

# MID1IP1 and CCT2 in HIV-1 Transduction

Marina Ermakova

Submitted in partial fulfillment of the  
requirements for the degree of  
Doctor of Philosophy  
under the Executive Committee  
of the Graduate School of Arts and Sciences

COLUMBIA UNIVERSITY

2020

© 2020

Marina Ermakova

All Rights Reserved

## **Abstract**

### MID1IP1 and CCT2 in HIV-1 Transduction

Marina Ermakova

HIV-1 completes its life cycle by coopting host proteins. Hundreds of proteins have been identified as potential host factors functioning in viral infection through screens, two of which are MID1IP1 and CCT2. Little is known about MID1IP1, but its localization to microtubules may suggest a cytoskeletal function and a possible role in microtubule transport of HIV-1 viral cores. We use the CRISPR/Cas9 system to create frameshift mutations in MID1IP1 in 293 cells and find that these mutations do not produce effects on HIV-1 transduction in experiments capable of assaying for completion of the life cycle from initial entry into host cells to gene expression. Furthermore, we were unable to find an effect on the staining for markers of microtubule stability using Western blots as a result of the mutations in these cells. CCT2 is a component of the TRiC/CCT protein folding complex whose substrates include actin and tubulin, which also suggests that CCT2 might function in the HIV-1 life cycle in a cytoskeleton-dependent manner. siRNA knockdowns in TE671 cells demonstrate a slight effect on HIV-1 transduction. Our data does not support a role for MID1IP1 in the entry stage of the HIV-1 life cycle, but does suggest CCT2 may be a potential candidate for further study.

# Table of Contents

List of Figures .....	iii
List of Tables .....	v
Acknowledgments.....	vi
Chapter 1: Introduction.....	1
Retrovirology .....	1
HIV-1/AIDS and the Retrovirus Family.....	1
Genomic Content of HIV-1 .....	4
The HIV-1 Life cycle.....	8
Host proteins in the HIV-1 Life Cycle.....	14
Microtubules in the Entry Stage of HIV-1 Infection .....	16
MID1IP1 and MID1.....	24
siRNA Screens Identifying MID1IP1 as a Potential Host Protein Involved in HIV-1 Infection .....	24
MID1IP1 .....	25
MID1 and the TRIM Family.....	26
MID1 Interactions.....	29
Opitz syndrome.....	33
CCT2, chaperonin-containing TCP-1 subunit 2, a component of a major multisubunit chaperone complex .....	38

An siRNA Screen Identifying CCT2 as a Potential Host Protein Involved in HIV-1	
Infection .....	38
CCT2 and the TRiC/CCT Complex.....	40
TRiC/CCT subunits .....	40
Protein targets folded by TRiC/CCT .....	43
Conclusion .....	45
Chapter 2: MID1IP1 Does Not Function in the Early Phase of the HIV-1 Viral Life Cycle through Viral Gene Expression.....	
	46
Chapter 3: Markers of Microtubule Stability Do Not Reveal Functions of MID1IP1 at Endogenous Levels in 293 CRISPR Cells .....	
	76
Chapter 4: Chaperonin Component CCT2 May Function in the Early Phase of the HIV-1 Viral Life Cycle through Viral Gene Expression.....	
	80
Chapter 5: Discussion .....	
	86
MID1IP1 and MID1 in Retroviral Infection.....	86
Potential Microtubule-Related Functions for MID1IP1 .....	87
Potential Interactions Between MID1 and MID1IP1.....	90
CCT2 in Retroviral Infection .....	92
Conclusion .....	95
Chapter 6: Materials and Methods .....	
	96
References.....	104

## List of Figures

Figure 1.1: DNA sequence of the HIV-1 provirus.....	5
Figure 1.2: Schematic of an HIV-1 viral particle .....	7
Figure 1.3: Representation of the HIV-1 viral life cycle .....	9
Figure 1.4: MID1 domains.....	27
Figure 2.1: Representation of the HIV-1 machinery plus luciferase reporter encoded in pNL4-3.Luc.R-E-.....	49
Figure 2.2: siRNA knockdown of MID1IP1 in TE671 cells .....	50
Figure 2.3: siRNA knockdown of MID1 does not produce an effect on luciferase reporter expression in TE671 cells .....	51
Figure 2.4: Genomic DNA in 293 CRISPR cells for MID1IP1 .....	55
Figure 2.5: Coding sequences for MID1IP1 CRISPR mutants produce frameshift mutations and premature stop codons .....	58
Figure 2.6: Three 293 CRISPR cell lines do not contain a wild-type copy of <i>MID1IP1</i> .....	61
Figure 2.7: VSV-G pseudotyped HIV-1 reporter viruses do not indicate a change in infectivity between CRISPR and control cells .....	64
Figure 2.8: Amphotropic pseudotyped HIV-1 reporter viruses do not indicate a change in infectivity between CRISPR and control cells.....	65
Figure 2.9: VSV-G pseudotyped MLV reporter viruses do not indicate a change in infectivity between CRISPR and control cells .....	66

Figure 2.10: Flow chart of experimental procedure for overexpression of MID1 and MID1IP1  
..... 70

Figure 2.11: Overexpression of MID1IP1 and MID1 does not produce an effect on HIV-1  
infectivity in TE671 cells..... 71

Figure 2.12: Overexpression of MID1IP1 and MID1 does not produce an effect on HIV-1  
infectivity in HeLa cells..... 72

Figure 3.1: Markers of microtubule stability in 293 CRISPR cells..... 77

Figure 4.1: siRNA knockdown of CCT2 in TE671 cells..... 84

## List of Tables

Table 1.1: Retroviruses .....	3
Table 1.2: Regulators of Microtubule Stability in HIV-1 Infection .....	23
Table 1.3: Select Proteins that Interact with the TRiC/CCT Complex.....	44

## **Acknowledgments**

This graduate experience has facilitated my growth in ways I could never have predicted at the start of it. So many people deserve my thanks for the part they played in getting me to this point, first and foremost of which is my advisor, Stephen Goff. I have to thank him not only for his own gentle encouragement and willingness to provide advice whenever his students come to him with questions, but also for creating such a positive working environment. I learned to do more on my own than I thought I could, but I could always rely on the expertise of anyone else in the lab when I needed it.

That leads me to thanking the rest of the lab, members past and present. I especially thank: Yosef Sabo, with whom I've consulted regularly, for always being ready to help. Kenia de los Santos, for being a voice of support for all of these years. Marlene Arroyo, for sharing this graduate student experience with me and being someone I could talk to about it. Gary Wang, for taking the initiative in checking up on me and my progress. Sedef Tinaztepe, for training me when I first came to the lab and easing me into a new phase of my life. Martine Lecorps, for all of her encouragement. It would be impossible to detail everything every member of this lab has done for me all of the years, but I'd like to express my gratitude to everyone who's been a part of my experience--Yiping Zhu, Oya Cingoz, Michael Metzger, Andreia Lee, Daniel Griffin, Angela Erazo, Cheng Wang, Silje Krokeide, Jeffrey Sebrow, Martha de los Santos, and Helen Wang. All of you played a part in making this lab what it is.

I am also grateful to my committee members, Tim Bestor and Richard Vallee. Thank you for all of the guidance and support you have provided, as well as the time you have put into making sure I was headed in the right direction.

Thank you to Cathy Mendelsohn, Vincent Racaniello, and Mojgan Naghavi for agreeing to join my thesis committee.

I'm grateful to my department, Genetics and Development, for the opportunity to be here and the support throughout the years. I would like to thank Stacy Warren in particular for being a huge help since the beginning. In addition, I'd like to thank my classmates for being an integral part of my graduate career, especially Yelena and Fraulin. You have been a source of support and understanding. And Yelena--you've been a great friend and I miss having you around.

My (unfortunately now former) roommates, Beth and Katherine, have been such a huge part of my graduate life. The two of you are the only ones who can understand what it's meant for us to live together all of these years, going through all of the same stages of graduate school at the same time.

And finally, my family and the rest of my friends. I have been incredibly lucky to find the support network that I've had throughout my life. My parents, my sister, the friends I've kept throughout the years--I always know that all of you are there, and it's a huge weight off my shoulders even as I devote myself to something as consuming as graduate life. I love you all. Thanks for putting up with my hectic schedule.

Mom and dad--Спасибо.

# Chapter 1: Introduction

## Retrovirology

### HIV-1/AIDS and the Retrovirus Family

Human immunodeficiency virus type-1 (HIV-1) causes acquired immunodeficiency syndrome (AIDS), a disease that weakens the immune system by depleting CD4<sup>+</sup> T cells, conferring vulnerability to opportunistic diseases (Fauci and Desrosiers, 1997). HIV-1 belongs to the retrovirus family, which is characterized by reverse transcription of viral RNA and integration of viral DNA into the host genome. Retroviral particles are enveloped, with their positive sense ssRNA genomes contained in the core (Vogt, 1997a).

Viruses that replicate through reverse transcription have recently been organized into six families (*Retroviridae*, *Caulimoviridae*, *Hepadnaviridae*, *Metaviridae*, *Pseudoviridae*, and *Belpaoviridae*). Excluding *Hepadnaviridae*, these families are grouped into the *Ortervirales* order due to their evolutionary relatedness (Krupovic et al., 2018). The retrovirus family is divided into two subfamilies (Table 1.1). The *Orthoretrovirinae* subfamily contains six genera, one of which is the lentivirus genus, which includes HIV-1, the virus of most importance to this study (Stoye et al., 2012). The *Spumaretrovirinae* subfamily previously contained one genus, but has been recently reorganized into five genera (Khan et al., 2018). Spumaviruses are atypical retroviruses that reverse transcribe their RNA during or after viral particle formation, whereas orthoretroviruses initiate reverse transcription after infection. Spumaviruses are the only

retroviruses to have a second transcriptional promoter, and budding is dependent on envelope proteins rather than the Gag protein (Lindemann and Rethwilm, 2011; Linial, 1999).

