=NUP93 PE=1 SV=2 - [NUP93_HUM AN]		0.00	11.78	5.98
Q96S55	ATPase WRNIP1	9.56	5.86	6.10	4.06

	OS=Homo sapiens GN=WRNIP1 PE=1 SV=2 - [WRIP1_HUMAN]				
P04843	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1 OS=Homo sapiens GN=RPN1 PE=1 SV=1 - [RPN1_HUMAN]	5.80	3.79	10.96	5.77
Q16891	Mitochondrial inner membrane protein OS=Homo sapiens GN=IMMT PE=1 SV=1 - [IMMT_HUMAN]	5.81	4.35	9.17	5.54
O76031	ATP-dependent Clp protease ATP-binding subunit clpX-like, mitochondrial OS=Homo sapiens GN=CLPX PE=1 SV=2 - [CLPX_HUMAN]	6.16	3.63	6.38	3.95
Q9Y265	RuvB-like 1 OS=Homo sapiens GN=RUVBL1 PE=1 SV=1 - [RUVB1_HUMAN]	4.98	5.48		0.00

P21022	Metalloendopept idase G1 OS=Vaccinia virus (strain Copenhagen) GN=G1L PE=3 SV=1 - [G1_VACCC]	7.79	5.41	8.26	3.38
Q9Y230	RuvB-like 2 OS=Homo sapiens GN=RUVBL2 PE=1 SV=3 - [RUVB2_HUM AN]	5.18	5.18		
Q15645	Pachytene checkpoint protein 2 homolog OS=Homo sapiens GN=TRIP13 PE=1 SV=2 - [PCH2_HUMA N]	5.71	5.09		0.00
P28288	ATP-binding cassette sub-family D member 3 OS=Homo sapiens GN=ABCD3 PE=1 SV=1 - [ABCD3_HUM AN]	8.83	4.86	11.61	3.49
O00232	26S proteasome non-ATPase regulatory subunit 12 OS=Homo sapiens GN=PSMD12 PE=1 SV=3 - [PSD12_HUMA N]	8.27	4.82		0.00
P33993	DNA replication	5.05	2.92	5.04	3.06

	licensing factor MCM7 OS=Homo sapiens GN=MCM7 PE=1 SV=4 - [MCM7_HUMAN]				
P08237	6-phosphofructokinase, muscle type OS=Homo sapiens GN=PFKM PE=1 SV=2 - [K6PF_HUMAN]	9.86	4.62	6.15	2.95
P34932	Heat shock 70 kDa protein 4 OS=Homo sapiens GN=HSPA4 PE=1 SV=4 - [HSP74_HUMAN]	8.38	4.52		0.00
Q00839	Heterogeneous nuclear ribonucleoprotein U OS=Homo sapiens GN=HNRNPU PE=1 SV=6 - [HNRPU_HUMAN]	12.76	4.48		0.00
P61619	Protein transport protein Sec61 subunit alpha isoform 1 OS=Homo sapiens GN=SEC61A1 PE=1 SV=2 - [S61A1_HUMAN]	5.11	4.41		0.00
P35637	RNA-binding protein FUS	5.23	4.37		

	OS=Homo sapiens GN=FUS PE=1 SV=1 - [FUS_HUMAN]				
P33053	DNA-directed RNA polymerase 147 kDa polypeptide OS=Variola virus (isolate Human/India/Ind3/1967) GN=RPO147 PE=3 SV=1 - [RP147_VAR67]	8.86	2.57	17.01	4.20
A0FGR8	Extended synaptotagmin-2 OS=Homo sapiens GN=ESYT2 PE=1 SV=1 - [ESYT2_HUMAN]	12.53	4.13	5.13	2.82
P17987	T-complex protein 1 subunit alpha OS=Homo sapiens GN=TCP1 PE=1 SV=1 - [TCPA_HUMAN]	5.36	3.96		0.00
Q92973	Transportin-1 OS=Homo sapiens GN=TNPO1 PE=1 SV=2 - [TNPO1_HUMAN]	8.55	3.79		0.00
P17474	DNA-directed RNA polymerase 132 kDa polypeptide OS=Cowpox virus (strain	14.09	3.78		0.00