The lentivirus genus is associated with slow-progressing pathogenesis in mammalian hosts. The similarity of their regulatory and accessory proteins as well as the distinct cone shape of their mature capsid cores distinguish lentiviruses from other retroviruses (Fauci and Desrosiers, 1997; Stoye et al., 2012; Swanstrom and Wills, 1997). Viruses in this genus exhibit tropism for macrophages or lymphocytes (Fauci and Desrosiers, 1997; Narayan and Clements, 1989). CD4<sup>+</sup> T cells and macrophages are the primary cell types infected by HIV-1 *in vivo* (Gaudin et al., 2013).

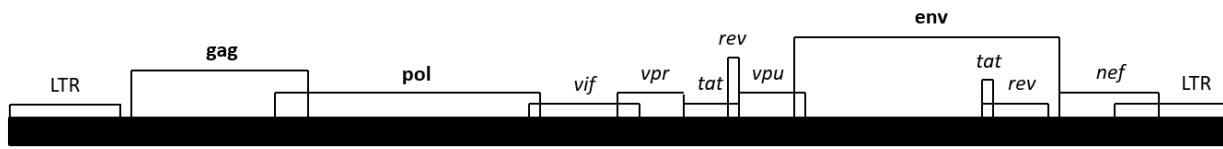
**Table 1.1: Retroviruses**

<b>Subfamily</b>	<b>Genus</b>	<b>Example Species</b>
<b>Orthoretrovirinae</b> (Stoye et al., 2012)	Alpharetrovirus	Avian leukosis virus Avian myeloblastosis virus Rous sarcoma virus
	Betaretrovirus	Jaagsiekte sheep retrovirus Mason–Pfizer monkey virus Mouse mammary tumor virus Squirrel monkey retrovirus
	Gammaretrovirus	Feline leukemia virus Gibbon ape leukemia virus Guinea pig type-C oncovirus Moloney murine sarcoma virus Murine leukemia virus Reticuloendotheliosis virus Viper retrovirus
	Deltaretrovirus	Bovine leukemia virus Primate T-lymphotropic virus 1 Primate T-lymphotropic virus 2 Primate T-lymphotropic virus 3
	Epsilonretrovirus	Walleye dermal sarcoma virus Walleye epidermal hyperplasia virus 1 Walleye epidermal hyperplasia virus 2
	Lentivirus	Bovine immunodeficiency virus Caprine arthritis encephalitis virus Equine infectious anemia virus Feline immunodeficiency virus Human immunodeficiency virus 1 Human immunodeficiency virus 2 Puma lentivirus Simian immunodeficiency virus Visna/maedi virus
<b>Spumaretrovirinae</b> (Khan et al., 2018)	Bovispumavirus	Bovine foamy virus
	Equispumavirus	Equine foamy virus
	Felispumavirus	Feline foamy virus Puma feline foamy virus
	Prosimiispumavirus	Brown greater galago prosimian foamy virus
	Simiispumavirus	Central chimpanzee simian foamy virus Eastern chimpanzee simian foamy virus Grivet simian foamy virus Japanese macaque simian foamy virus Rhesus macaque simian foamy virus Taiwanese macaque simian foamy virus

## Genomic Content of HIV-1

Retroviruses may be simple or complex. The genomes of the simple retroviruses contain only the three genes which code for the structural and enzymatic components of the virus: *gag*, *pol*, and *env*. The prototypical example of a simple retrovirus is the Moloney murine leukemia virus (MLV) which causes T-cell lymphoma in mice through the activation of proto-oncogenes (Braoudaki and Tzortzatou-Stathopoulou, 2011; Steffen, 1984). The genomes of complex retroviruses such as HIV-1 contain additional regulatory and accessory genes (Figure 1.1).

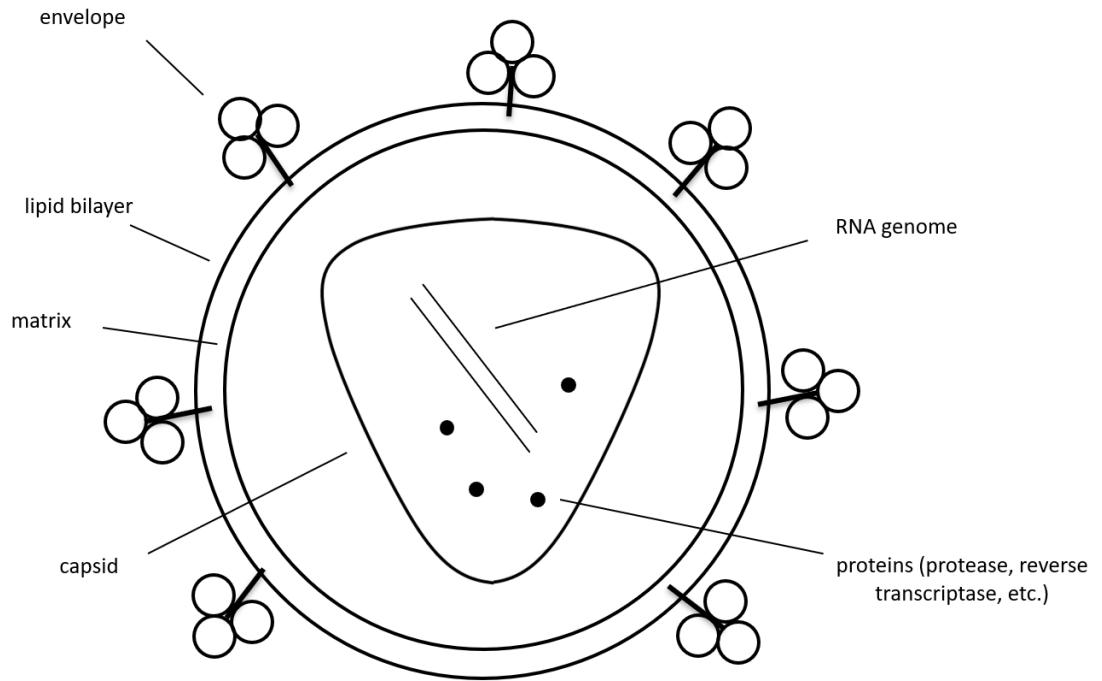
The HIV-1 RNA genome is approximately 9.2 kb in length. The full-length unspliced RNA serves as the mRNA for the translation of the Gag and Gag-Pol polyproteins. HIV-1 *gag* encodes the matrix, capsid, nucleocapsid, and p6 proteins, which are initially translated as the Gag polyprotein precursor. The Gag precursor contains the structural components of the viral particles, and is responsible for assembling budding viral particles and forming the capsid. *pol* codes for protease, reverse transcriptase, and integrase in a different reading frame from *gag*. (In other retroviruses, protease may alternatively be coded for as part of the *gag* gene, or in its own separate reading frame.) During translation of the Gag polyprotein, a ribosomal frameshift will occur approximately 5% of the time to produce the Gag-Pol polyprotein. Both the Gag and Gag-Pol polyproteins are incorporated into virion particles during assembly, and are later cleaved by the viral protease into individual components. The *env* gene encodes the surface and transmembrane proteins of the viral envelope, which are also initially translated as a polyprotein precursor but then cleaved by cellular proteases during transit through the endoplasmic reticulum and golgi apparatus (Vogt, 1997b).



**Figure 1.1: DNA sequence of the HIV-1 provirus.** A representation of the HIV-1 DNA for the provirus which is integrated into host genomes. The *gag*, *pol*, and *env* genes common to all retroviruses are in bold while the regulatory and accessory genes present in HIV-1 are in italics.

Six additional genes are present in the HIV-1 genome: *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu*. The functions of their gene products will be addressed in the discussion of the HIV-1 life cycle, although it should be noted that many of these HIV-1 proteins have several functions and affect multiple cellular processes in infected cells. Briefly, Tat enhances transcription of the provirus, Rev functions in nuclear export (Faust et al., 2017), Nef facilitates penetration of the cortical actin network and regulates surface proteins (Campbell et al., 2004; Pereira and daSilva, 2016), and Vif antagonizes the function of host APOBEC3 restriction factors (Faust et al., 2017). Vpr induces cell cycle arrest at the G2 phase in T cells (which corresponds to increased viral transcription) (Faust et al., 2017; Le Rouzic and Benichou, 2005), functions in nuclear entry, and mediates the incorporation of DNA repair protein UNG2 into viral particles (which correlates to a 4-fold decrease in mutation rate in dividing cells and an 18-fold decrease in non-dividing macrophages) (Chen et al., 2004; Le Rouzic and Benichou, 2005). Vpu downregulates host proteins to facilitate viral budding (Faust et al., 2017).

The 5' region of genomic RNA contains two noncoding elements or sequence blocks, dubbed R and U5. The 3' region contains noncoding elements U3 and R. The repeated R region at both ends of the RNA allows complementary DNA synthesized from the 5' region during reverse transcription to translocate to the 3' end and then to use the genomic RNA as a template for continued DNA synthesis. This process will be detailed further in the discussion of the life cycle, but reverse transcription of the viral RNA results in a double-stranded viral DNA with the elements U3-R-U5, termed long terminal repeats (LTRs), flanking both ends of the DNA (Hu and Hughes, 2012). The LTRs contain a transcriptional promoter and regulatory elements, as well as sequences at the tips needed for DNA integration into the host genome (Vogt, 1997b).

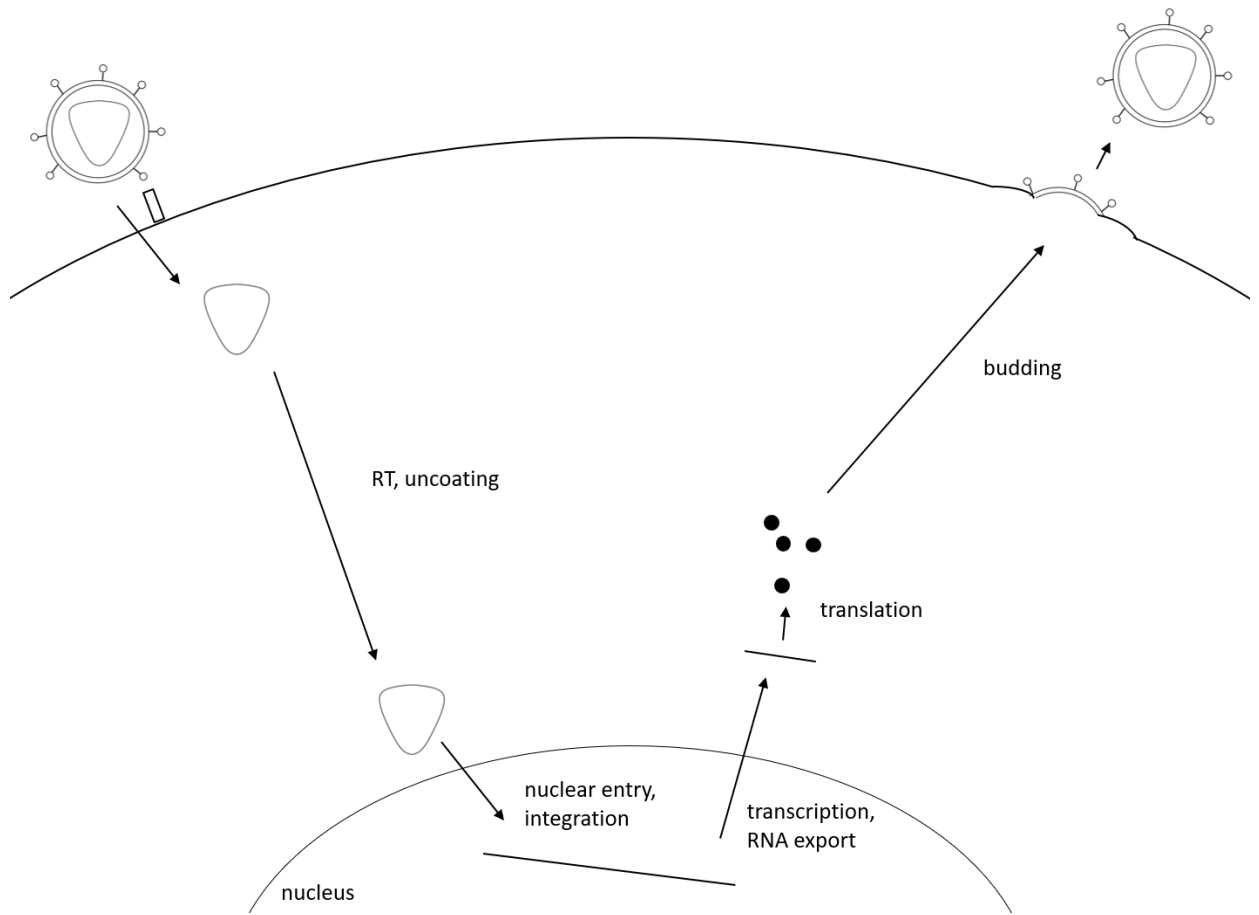


**Figure 1.2: Schematic of an HIV-1 viral particle.** The RNA genome and assorted proteins are encapsulated by a conical capsid layer, which is inside of the matrix layer and lipid bilayer. Envelope proteins are present on the outside of the lipid bilayer.

## **The HIV-1 Life cycle**

An infectious HIV-1 particle (Figure 1.2) consists of a lipid bilayer studded with trimeric envelope proteins on the outside and a layer of matrix protein multimers on the inside. Multimers of the viral capsid protein form a cone-shaped viral core beneath the matrix layer (Briggs and Krausslich, 2011). Inside the core are two copies of the viral RNA genome bound by nucleocapsid proteins, the viral replication enzymes integrase and reverse transcriptase, accessory viral proteins such as Nef and Vpr, and an array of host RNAs including tRNAs and 7SL RNA (Telesnitsky and Wolin, 2016). Cellular proteins from the host cells in which particles were produced are also incorporated into viral particles, though the importance of most of these proteins for the viral life cycle is unknown (Goff, 2018; Sundquist and Krausslich, 2012).

The life cycle of retroviruses can be sharply divided into two discrete phases. HIV-1 particles enter cells to generate a provirus DNA integrated into the host genome during the early phase of the life cycle, and the cell then forms and releases virion particles capable of infecting other cells during the late phase (Figure 1.3). The envelope proteins of infectious particles bind the CD4 receptor and a co-receptor (CCR5 or CXCR4) (Berger et al., 1999; Sattentau and Weiss, 1988) at the cell surface. The interaction of the viral envelope with a receptor induces signaling cascades that result in actin-dependent clustering of CD4 and co-receptors, facilitating entry of the viral core into the cell (Fackler and Krausslich, 2006; Stolp and Fackler, 2011). The viral particle releases its core into the cytoplasm through membrane fusion, which may happen either at the plasma membrane or after endocytosis. HIV-1 is capable of both mechanisms of entry (Schaeffer et al., 2001), though there has been some discussion about which of these pathways leads to productive infection, and there may be cell type-dependent differences (Aggarwal et al., 2017; Herold et al., 2014).



**Figure 1.3: Representation of the HIV-1 viral life cycle.** The entry of HIV-1 particles into cells is mediated by interaction of the envelope proteins with host cell receptors. The capsid enters the cytoplasm and travels towards the nucleus. Reverse transcription of the viral RNA genome and uncoating of the capsid layer occur while the viral particle is in the cytoplasm, followed by nuclear entry. The viral DNA is integrated into the host genome, after which the provirus is transcribed and translated. Viral proteins and RNA bud out of the lipid bilayer at the cell periphery to form viral particles.

The actin network near the plasma membrane presents a barrier for viral cores after fusion at the plasma membrane, which is alleviated by the function of Nef (Stolp and Fackler, 2011). Infection is inhibited in Nef-deficient HIV-1, but this defect is rescued by drug-mediated actin depolymerization *in vitro* (Campbell et al., 2004). Pseudotyping virions with pH-dependent envelopes such as VSV-G allows HIV-1 to bypass the need for Nef-mediated actin disruption, while HIV-1 particles with the pH-independent amphotropic MLV envelope exhibit the same defect in response to Nef-deficiency and the same rescue in response to actin disruption.

The early post-fusion stage of infection consists of transport of the viral genome to the nucleus, possible partial uncoating of the capsid layer, and reverse transcription. The viral core is referred to as the reverse transcription complex (RTC) prior to completion of reverse transcription, and the pre-integration complex (PIC) thereafter (Goff, 2007). Reverse transcription is initiated from a tRNA bound to the primer binding site (PBS), producing a short DNA sequence called minus-strand strong stop (MSS) DNA. This sequence is able to bind the complementary repeated sequence in the 3' R region of the viral RNA, priming the synthesis of the longer minus strand HIV-1 DNA. Meanwhile, the RNase H activity of reverse transcriptase is responsible for the degradation of RNA found in an RNA-DNA duplex, excepting two resistant polypurine tracts (PPTs) which serve as primers for plus strand DNA synthesis. The PBS sequences at the 3' end of the plus strand DNA (copied from the retained primer tRNA) is able to bind to the complementary sequence of the minus strand PBS, with both strands then serving as primers and templates for the synthesis of full-length linear double-stranded DNA. MSS and total viral DNA may be assayed using PCR and serve as a readout for the synthesis of early and late reverse transcription products (Goff, 2018; Hu and Hughes, 2012).

Between entry of the viral core into the cellular cytoplasm and trafficking into the nucleus, the RTC/PIC will gradually lose capsid protein through a process called uncoating, though some capsid will remain associated with the complex even after nuclear entry (Arhel, 2010; Goff, 2007). There is evidence that reverse transcription and uncoating are interlinked. Point mutations in capsid proteins that affect the stability or structure of the viral core blocked HIV-1 infection, often at or before reverse transcription (Forshey et al., 2002; Tang et al., 2001). Conversely, defects in reverse transcription increased viral core stability or delayed uncoating relative to controls (Hulme et al., 2015; Hulme et al., 2011; Yang et al., 2013).

The PIC is trafficked into the nucleus through nuclear pores, allowing HIV-1 to infect non-dividing cells as well as dividing cells--unlike the simple retroviruses such as the murine leukemia viruses, which can only infect dividing cells upon nuclear envelope breakdown (Lewis et al., 1992; Lewis and Emerman, 1994). Capsid binds several proteins involved in nuclear entry, including members of the nuclear pore complex (NPC) (Ambrose and Aiken, 2014). Nuclear membrane associated transmembrane proteins SUN1 and SUN2 might affect nuclear entry in a redundant manner. The overexpression of both proteins, or the knockout of SUN2 but not SUN1, can produce reductions in the percentage of GFP positive cells when infected with several strains of GFP containing HIV-1 and defects in nuclear entry as measured by 2-LTR circles (Schaller et al., 2017). Once in the nucleus, a portion of the linear viral DNA will be circularized by the nonhomologous end joining (NHEJ) pathway to form 2-LTR circles, or by the homologous recombination (HR) pathway to form one-LTR circles. These circles are not productive for infection, but may be assayed using PCR as a readout of successful nuclear import (Goff, 2018).

The integrase protein is responsible for integrating the linear viral DNA into the host genome to form a provirus. Lens epithelium-derived growth factor (LEDGF)/p75 functions as a

cofactor for the HIV-1 integrase by enhancing its activity and tethering integrase to host chromatin. This protein has only been shown to interact with integrase from lentiviral retroviruses (Debyser et al., 2015; Engelman and Cherepanov, 2008). Other host proteins serve similar functions for other retroviruses, such as the bromodomain and extraterminal (BET) family proteins BRD2, BRD3, and BRD4, which target MLV integration (De Rijck et al., 2013; Gupta et al., 2013; Sharma et al., 2013). Provirus DNA may be detected by PCR utilizing primers for human genomic Alu sequence repeats and the LTR, providing a readout for successful integration (Butler et al., 2001). New methods involving oligonucleotide hybridization to enrich for viral sequences before PCR have been developed for low abundance of proviral DNA, and to avoid bias toward integration events near Alu repeats (Iwase et al., 2019; Katsuya et al., 2019).