	Brighton Red) GN=RPO132 PE=3 SV=1 - [RP132_CWPXB]				
P27708	CAD protein OS=Homo sapiens GN=CAD PE=1 SV=3 - [PYR1_HUMAN]	15.55	3.06	5.59	1.21
Q15029	116 kDa U5 small nuclear ribonucleoprotein component OS=Homo sapiens GN=EFTUD2 PE=1 SV=1 - [U5S1_HUMAN]	8.38	3.50		0.00
Q96P70	Importin-9 OS=Homo sapiens GN=IPO9 PE=1 SV=3 - [IPO9_HUMAN]	8.80	3.46		0.00
O57207	RNA polymerase-associated transcription-specificity factor RAP94 OS=Vaccinia virus (strain Ankara) GN=RAP94 PE=2 SV=1 - [RAP94_VACCA]		0.00	9.00	3.40
O75643	U5 small nuclear ribonucleoprotein 200 kDa	16.65	3.28		0.00

	helicase OS=Homo sapiens GN=SNRNP200 PE=1 SV=2 - [U520_HUMA N]				
P46459	Vesicle-fusing ATPase OS=Homo sapiens GN=NSF PE=1 SV=3 - [NSF_HUMAN]		0.00	6.35	3.09
Q14974	Importin subunit beta-1 OS=Homo sapiens GN=KPNB1 PE=1 SV=2 - [IMB1_HUMA N]		0.00	4.93	3.08
Q14204	Cytoplasmic dynein 1 heavy chain 1 OS=Homo sapiens GN=DYNC1H1 PE=1 SV=5 - [DYHC1_HUM AN]	30.86	2.48	13.72	1.27
Q66K14	TBC1 domain family member 9B OS=Homo sapiens GN=TBC1D9B PE=1 SV=3 - [TBC9B_HUM AN]		0.00	8.59	2.80
P19338	Nucleolin OS=Homo sapiens GN=NCL PE=1 SV=3 - [NUCL_HUMA N]	5.84	2.68		0.00

Q29RF7	Sister chromatid cohesion protein PDS5 homolog A OS=Homo sapiens GN=PDS5A PE=1 SV=1 - [PDS5A_HUMAN]		0.00	7.42	2.24
Q15386	Ubiquitin-protein ligase E3C OS=Homo sapiens GN=UBE3C PE=1 SV=3 - [UBE3C_HUMAN]	4.97	2.03		0.00
O95373	Importin-7 OS=Homo sapiens GN=IPO7 PE=1 SV=1 - [IPO7_HUMAN]	5.32	2.02	8.31	2.02
Q9NTJ3	Structural maintenance of chromosomes protein 4 OS=Homo sapiens GN=SMC4 PE=1 SV=2 - [SMC4_HUMAN]		0.00	5.18	1.86
A6NKG5	Retrotransposon -like protein 1 OS=Homo sapiens GN=RTL1 PE=2 SV=3 - [RTL1_HUMAN]		0.00	5.51	1.69
Q9Y6D5	Brefeldin A-inhibited guanine nucleotide-	5.89	1.62		0.00

	exchange protein 2 OS=Homo sapiens GN=ARFGEF2 PE=1 SV=3 - [BIG2_HUMA N]			
Q5UIP0	Telomere-associated protein RIF1 OS=Homo sapiens GN=RIF1 PE=1 SV=2 - [RIF1_HUMAN]	7.60	1.54	0.00
O95071	E3 ubiquitin-protein ligase UBR5 OS=Homo sapiens GN=UBR5 PE=1 SV=2 - [UBR5_HUMA N]	5.26	0.86	0.00
Q13315	Serine-protein kinase ATM OS=Homo sapiens GN=ATM PE=1 SV=3 - [ATM_HUMA N]	5.40	0.72	

Table A.2 VACV mass spectrometry results of FLAG-p28 and FLAG-p28(C173S/C176S) pull-downs.

Accession	Description	Score VACV-FLAG p28 (C173S/C176S)	Coverage VACV-FLAG p28 (C173S/C176S)	Score VACV-FLAG-p28	Coverage VACV-FLAG-p28
Q6RZT3	RPXV008 OS=Rabbitpox virus GN=RPXV008 PE=4 SV=1 - [Q6RZT3_9POXV]	1655.94	49.17	1875.01	57.02
A9J0R8	20k virion core protein OS=Vaccinia virus (strain Ankara) GN=CVA138 PE=4 SV=1 - [A9J0R8_VACCA]	16.85	33.85	50.76	46.35
H2DZ22	Late transcription elongation factor OS=Vaccinia virus GN=VAC_DPP11_091 PE=4 SV=1 - [H2DZ22_9POXV]	11.92	19.09	25.83	31.82
A4GDH7	Major core protein p4b OS=Vaccinia virus GN=List118 PE=4 SV=1 - [A4GDH7_9POXV]	138.32	26.40	425.45	34.16
L7QJK6	Core protein OS=Vaccinia virus GN=A12L PE=4 SV=1 -	16.85	34.39		0.00