In the exit stage of the HIV-1 life cycle, the promoter in the 5' LTR region of the provirus drives transcription by RNA polymerase II (RNAP II). Efficient production of all viral RNAs requires HIV-1 Tat to bind the transactivation response element (TAR) in the 5' end of the nascent viral RNA and recruit the positive transcription elongation factor b (P-TEFb) complex, including a kinase activity that phosphorylates the RNAP II C-terminal domain and releases paused RNAP II. A mixture of as many as a hundred distinct fully spliced, partially spliced, and unspliced RNAs are produced in the nucleus. The complex array of mRNAs is the product of many alternative splicing choices, determined by regulatory sequence elements near the various splice acceptor sites scattered along the viral RNA (Emery et al., 2017; Takata et al., 2018). All the mRNAs are polyadenylated at the 3' end, at the same position, at the R-U5 boundary in the 3' LTR.

Fully spliced viral mRNAs are exported in the same manner as cellular mRNA and serves as a template for the translation of Tat, Rev, and Nef (Faust et al., 2017). Nef, which is highly expressed during early infection, regulates the expression of multiple surface proteins. CD4 receptors, major histocompatibility complex class I (MHC-I) molecules, and restriction factors serine incorporator 3 (SERINC3)/ serine incorporator 5 (SERINC 5) are among the targets downregulated by Nef (Pereira and daSilva, 2016). Newly translated Rev is transported into the nucleus, where it binds the Rev response element (RRE) of unspliced and partially spliced viral RNA, mediating their nuclear export (Faust et al., 2017; Malim et al., 1989).

Env, Vif, Vpr, and Vpu are translated from partially spliced mRNAs (Sundquist and Krausslich, 2012). Translation of the transcript coding for Env and Vpu occurs at the rough ER (Sundquist and Krausslich, 2012), where Vpu downregulates newly synthesized CD4 (Dube et al., 2010). Finally, unspliced RNA serves as both genomic RNA and a template for Gag and Gag-Pol translation. Gag and Gag-Pol polyproteins assemble at the plasma membrane and incorporate other necessary components (such as genomic viral RNA and envelope proteins) into budding viral particles. The HIV-1 p6 domain of the Gag polyprotein recruits host ESCRT (endosomal sorting complexes required for transport) machinery to release viral particles from the plasma membrane through membrane fission (Sundquist and Krausslich, 2012). Vpu also functions in viral budding in a cell type-dependent manner by inhibiting the activity of the antiviral protein Tetherin (which tethers viral particles to the plasma membrane) (Dube et al., 2010; Sundquist and Krausslich, 2012).

The HIV-1 particles which bud out of the plasma membrane are immature and noninfectious (Briggs and Krausslich, 2011). These particles mature when protease cleaves the Gag/Gag-Pol polyproteins into individual matrix, capsid, nucleocapsid, p6, protease, reverse

transcriptase, and integrase proteins. The ensuing structural rearrangement of the capsid proteins produces the cone shape characteristic of HIV-1 particles (Sundquist and Krausslich, 2012), and activates the infectivity of the particles.

### **Host proteins in the HIV-1 Life Cycle**

A substantial number of host proteins are recruited by HIV-1 at nearly all steps of the viral life cycle. siRNA screens have implicated hundreds of host proteins with potential roles in the HIV-1 life cycle (Brass et al., 2008; Konig et al., 2008; Zhou et al., 2008; Zhu et al., 2014), as have mass spectrometry and yeast two-hybrid studies assaying for interactions between viral and host proteins (Bushman et al., 2009; Chertova et al., 2006; Fellay et al., 2007; Kalpana et al., 1994; Luban et al., 1993). These screens likely produce candidate lists which contain many false positives, but the roles of many cellular proteins in HIV-1 infection have been confirmed. Some of these proteins will have cell-type dependent functions, such as the microtubule motor kinesin KIF3A, which functions in the exit stage of HIV-1 in primary macrophages but not T cells (Gaudin et al., 2012).

Host proteins recruited by HIV-1 function at every step of the viral life cycle. For example, Cyclophilin A (CypA) binds the capsid protein and may regulate the uncoating process or compete with a restriction factor that might otherwise bind the capsid (Kim et al., 2019; Luban et al., 1993). During the exit phase of the life cycle, HIV-1 recruits the machinery of the ESCRT pathway to release viral particles from host membranes (Votteler and Sundquist, 2013). This machinery normally functions in membrane fission of vesicles budding into endosomes to form multivesicular bodies (MVBs) which will then fuse with lysosomes. Similarly, the formation of the autophagosome during autophagy and of extracellular vesicles also utilize ESCRT proteins.

These proteins are responsible for repair of damage to several membranes, such as the plasma membrane and lysosomal membrane. Cell division also relies on ESCRT proteins to seal the nuclear envelope and for the abscission of daughter cells during cell division (Carlton and Martin-Serrano, 2007; Hurley, 2010; Hurley and Hanson, 2010; Vietri et al., 2020).

Dozens of proteins responsible for regulating the cytoskeletal actin network are exploited by HIV-1 to complete its life cycle. These include proteins which bind both plasma membrane surface proteins and F-actin such as filamin-A, inducers of actin polymerization such as WAVE2 and Arp2/3 (Spear et al., 2014), and inducers of actin depolymerization such as cofilin. Env, Gag, Nef, and Tat all participate in managing actin dynamics, first by inducing polymerization to facilitate clustering of CD4 and coreceptors at the cell surface, then by prompting depolymerization to allow penetration of the cortical actin network (Lehmann et al., 2011; Ospina Stella and Turville, 2018; Taylor et al., 2011). Regulator of actin dynamics Slit2 blocks HIV-1 infection by opposing Env-induced signaling (Anand et al., 2013).

While there is contradictory evidence for the necessity of actin in budding of viral particles out of the cytoplasm, it is required for cell-to-cell transfer. In cell-to-cell transfer, infected cells form a point of contact called a virological synapse (VS) with an uninfected cell to mediate transfer of viral particles. Both the actin and microtubule network function in polarized budding of viral particles at the VS, while actin mediates the clustering of HIV-1 receptors at the VS in uninfected cells (Lehmann et al., 2011; Ospina Stella and Turville, 2018; Taylor et al., 2011). PACSIN2 is a regulator of actin dynamics used by HIV-1 in cell-to-cell transfer, but not other stages of the life cycle (Popov et al., 2018). Furthermore, Nef reduces cell motility in humanized mouse models by inhibiting actin dynamics through its association with p21-activated kinase 2 (PAK2). Viruses with mutant Nef unable to associate with PAK2 initially

outcompete viruses with wild-type Nef, but the opposite is true at later time points corresponding to increased immune response (Usmani et al., 2019).

Many host proteins are incorporated into viral particles during assembly, though few have known functions. Those include the helicase protein Upf1, RNA-binding protein HuR, and the previously mentioned DNA repair protein UNG2 (Chen et al., 2004; Lemay et al., 2008; Serquina et al., 2013). Proteins incorporated into viral particles may also function in cellular defense. The APOBEC3 family of proteins are restriction factors that mutate retroviruses by deaminating cytosines in minus strand ssDNA intermediates, thus targeting the reverse transcription step of the HIV-1 life cycle (Harris et al., 2003). HIV-1 RNA or viral nucleocapsid-bound cellular RNA can bind several APOBEC3 proteins, recruiting them into assembling viral particles. HIV-1 accessory protein Vif inhibits the function of host cell APOBEC3 proteins primarily through the recruitment of an E3 ligase complex to mediate their degradation (Conticello et al., 2003; Feng et al., 2014).

### **Microtubules in the Entry Stage of HIV-1 Infection**

Microtubules function in cell motility, in determining cell shape, and in promoting mitosis and intracellular transport. They consist of  $\alpha$ -tubulin and  $\beta$ -tubulin heterodimers that maintain the same directionality as they are added to pre-existing microtubule oligomers, forming distinct plus and minus ends. This orientation determines the direction of transport for motor proteins. Microtubules primarily grow from the plus end while their minus end is anchored in a microtubule-organizing centre (MTOC), often located at the centrosome. The nucleation of microtubules at the MTOC typically occurs after the assembly of  $\gamma$ -tubulin ring

complexes ( $\gamma$ TuRCs), which provide scaffolds for  $\alpha$ -tubulin and  $\beta$ -tubulin from which heterodimers grow (Bartolini and Gundersen, 2010; Lin et al., 2015; Zheng et al., 1995).

Microtubules polymerize and depolymerize in a dynamic fashion. The shift from growth to shrinkage is termed catastrophe, while the shift from shrinkage to growth is known as rescue. The dynamic microtubules have half-lives of under 10 minutes (Bartolini and Gundersen, 2010). A subset of microtubules remain stable for longer periods of time (on the order of hours rather than minutes), and these stable microtubules have post-translational modifications along their length.

Acetylation of tubulin is widely used as a marker for microtubule stability, and there is evidence that acetylation confers resistance to damage from mechanical stress (Portran et al., 2017; Xu et al., 2017). Detyrosination (removal of the C-terminal tyrosine) has also been associated with longer-lived microtubules (Gundersen et al., 1987; Janke and Bulinski, 2011; Khawaja et al., 1988; Li and Gundersen, 2008; Song and Brady, 2015). There is evidence that these modifications may correlate with preferential association with certain motors--for example, several experiments indicate kinesin-1 may have a preference for detyrosinated tubulin, while kinesin-3 may have a preference for tyrosinated tubulin (Cai et al., 2009; Guardia et al., 2016; Konishi and Setou, 2009; Sirajuddin et al., 2014; Tas et al., 2017). Unlike dynamic microtubules, the plus ends of detyrosinated microtubules remain static, without experiencing growth or shrinkage (Bartolini and Gundersen, 2010). Plus-end tracking proteins (+TIPs) are recruited to the plus ends of microtubules, from which the +TIP end-binding protein 1 (EB1) cooperates with Kif4 to stabilize microtubules as part of the Rho-Dia-EB1 pathway (Morris et al., 2014). All these factors may be important for virus infection.

The dynein family, made up of microtubule motor proteins, consists of axonemal dyneins (which move cilia and flagella) and cytoplasmic dynein (which transport cargo) (Hook and Vallee, 2012). Cytoplasmic dynein further divides into cytoplasmic dynein 1 and cytoplasmic dynein 2, the latter of which is also associated with cilia and flagella. Cytoplasmic dynein 1 is responsible for the minus end-directed transport of cargo from the cell periphery along microtubules (retrograde transport) toward the MTOC in all cells (Gaudin et al., 2013; Hook and Vallee, 2012; Hook and Vallee, 2006; Vallee et al., 2012).

Dynein plays a major role in HIV-1 infection (Arriagada, 2017; Dharan and Campbell, 2018). Disruption of dynein transport by microinjection into cells of antibodies to the intermediate chain of the dynein motor complex led to accumulation of viral particles at the cell periphery one hour post-infection (McDonald et al., 2002). Similar results were attained through the siRNA knockdown of the dynein heavy chain (Pawlica and Berthoux, 2014), but not from siRNA knockdowns of other components of the dynein complex (Carnes et al., 2018). Other studies suggest roles for dynein light chains in HIV-1 (Caly et al., 2016; Desfarges et al., 2009; Jayappa et al., 2015) or MLV infection (Opazo et al., 2017). siRNA knockdown of the dynein heavy chain, dynein motor adaptor BICD2, and several components of the dynactin complex, which is required for cytoplasmic dynein function as a processivity factor (King and Schroer, 2000), correlated with decreases in both infectivity and the appearance of 2-LTR circles (Carnes et al., 2018). CRISPR depletion of BICD2 in several cell lines, including Jurkat T cells, also reduced infection by single-cycle reporter viruses and formation of 2-LTR circles (Dharan et al., 2017).

Motors responsible for plus end trafficking along microtubules (anterograde transport) belong to the kinesin superfamily (Verhey and Hammond, 2009). Cellular cargo is often bound

by both dyneins and plus end-directed kinesins at the same time, resulting in bidirectional transport with net movement in one direction (Hancock, 2014). This bidirectionality with net movement towards the cell center has been reported for retroviral trafficking during the entry phase of the life cycle (Gaudin et al., 2013). siRNA knockdown of the kinesin-1 adaptor Fasciculation and Elongation Factor zeta 1 (FEZ1) as well as the kinesin-1 heavy chain isoforms Kif5A or Kif5B decreased expression of single-cycle HIV-1-based reporter virus and inhibited transport of viral particles to the nucleus. Kif5A and Kif5B knockdowns also reduced levels of 2-LTR circles (but not MSS or total viral DNA) (Malikov et al., 2015). There is evidence of roles for microtubules, including the functions of both cytoplasmic dynein and kinesin-1, in the life cycles of other viruses as well (Arriagada, 2017; Berka et al., 2013; Clark et al., 2013; Dodding and Way, 2011; Gladue et al., 2011; Mabit et al., 2002; Smith et al., 2001; Su et al., 2010; Zhou et al., 2018).

There is also evidence that uncoating of the capsid core is reliant on microtubules. Lukic et al. (2014) tested the effects of nocodazole treatment on uncoating using the cyclosporine A (CsA) washout assay, an *in situ* uncoating assay, and the so-called “fate of capsid” assay. CsA is a drug that prevents the restriction factor TRIM-CypA from binding to the viral capsid to inhibit infection. Only viral cores with an intact capsid are vulnerable to TRIM-CypA restriction, such that the extent of uncoating may be estimated by susceptibility to TRIM-CypA after CsA washout at various time points post-infection (Hulme and Hope, 2014). The *in situ* uncoating assay uses GFP-labeled Vpr (which labels the viral core) to detect the amount of capsid staining associated with individual virions post-fusion. Pre- and post-fusion virions were determined by the presence or absence of mCherry, which had been fused with a membrane binding sequence and incorporated into viral particles (Campbell et al., 2007). The fate of capsid assay consists of

centrifuging cell lysates through a sucrose cushion to separate intact capsid cores from soluble capsid proteins.

Lukic et al. (2014) found that uncoating is delayed by nocodazole treatment using these three assays. This study determined that treatment with an inhibitor of dynein motor function (ciliobrevin D) and siRNA knockdown of cytoplasmic dynein heavy chain (DYNC1H1) both delayed uncoating using the CsA washout assay and *in situ* uncoating assay respectively. They also found siRNA knockdown of kinesin KIF5B, strangely, to initially enhance uncoating at their first time point post-infection and then to delay it in subsequent time points relative to controls in an *in situ* uncoating assay (Lukic et al., 2014). Malikov and Naghavi (2017) found that siRNA knockdowns of both KIF5B and FEZ1 delayed uncoating using *in situ* and fate-of-capsid assays. Dharan et al. (2016) used an *in situ* uncoating assay to determine that siRNA knockdowns of both KIF5B and Nup358 (which is both a KIF5B cargo adapter and nuclear pore complex protein) delayed uncoating and caused the accumulation of viral cores outside the nucleus. HIV-1 cores colocalized with Nup358 in the cytoplasm, but this colocalization was not seen in cells with KIF5B knockdowns. Other studies also found that inhibiting dynein motor function increased pelletable capsid in a fate of capsid assay (Pawlica and Berthoux, 2014), and that depleting the dynein motor adaptor BICD2 delays uncoating in an *in situ* uncoating assay using GFP-labelled integrase (Dharan et al., 2017).

Drugs affecting the stability of microtubules have only slight effects on HIV-1 infectivity. Several such drugs have been tested. Paclitaxel/taxol binds directly to microtubules and prevents depolymerization (Schiff et al., 1979) Colchicine, nocodazole, and vinblastine bind microtubules and interfere with polymerization (Banerjee, 1997; Luduena and Roach, 1991; Wilson et al., 1982). Treatment with taxol produced a two fold increase in infectivity in Rev-

CEM T cells, while the effects produced by the other three drugs were even smaller, and likely were not significant (Yoder et al., 2011). Another study found 2-3 fold reductions in HIV-1 infectivity upon nocodazole treatment in 293A and CHME3 cells (Sabo et al., 2013). While treatment with actin polymerization inhibitors like cytochalasin D (CCD) prior to infection reduced HIV-1 infectivity and matrix protein staining in the cytoskeletal subfraction of cell lysates, treatment with nocodazole did not have these effects (Bukrinskaya et al., 1998).

The limited impact of taxol treatment on HIV-1 infection may be explained by the effect of infection on the microtubule network. Cells infected by HIV-1 showed an increase in staining for acetylated tubulin, demonstrating that HIV-1 stabilizes microtubules upon infection. This effect occurs in several cell lines, including primary macrophages and Jurkat cells. As both HIV-1 infection and taxol treatment stabilize microtubules, the effect of taxol may be partially redundant. The stabilization of microtubules by taxol may simply not create structures that block infection.

Furthermore, the three microtubule-disrupting drugs used in these studies function by preventing assembly (at the concentrations used in those particular experiments). As such, preexisting stable microtubules may persist in these cells despite drug treatment, and thus allow for near-normal infection for a period of time. Correspondingly, Sabo *et al.* (2013) found that viral particles labeled with GFP-Vpr colocalized with stable microtubules even after nocodazole treatment in immunofluorescence experiments, suggesting that virus can traffic on microtubules even with drug treatment (Naghavi, 2014).

Several proteins which influence microtubule stability do affect HIV-1 infection (Dharan and Campbell, 2018). Moesin crosslinks actin filaments to the plasma membrane and negatively regulates microtubule stability. Its knockdown increases infection efficiency of HIV-1 and MLV,

while its overexpression blocks infection (Naghavi et al., 2007). EB1 recruits plus-end tracking proteins (+TIPs) to microtubules to promote microtubule stability. Depletion of EB1 inhibits infection, as does depletion of EB1 binding protein Kif4 (Sabo et al., 2013). The knockdowns of MAP1A and MAP1S, which bind to and stabilize microtubules, also inhibit HIV-1 infection (Fernandez et al., 2015). Further proteins that both regulate microtubule stability and impact infection are listed in Table 1.2.

**Table 1.2: Regulators of Microtubule Stability in HIV-1 Infection**

<b>Protein</b>	<b>Microtubule Related Functions</b>	<b>Effect on HIV-1 Infection</b>	<b>References</b>
<b>histone deacetylase 6 (HDAC6)</b>	Deacetylation of targets such as $\alpha$ -tubulin	Negative	(Valenzuela-Fernandez et al., 2005)
<b>Moesin</b>	Negatively regulates microtubule stability	Negative	(Capalbo et al., 2011; Naghavi et al., 2007)
<b>Ezrin</b>	Negatively regulates microtubule stability	Negative	(Haedicke et al., 2008)
<b>end-binding protein 1 (EB1)</b>	Regulates microtubule stabilization by recruiting plus-end tracking proteins (+TIPs)	Positive	(Sabo et al., 2013)
<b>kinesin family member 4 (Kif4)</b>	Regulates microtubule stabilization by binding EB1	Positive	(Sabo et al., 2013)
<b>microtubule associated protein 1A (MAP1A)</b>	Binds and stabilizes microtubules	Positive	(Fernandez et al., 2015)
<b>microtubule associated protein 1S (MAP1S)</b>	Binds and stabilizes microtubules	Positive	(Fernandez et al., 2015)
<b>diaphanous related formin 1 (Dia1)</b>	Functions in microtubule stabilization	Positive	(Delaney et al., 2017)
<b>diaphanous related formin 2 (Dia2)</b>	Functions in microtubule stabilization	Positive	(Delaney et al., 2017)
<b>microtubule associated protein 4 (MAP4)</b>	Binds and stabilizes microtubules	Positive	(Gallo and Hope, 2012)

## **MID1IP1 and MID1**

### **siRNA Screens Identifying MID1IP1 as a Potential Host Protein Involved in HIV-1**

#### **Infection**

MID1IP1 has been implicated as a host factor necessary for the entry phase of the HIV-1 life cycle by two siRNA screens, one of which was conducted in TZM-bl cells and the other in 293T cells. Brass et al. (2008) conducted a screen by assaying for levels of the capsid protein (or p24) within TZM-bl cells (derived from HeLa cells, expressing CD4 and CCR5) after infection by wild-type replication-competent HIV-1 strain IIIB. siRNA pools were used to knock down host genes, after which the cells were infected with HIV-IIIB. Cells were stained for p24 at 48 hours post-infection, which is sufficient time for the translation of Gag in cells infected by the initial inoculum. There were 358 hits in which the percentage of p24 staining cells was reduced by at least two standard deviations, 273 of which were confirmed with a follow-up experiment. 36 of the hits, including cell receptor CD4, were already known to affect the life cycle. 14 of them had functions related to binding of transcription factors, 12 to GTP binding, and 12 to actin binding. Hits known to localize to microtubules included MAP4, KIF3C, and TUBAL3. MID1IP1 was also a hit in this screen. 33% as many of the infected cells were found to stain for p24 after MID1IP1 was knocked down, relative to controls.