	[L7QJK6_9POX V]			
Q8JLG8	EEV phospholipase OS=Ectromelia virus GN=EVM036 PE=4 SV=1 - [Q8JLG8_9POX V]	75.29	33.33	0.00
H2DYZ1	Palmytilated EEV membrane protein OS=Vaccinia virus GN=VAC_DPP2 1_062 PE=4 SV=1 - [H2DYZ1_9POX V]	0.00	54.47	31.18
Q9JFD6	TE4L OS=Vaccinia virus (strain Tian Tan) PE=4 SV=1 - [Q9JFD6_VACC T]	6.13	30.86	
M9WG49	Tyr/Ser protein phosphatase OS=Vaccinia virus GN=VACV_TT1 0_120 PE=4 SV=1 - [M9WG49_9PO XV]	6.90	12.28	14.61 26.90
A0ES19	Hydroxysteroid dehydrogenase OS=Vaccinia virus GN=VAC_DPP1 3_181 PE=3 SV=1 - [A0ES19_9POX V]	20.09	18.79	15.68 15.32
Q89207	36kD late protein (Fragment)	45.09	25.94	12.87 19.80

	OS=Vaccinia virus PE=4 SV=1 - [Q89207_9POXV]				
A9J0X0	EEV membrane glycoprotein OS=Vaccinia virus (strain Ankara) GN=CVA165 PE=4 SV=1 - [A9J0X0_VACCA]	20.02	25.60	24.47	25.60
Q0GNZ8	HSPV087 OS=Horsepox virus PE=4 SV=1 - [Q0GNZ8_HSPV]	26.27	23.08	27.76	20.38
Q85331	RNA polymerase 22 kD subunit (Fragment) OS=Vaccinia virus PE=4 SV=1 - [Q85331_9POXV]	7.73	11.17	13.71	21.79
A9J1K3	Putative uncharacterized protein CVA064 OS=Vaccinia virus (strain Ankara) GN=CVA064 PE=4 SV=1 - [A9J1K3_VACCA]	35.48	20.35	23.75	13.98
B9U1U2	Thymidylate kinase OS=Vaccinia virus GLV-1h68 GN=GL234 PE=4 SV=1 - [B9U1U2_9POXV]	3.98	7.35	18.44	20.10

O57223	Major core protein P4a OS=Vaccinia virus GN=MVA121L PE=4 SV=1 - [O57223_9POXV]	30.85	9.32	0.00
M9WH30	Uncharacterized protein OS=Vaccinia virus GN=VAC_TP3_1 35 PE=4 SV=1 - [M9WH30_9PO XV]	0.00	15.05 13.84	
A4GDI5	Putative uncharacterized protein OS=Vaccinia virus GN=List126 PE=4 SV=1 - [A4GDI5_9POX V]	13.94	17.61	0.00
A9J0Q2	Core protein required for morphogenesis OS=Vaccinia virus (strain Ankara) GN=CVA131 PE=4 SV=1 - [A9J0Q2_VACC A]	21.58	16.94 8.76	9.68
B9U1N1	Precursor p4a of core protein 4a OS=Vaccinia virus GLV-1h68 GN=GL174 PE=4 SV=1 - [B9U1N1_9POX V]	30.85	9.32	0.00
A4GDB5	DNA-dependent RNA polymerase subunit rpo30 OS=Vaccinia	9.12	16.22 8.30	12.74

ID	Description				
	virus GN=List056 PE=4 SV=1 - [A4GDB5_9POXV]				
Q9JF94	TA5L OS=Vaccinia virus (strain Tian Tan) PE=4 SV=1 - [Q9JF94_VACCT]		0.00	10.19	14.71
A0ERU7	DNA-dependent RNA polymerase subunit rpo147 OS=Vaccinia virus GN=VAC_DPP16_109 PE=4 SV=1 - [A0ERU7_9POXV]	31.71	11.12	34.25	8.55
H2DWL6	Putative uncharacterized protein OS=Vaccinia virus GN=VAC_DPP16_184 PE=4 SV=1 - [H2DWL6_9POXV]		0.00	14.86	13.52
H2DUQ8	ER-localized MP OS=Vaccinia virus GN=VAC_DPP12_075 PE=4 SV=1 - [H2DUQ8_9POXV]	7.28	12.82		
Q80DT6	A42R protein OS=Cowpox virus GN=A42R PE=4 SV=1 - [Q80DT6_COWPX]		0.00	3.29	12.73
B9U1G9	Myristylprotein	6.27	9.41		0.00

	OS=Vaccinia virus GLV-1h68 GN=GL113 PE=4 SV=1 - [B9U1G9_9POXV]				
Q77TL1	TG8L OS=Vaccinia virus (strain Tian Tan) PE=4 SV=1 - [Q77TL1_VACCT]			4.89	10.78
A0ES09	VACV-DUKE-168 OS=Vaccinia virus GN=VAC_DPP21_171 PE=4 SV=1 - [A0ES09_9POXV]		0.00	7.18	10.65
B9U1D4	Putative uncharacterized protein OS=Vaccinia virus GLV-1h68 GN=GL079 PE=4 SV=1 - [B9U1D4_9POXV]	16.38	10.58		0.00
A0ERZ3	DNA-directed RNA polymerase OS=Vaccinia virus GN=VAC_DPP9_155 PE=3 SV=1 - [A0ERZ3_9POXV]	28.31	10.40	19.11	7.56
L7QJ25	Core protein vp8 OS=Vaccinia virus GN=L4R PE=4 SV=1 - [L7QJ25_9POXV]	4.74	10.36	4.49	10.36
Q8JLC9	EVM086	13.74	7.68	16.78	7.81

	OS=Ectromelia virus GN=EVM086 PE=4 SV=1 - [Q8JLC9_9POX V]				
F1DIT6	Myristylprotein OS=Monkeypox virus PE=4 SV=1 - [F1DIT6_MONP V]		0.00	5.59	7.94
Q8JLG2	EVM045 OS=Ectromelia virus GN=EVM045 PE=4 SV=1 - [Q8JLG2_9POX V]	6.97	8.76		0.00
H2DS96	Insulin metalloproteinase -like OS=Vaccinia virus GN=VAC_DPP2 1_089 PE=4 SV=1 - [H2DS96_9POX V]	11.45	8.63	16.20	7.11
A0ERR7	VACV-DUKE-076 OS=Vaccinia virus GN=VACV-DUKE-076 PE=4 SV=1 - [A0ERR7_9POX V]	8.27	5.71	14.15	7.06
Q80DS5	DNA ligase OS=Cowpox virus GN=A53R PE=3 SV=1 - [Q80DS5_COWP X]	7.27	5.25		0.00
A0ES25	DNA ligase OS=Vaccinia virus		0.00	2.20	3.80

	GN=VACV-DUKE-184 PE=3 SV=1 - [A0ES25_9POXV]				
Q80E18	G12L protein OS=Cowpox virus GN=G12L PE=4 SV=1 - [Q80E18_COWPX]	13.87	7.10	7.06	5.68
A4GDJ3	DNA helicase OS=Vaccinia virus GN=List134 PE=4 SV=1 - [A4GDJ3_9POXV]	6.80	7.10		
E2CZJ5	M106L OS=Myxoma virus GN=m106L PE=4 SV=1 - [E2CZJ5_9POXV]		0.00	1.82	6.91
A4GDP6	Serine/threonine protein kinase OS=Vaccinia virus GN=List189 PE=4 SV=1 - [A4GDP6_9POXV]	5.71	6.01		0.00
B9U1H7	Beta-D-galactosidase OS=Vaccinia virus GLV-1h68 GN=lacZ PE=3 SV=1 - [B9U1H7_9POXV]	4.73	2.26	17.96	5.89
A0ERY5	VACV-DUKE-144 OS=Vaccinia virus GN=VACV-DUKE-144 PE=4 SV=1 - [A0ERY5_9POXV]		0.00	3.70	5.57

A0ERX5	VACV-DUKE-134 OS=Vaccinia virus GN=VACV-DUKE-134 PE=4 SV=1 - [A0ERX5_9POXV]	3.62	2.82	6.04	3.94
H2DY34	Ankyrin-like OS=Vaccinia virus GN=VAC_DPP19_026 PE=4 SV=1 - [H2DY34_9POXV]			3.45	5.36
B2CWP7	M140R OS=Myxoma virus GN=m140R PE=4 SV=1 - [B2CWP7_9POXV]		0.00	3.34	4.16
A0ERZ7	Cowpox A-type inclusion protein OS=Vaccinia virus GN=VAC_DPP15_159 PE=4 SV=1 - [A0ERZ7_9POXV]	13.11	3.33		0.00
Q80E23	C3L protein OS=Cowpox virus GN=C3L PE=4 SV=1 - [Q80E23_COWPX]		0.00	1.61	2.52