König et al. (2008) screened for host genes affecting the early stages of HIV infection using a single-cycle reporter virus. 293T cells were transfected with 2 siRNAs targeting the same gene per well (for a total of 6 different siRNAs across 3 wells) for initial screening or individual siRNAs for confirmation, then infected with an HIV-1 luciferase reporter virus pseudotyped with

glycoprotein G from vesicular stomatitis virus (VSV-G). 295 genes were identified whose knockdown inhibited infection by at least 45% when targeted by at least two siRNAs.

The hits included 26 genes that have known functions in the HIV-1 life cycle or were reported by Brass et al. (2008) in their screen. Many genes involved in functions such as DNA repair, transcription, nucleic acid binding, and zinc finger binding (including several TRIM genes) were reported as hits. Microtubule related hits included MAP4, KIF3A, Tripartite motif-containing 55 (TRIM55), Dynamin 2 (DNM2), Tubulin polymerization promoting protein (TPPP), Microtubule associated monooxygenase, calponin and LIM domain containing 3 (MICAL3), and MID1 interacting protein 1 (MID1IP1). Knockdown of MID1IP1 with 2 siRNAs produced an average reporter expression of 10% relative to the negative control. Similar results for MID1IP1 have been found in a parallel screen for murine leukemia virus, which produced an average 9 fold reduction in reporter expression relative to the negative control, implying a general mechanism that may affect the life cycles of multiple viruses. The design of the MLV screen mirrored that of the HIV-1 screen, except that cells were infected with a VSV-G pseudotyped MLV reporter virus. 80% of the hits for the HIV-1 screen were also hits in the MLV screen.

## **MID1IP1**

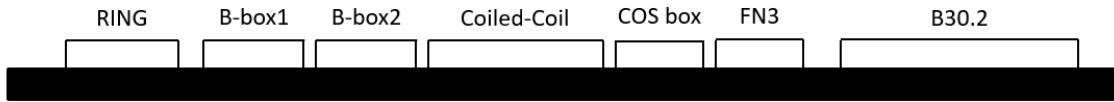
There have been only a limited number of studies pertaining to the function of MID1IP1. The 20-kDa protein was first identified as a protein interacting with MID1, in a screen conducted to explore the role of MID1 mutations in a developmental disorder, Opitz syndrome (OS) (Li et al., 2016). The same study determined that MID1 recruits MID1IP1 to microtubules (Berti et al., 2004). Berti et al. (2004) suggest the overexpression of both MID1 and MID1IP1 could induce

stable microtubule bundles resistant to nocodazole-induced depolymerization, implying a potential role for MID1IP1 in microtubule stability.

MID1IP1 has also been reported to function in lipogenesis by inducing the polymerization of acetyl-coenzyme A carboxylase and increasing its activity (Kim et al., 2010). How this might relate to its role in microtubule function is unclear. Two studies of a MID1IP1 homolog in zebrafish, Mid1ip11, found that embryos from females with a maternal effect mutation in the gene have defects in F-actin and microtubule reorganization during cytokinesis at the egg-to-embryo transition (Eno and Pelegri, 2018; Eno et al., 2016).

### **MID1 and the TRIM Family**

MID1 (also known as TRIM18) (Figure 1.4) belongs to a family of TRIM/RBCC proteins that contain N-terminal tripartite motifs consisting of RING finger, B-box, and coiled-coil domains. The RING domain is responsible for E3 ligase activity, while both the RING and B-box domains are zinc binding domains (McNab et al., 2011). MID1 has two B-box domains, which are capable of monoubiquitination without RING function and enhance polyubiquitination by the RING domain *in vitro* (Han et al., 2011b). The coiled-coil domain functions in protein-protein interactions and facilitates homo- and hetero-meric protein binding to other MID1 proteins or the MID1 homolog MID2 (also known as TRIM1) (Short et al., 2002). Both the RING and coiled-coiled domains are found in many proteins, while B-box domains are unique to the TRIM family.



**Figure 1.4: MID1 domains.** A representation of the domains in MID1. The RING domain has E3 ligase activity. The B-boxes are zinc binding domains while the coil-coiled, FN3, and B30.2 domains function in protein binding. The COS box binds microtubules.

TRIM proteins are divided into subgroups based on the composition of their C-terminal domains (McNab et al., 2011). Both MID1 and MID2 have a COS box motif, a fibronectin type III (FN3) motif, and B30.2-like domain (Short and Cox, 2006). MID2 has 83% similarity and 76% identity with MID1 (Perry et al., 1999; Yap et al., 2004). Both proteins form homo- and heterodimers, bind the  $\alpha$ 4 subunit of protein phosphatase 2A (PP2A) (Short et al., 2002), and co-localize with microtubules (Cainarca et al., 1999; Perry et al., 1999; Schweiger et al., 1999). The COS box motif is responsible for microtubule binding (Short and Cox, 2006; Wright et al., 2016), while FN3 (Campbell and Spitzfaden, 1994) and B30.2 domains (D'Cruz et al., 2013) are involved in protein-protein interactions.

There are about a hundred human TRIM family proteins (Han et al., 2011a), and several of them have been demonstrated to exhibit anti-viral restriction activity. The most extensively studied member of the family is TRIM5. TRIM5 $\alpha$  from many species strongly inhibits the infection of several retroviruses (Colomer-Lluch et al., 2018). The rhesus monkey  $\alpha$  isoform of TRIM5 restricts HIV-1, while human TRIM5 has milder effects and only at high overexpression levels (Stremlau et al., 2004; Uchil et al., 2008; Yap et al., 2004). TRIM5 $\alpha$  is responsible for the resistance of rhesus monkeys to HIV-1 in a microtubule-dependent mechanism (Pawlica et al., 2014). It binds the retroviral capsid, potentially affecting uncoating (Sastri and Campbell, 2011; Sebastian and Luban, 2005). There seem to be two steps early in infection that are blocked by TRIM5 $\alpha$ : there is one block in reverse transcription, and a second block after viral DNA synthesis preventing normal nuclear entry of the PIC. The block to reverse transcription is relieved by treatment with proteasome inhibitors such as MG132, while nuclear entry (using 2-LTR circle formation as a readout) remains defective, suggesting distinctive effects at these two steps of the HIV-1 life cycle (Wu et al., 2006).

TRIM22 also restricts HIV-1 infection, a function dependent on its ubiquitin ligase activity (Barr et al., 2008). TRIM11 also increases uncoating and decreases reverse transcription (Yuan et al., 2016). Depletion of TRIM37 in cells producing virus (but not cells infected with a single-cycle reporter virus) inhibits the formation of infectious virus, suggesting a supportive role for this family member. TRIM37 was found to be incorporated into viral particles (Tabah et al., 2014).

Uchil et al. (2008) transiently expressed 36 human TRIMs and 19 mouse TRIMs in HEK293 cells and tested the effects on viral entry by infection with a single-cycle reporter virus. HIV-1 infection was inhibited by both rhesus monkey TRIM5 (38 fold inhibition) and human TRIM5 (5 fold inhibition), though the block by the rhesus protein was much stronger. The overexpression of three human TRIMs (TRIM5, TRIM26, TRIM31) and four mouse TRIMs were found to inhibit HIV-1 infection, while one human TRIM (TRIM38) and one mouse TRIM enhanced infection. Neither MID1 or MID2 affected HIV-1 infection in these experiments.

The effect of the expression of these TRIM proteins on MLV was also tested. Infection of cells overexpressing MID2 with an N-tropic MLV reporter virus produced a 15 fold inhibition, while the same experiment done with MID1 overexpression had no effect. MID2 overexpression did not have an effect on B-tropic MLV or HIV-1 infection. Human TRIM5 affected N-tropic MLV (an 18-fold effect), but not B-tropic MLV.

### **MID1 Interactions**

The molecular details of MID1 functions are not very clear. Its identity as a TRIM protein suggests that it might act as an E3 ubiquitin ligase to mediate the polyubiquitylation and degradation of selected target proteins. MID1 has been shown to negatively regulate PP2A

activity, through its interaction with the PP2A regulatory subunit  $\alpha 4$ . It was initially reported that MID1 polyubiquitinated the catalytic subunit of PP2A (PP2Ac), targeting it for degradation (Troddenbacher et al., 2001). Later studies reported that MID1 monoubiquitinated  $\alpha 4$  (McConnell et al., 2010) (Han et al., 2011b), leading to the cleavage of the C-terminal domain of  $\alpha 4$  and elimination of its ability to bind MID1. The full-length, unubiquitinated  $\alpha 4$  protects PP2Ac from polyubiquitination of PP2Ac by MID1 and proteasomal degradation, while cleaved  $\alpha 4$  correlates to the downregulation of PP2Ac, possibly mediated by a different E3 ubiquitin ligase (Du et al., 2014; Kong et al., 2009; LeNoue-Newton et al., 2011; Watkins et al., 2012). Du et al. (2013) report that MID1 is also capable of polyubiquitinating  $\alpha 4$  *in vitro*.

There are several potential consequences of the MID1-PP2A interaction. Regardless of the mechanism by which MID1 downregulates PP2A activity, the  $\alpha 4$  subunit facilitates the interaction between MID1 and PP2A (McConnell et al., 2010). *In vitro* interaction of C-terminal fragments of  $\alpha 4$  with MID1 fragments that contain RING-Bbox domains decreased polyautoubiquitination of MID1 (Han et al., 2011b), suggesting that  $\alpha 4$  may regulate the E3 ligase activity of MID1. The interaction of MID1 with the cytoskeleton is likely regulated by its phosphorylation, and PP2A may modulate this. Dynamic phosphorylation of MID1 by mitogen-activated protein (MAP) kinase regulates MID1 binding to the cytoskeleton, and the association with the  $\alpha 4$  subunit causes the dephosphorylation of MID1 and its displacement from microtubules (Liu et al., 2001). MID1 is also transported along microtubules in a dynein and kinesin motor dependent manner. Its transport, but not its association with microtubules, is inhibited by the knockdown of  $\alpha 4$  or treatment with PP2A inhibitors (Aranda-Orgilles et al., 2008a).

Some of MID1's functions may involve its interaction with PP2A. An embryonic fibroblast cell line derived from an OS patient (corresponding to a 4 bp deletion altering the C-terminus of MID1) (Schweiger et al., 1999) demonstrates upregulated microtubule-associated PP2A, which is no longer the case when wild type MID1 is added to the system. Proteins that immunoprecipitated with microtubules from this cell line were hypophosphorylated compared to controls (Trockenbacher et al., 2001). The phosphorylation of several microtubule-associated proteins is regulated by PP2A: Tau (Goedert et al., 1995; Watkins et al., 2012), MAP2 (Sanchez et al., 1996; Sontag et al., 2012), PRC1 (Cundell et al., 2013), and Kif4 (Bastos et al., 2014) are specific microtubule associated proteins known to be targeted for dephosphorylation by PP2A. Tau and MAP2 function in microtubule stability and are primarily expressed in neurons (Dehmelt and Halpain, 2005), while the dephosphorylation of PRC1 and Kif4 by two isoforms of PP2A has been studied mainly in the context of central spindle formation during anaphase.

MID1 may have effects through PP2A on other human diseases. Tau is hyperphosphorylated in the brains of Alzheimer's disease (AD) patients (Javadpour et al., 2019), and PP2A is responsible for approximately 70% of phosphatase activity targeting tau in human brain tissue (Liu et al., 2005). Decreased PP2A activity has been reported in AD, and one study found an increase in MID1 expression (Schweiger et al., 2017). PP2A also positively regulates tau kinase GSK3- $\beta$  activity and negatively regulates extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) signaling pathways (which are also implicated in tau hyperphosphorylation), indicating that the mechanism of PP2A in the hyperphosphorylation of tau may be complex. PP2A affects other disease-causing pathways. It regulates alpha-synuclein, whose accumulation is a feature of Parkinson's disease (PD) (Javadpour et al., 2019). It functions in insulin signaling and has been linked to diabetes. Additionally, its activity has been associated

with both tumor suppression and tumor progression (Baskaran and Velmurugan, 2018). MID1 may modulate any of these functions.

Regulation of PP2A places MID1 upstream of the mammalian target of rapamycin (mTOR) signaling pathway, which affects various functions in cells including metabolism, cytoskeletal rearrangement, and autophagy (Liu et al., 2011). PP2A positively or negatively affects the activity of several kinases, including MAPK, GSK3- $\beta$ , Protein kinase C (PKC), and Akt (PKB) (Javadpour et al., 2019). Hundreds of interaction partners have been identified or proposed for PP2A. The regulatory subunit (B) that targets PP2A to its substrates has 26 known variants with affinities to different targets (Wlodarchak and Xing, 2016). Though studies of  $\alpha$ 4-MID1 interactions have focused on PP2A due to its associations with OS, it should be noted that  $\alpha$ 4 binds the PP4 and PP6 phosphatases as well (Kong et al., 2009).

MID1- $\alpha$ 4 is the most extensively studied interaction for MID1, but there are other known binding partners. MID1 and MID2 both bind SPAG5 (which regulates microtubules during cell division), though only MID2 has been shown to ubiquitinate and regulate the turnover of this protein (Gholkar et al., 2016). BRCA2-associated factor 35 (BRAAF35), a DNA binding protein associated with cytokinetic and neuronal differentiation complexes, is ubiquitinated by MID1 and targeted for proteasomal degradation (Zanchetta et al., 2017). In addition to its association with the tumor suppressor BRCA2 (Marmorstein et al., 2001), BRAAF35 interacts with Kif4, a kinesin that also binds DNA and functions in homologous recombination (Lee and Kim, 2003; Wu et al., 2008). As previously mentioned, Kif4 plays a role in HIV-1 infection due to its role in microtubule stability, perhaps through its direct interactions with retroviral Gag proteins (Tang et al., 1999).

Co-immunoprecipitation experiments indicate that MID1 and  $\alpha 4$  form a complex with several proteins involved in translation or mRNA binding. These include elongation factor 1 $\alpha$  (EF-1 $\alpha$ ), Annexin A2 (ANXA2), receptor of activated protein kinase C1 (RACK1), nucleophosmin (NPM), and ribosomal proteins. MID1 also associates with G-rich RNA and with its own mRNA (Aranda-Orgilles et al., 2008b). A follow-up study found that MID1 and  $\alpha 4$  increase translation efficiency of mRNAs containing the purine-rich MID1 association sequence (MIDAS) motif. 73 mRNAs with this motif were identified, and the association of 7 of them with MID1 was confirmed through RNA-protective immunoprecipitation. 31 of the proteins produced by these mRNAs have unknown functions, while 27 function in ventral midline development or embryogenesis (Aranda-Orgilles et al., 2011). MID1 mRNA does not contain a MIDAS motif, suggesting that this is not an exhaustive list of mRNAs associated with this MID1-associated translation complex.

### **Opitz syndrome**

Opitz G/BBB syndrome (OS) is a familial inherited genetic disorder characterized by variable midline defects in early development. The disorder has X-linked and autosomal loci which are clinically indistinguishable. *MID1* is the causative gene for the X-linked form, while 22q11.2 deletions produce the autosomal dominant form (Cox et al., 2000; Li et al., 2016; Quaderi et al., 1997; Robin et al., 1995; Winter et al., 2016).

A study compiling the symptoms of 184 patients with confirmed mutations in the *MID1* gene found common symptoms to include facial abnormalities, hypospadias, laryngotracheal abnormalities, and intellectual disability. Hypertelorism, or aberrant spacing between the eyes, was present in 99% (109/110) of male patients and 90% (38/42) of female patients. Other

commonly reported symptoms in males presented at under a 5% incidence rate in female patients: for example, laryngotracheal abnormalities occur in 45-47% of male patients, but only 2% of female patients. There were 88 mutations in *MIDI* among these 184 cases, which included truncations, frameshift mutations, deletions, insertions, splice errors, missense mutations, and whole gene deletions. Other than the whole gene deletions, these mutations spanned most of the gene excluding the RING finger domain. Four mutations repeated in non-related individuals: whole gene deletion, two truncations, and one frameshift mutation that resulted in a premature stop codon (Li et al., 2016).

The presence of whole gene deletions for *MIDI* in some OS patients indicates that the pathology can result from total loss-of-function. One study found an association between truncation mutants and brain abnormalities, but no other correlation between the nature of the mutations and the resulting phenotypes (Fontanella et al., 2008). A later study including more cases did not find that the type or location of mutations correspond to nature or severity of symptoms (Maia et al., 2017).

As previously mentioned, investigators have described a cell line from an OS patient with upregulated microtubule associated PP2A, in which hypophosphorylated proteins coimmunoprecipitated with microtubules relative to controls (Schweiger et al., 1999; Trockenbacher et al., 2001). One *MIDI* mutation in the B-box1 domain found in OS patients significantly reduced the ability of MID1 to polyubiquitinate  $\alpha 4$  but did not affect E3 ligase activity in general or disturb the B-box1 domain structure (Wright et al., 2017). Lack of PP2A turnover remains a potential mechanism for the midline developmental defects observed in OS.

Cytoskeletal protein SPECC1L had been proposed as one causative gene for autosomal dominant OS (Kruszka et al., 2015; Winter et al., 2016), but a recent paper by Bhoj et al. (2018)

argues that despite phenotypic overlap between craniofacial abnormalities OS and mutant *SPECC1L* syndrome, there are other symptoms that distinguish the two conditions and *SPECC1L* mutations should not be categorized as OS. Furthermore, not all 22q11.2 deletions associated with OS encompass the *SPECC1L* gene (Winter et al., 2016). Nonetheless, the phenotypic overlap between *SPECC1L* and *MID1* mutations with respect to facial abnormalities may indicate effects on the same pathway from a mechanistic standpoint. *MID1* functions in multiple pathways, several of which may result in each of the different phenotypes associated with OS, and *SPECC1L* only needs to contribute to one of these pathways to explain partially overlapping clinical features.

Defects in multipotent stem cells called neural crest cells (NCCs) are associated with several conditions involving facial abnormalities, including deficiencies in *SPECC1L*. During development, NCCs arise from the border that separates the neural and the non-neural ectoderm coincident with the formation of the neural tube. NCCs undergo an epithelial to a mesenchymal transition and migrate to give rise to cell types in many tissues within the body. Most facial structures are derived from NCCs, specifically cranial neural crest cells (CNCCs) (Cordero et al., 2011; Jeong et al., 2004; Snider and Mishina, 2014; Winter et al., 2016).

*SPECC1L* functions in actin reorganization and microtubule stability, with RNAi knockdowns producing defects in cell adhesion and migration (Saadi et al., 2011). Wilson et al. (2016) found that *SPECC1L* function in CNCCs regulates adherens junctions (AJs), which are actin-associated protein complexes that prevent delamination of CNCCs. *SPECC1L* deficiency in mutant mice produced defects in neural tube closure and CNCC delamination. In U2OS cells with stable knockdowns for *SPECC1L*, staining for AJ components was abnormal. For two components ( $\beta$ -catenin and E-cadherin), the staining was expanded compared to controls at

confluency. This corresponded with a reduction in PI3K-AKT signaling, which is responsible for downregulating E-cadherin. The inhibition of this signaling pathway in wild-type cells reproduced the AJ defects while activation of PI3K-AKT signaling in knockdown cells rescued those defects, indicating that SPECC1L functions upstream of PI3K-AKT signaling in cell adhesion.

Notably, PP2A also functions upstream of AKT signaling, which provides one mechanism by which MID1 might affect the same pathway. Other potential mechanisms exist as well. PP2A directly dephosphorylates  $\beta$ -catenin, which is one of the AJ components found to have an increased distribution in SPECC1L deficient cells. As the phosphorylation of  $\beta$ -catenin results in ubiquitination and degradation, dephosphorylation protects it from degradation. PP2A can dephosphorylate  $\beta$ -catenin residues unconnected to degradation as well, but the effects of this are unknown. Through use of another B regulatory subunit that targets it to different substrates, PP2A may also negatively regulate  $\beta$ -catenin levels through the activation of glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ), which phosphorylates  $\beta$ -catenin. AKT (which is inhibited by PP2A) fits into the pathway by activating GSK3 $\beta$  and thus negatively regulating  $\beta$ -catenin (Wlodarchak and Xing, 2016).

Aberrant Hedgehog signaling in neural crest cells is also associated with facial abnormalities. The depletion of Kif3A, which functions in intraflagellar transport, in neural crest cells via conditional knockout mice produces an increase in Hedgehog signaling, greater proliferation of neural crest cells, and hypertelorism (Brugmann et al., 2010). The elimination of responsiveness to Hedgehog signaling in neural crest cells with conditional knockout mice null for Smoothed (Smo) produced severe facial defects that demonstrated Hedgehog signaling is required for proper formation of NCC-derived craniofacial structures (Jeong et al., 2004).

Hypertelorism is a symptom of Greig cephalopolysyndactyly syndrome, which is caused by mutations in a Hedgehog signaling pathway gene encoding the transcription factor GLI3 (Winter et al., 2016). Nuclear localization of GLI3 (and therefore its ability to act in transcription) was increased by overexpression of  $\alpha 4$  (which increases interactions between PP2Ac and MID1, downregulating the former) in HeLa cells. Correspondingly, nuclear localization of GLI3 decreased after treatment with rapamycin (which increases PP2A activity) or overexpression of the B-Box1 domain of MID1 (which competes with wild-type MID1 for  $\alpha 4$  binding) (Krauss et al., 2008).

## **CCT2, chaperonin-containing TCP-1 subunit 2, a component of a major multisubunit chaperone complex**

Another candidate gene explored here for a potential effect on the HIV-1 life cycle, in addition to MID1IP1, is CCT2. CCT2 is a subunit of the protein folding TRiC/CCT complex. An siRNA screen conducted to identify host factors coopted by HIV-1 to complete its life cycle indicated that CCT2 may play a role in infection.

### **An siRNA Screen Identifying CCT2 as a Potential Host Protein Involved in HIV-1 Infection**

An siRNA screen in HeLa P4/R5 cells (which express CD4 and encode  $\beta$ -gal under the control of the HIV LTR) implicated CCT2 as a potential host protein participating in HIV-1 infection (Zhou et al., 2008). siRNA pools (of 3 siRNAs targeting each gene, initially in one well then repeated in a second experiment in triplicate) were used to knock down target genes and cells were infected with the replication-competent HXB2 isolate of HIV-1, which is capable of producing infectious viral particles that continue to infect other cells. Infection of these cells will result in activation of the LTR- $\beta$ -gal reporter gene by the viral Tat protein. Assays for  $\beta$ -gal activity were conducted at 48 and 96 hours post-infection. The 48 hour time point provides a measurement for the entry stages of the life cycle in those cells that are infected by the initial inoculum, while the 96 hour time point measures all stages of the life cycle as a readout of subsequent rounds of infection, as the cells continue producing and releasing infectious particles. The initial and confirmation screens produced 931 hits.

These hits were evaluated further by collecting viral particles from the supernatant of HeLa P4/R5 cells which had been transfected with siRNAs then infected with virus. Fresh HeLa P4/R5 cells untreated with siRNAs were infected with these viral particles and assayed for  $\beta$ -gal activity as a measure of whether gene knockdowns impacted any stage of the HIV-1 life cycle. This additional assay confirmed 390 hits. A selection of these hits chosen for their expression in T cells or macrophages were tested for  $\beta$ -gal activity after knockdown with independent siRNAs, which further confirmed 232 hits.

One of these 232 hits was CCT2, a component of a multisubunit protein folding complex. CCT2 RNAi knockdown experiments using siRNA pools produced effects of resulted in 80% of the  $\beta$ -gal activity present in controls at 48 hours and 36% at 96 hours, while knockdown experiments using independent siRNAs produced 15%  $\beta$ -gal expression relative to controls at 48 hours and 14% at 96 hours. Infection of fresh cells with viral particles produced in siRNA-transfected cells produced a 47% effect. To test for potential effects on Tat-mediated activation of  $\beta$ -gal expression from the HIV-1 LTR, HeLa P4/R5 cells were treated with siRNAs and then transfected with a plasmid expressing Tat rather than infecting with virus. CCT2 was one of 81 hits that produced at least a 40% reduction in  $\beta$ -gal expression.

In the results of the siRNA screens above, one experiment (using siRNA pools) found a late effect for CCT2 on the HIV-1 life cycle but not an early effect, while another experiment (using independent siRNAs) found an early effect. The experiment testing for Tat-mediated  $\beta$ -gal expression suggested a direct effect on Tat-mediated expression from the viral LTR (which should appear as an early effect in experiments using viral infection to drive reporter expression). In sum, the data suggest the possibility that CCT2 may have multiple effects on the viral life cycle, which is plausible given its role in protein folding of multiple targets.

## **CCT2 and the TRiC/CCT Complex**

Molecular chaperones are required by many proteins *in vivo* to reach their native structure. They prevent misfolding and aggregation in partially synthesized polypeptides during translation at ribosomes and participate in the folding of newly synthesized proteins (Spiess et al., 2004; Young et al., 2004). Some molecular chaperones (such as the heat-shock proteins) are upregulated under stress conditions that might lead to the unfolding of proteins (Hartl and Hayer-Hartl, 2002). The TCP1 Ring Complex/Chaperonin Containing TCP1 (TRiC/CCT) complex belongs to the chaperonin family of molecular chaperones, which assemble into double ring structures, and is present in the cytoplasm of all eukaryotic cells.

Chaperonins have a hydrophobic inner ring to bind substrates that they fold in an ATP-dependent manner. They are divided into two groups by sequence homology, with Group I encompassing chaperonins in bacteria and mitochondria and with Group II encompassing chaperonins in archaea and eukaryotes. One of the most studied chaperonins is bacterial GroEL, which belongs to Group I. GroEL forms a homo-oligomeric complex of 14 subunits. Unlike GroEL, TRiC/CCT contains eight different subunits. One of each of these subunits form a ring, and two rings assemble to form a 16 subunit complex (Horwich et al., 2007; Stoldt et al., 1996; Vallin and Grantham, 2019).

### **TRiC/CCT subunits**

Eight subunits (named CCT1 through CCT8) make up the TRiC/CCT complex (Kubota et al., 1994; Kubota et al., 1995; Rommelaere et al., 1993), though the CCT1 subunit is more commonly known as TCP-1. In yeast, all of the subunits are essential (Dekker et al., 2008; Stoldt

et al., 1996). The eight TRiC complex subunits share conserved sequences in the ATP-binding domains but diverge in their polypeptide-binding regions (Kim et al., 1994). Studies have produced several different models for the order in which CCT subunits are arranged in the complex (Cong et al., 2010; Dekker et al., 2011; Kalisman et al., 2012; Leitner et al., 2012; Liou and Willison, 1997). Each of these subunits are thought to bind different TRiC/CCT substrates.

The TRiC/CCT complex and several of its subunits have been associated with various diseases. The complex prevents the aggregation of huntingtin protein characteristic of Huntington's disease, and the overexpression of yeast CCT1 or CCT4 alone (but not the other subunits) in mammalian neurons reduced huntingtin aggregation (Tam et al., 2006). CCT1 and CCT2 were both found to be essential in breast cancer cells, suggesting they may support transformed cell survival (Guest et al., 2015). The complex may include substrates important for oncogenesis; for example, CCT3 binds to the TRiC/CCT substrate and oncoprotein STAT3 (Kasembeli et al., 2014). Various of the subunits may have tissue-specific substrates. CCT4 and CCT5 mutations have been associated with sensory neuropathy (Bouhouche et al., 2006; Lee et al., 2003).

The TRiC/CCT complex and several subunits have also been implicated as involved in the life cycles of various viruses. The PB2 subunit from several subtypes of the influenza A RNA polymerase coimmunoprecipitates with CCT2, while a CCT2 knockdown in A549 cells produces an approximately 2 log difference in influenza virus titers as scored by plaque assays. Influenza A forms ribonucleoprotein complexes of its genomic RNA with the three subunits of RNA polymerase – PB1, PB2, and PA – and nucleoprotein which are released into the cytoplasm of host cells during entry, so ribonucleoprotein reconstitution assays were conducted in 293T cells with a CCT2 knockdown by transfecting them with plasmids expressing these viral proteins

and a viral RNA reporter gene. These assays found that PB2 protein and viral RNA levels are reduced in knockdown cells (Fislova et al., 2010). Yeast 2-hybrid screening and coimmunoprecipitation assays indicated that the Mason-Pfizer monkey virus p4 domain of the Gag polyprotein interacts with CCT3 (Hong et al., 2001). CCT3 knockdown produced by stably expressed shRNA in N2a (mouse neuroblastoma) cells results in a decrease in rabies virus (RABV) nucleoprotein (N) expression and an 80% decrease in genomic RNA upon infection with rabies virus (Zhang et al., 2013). siRNA knockdown of CCT5 in Huh-7 cells produced a 25-35% decrease in hepatitis C virus RNA levels after acute HCV infection, and fresh cells infected with the supernatant from infected knockdown cells demonstrated as much as a 4-fold reduction of core viral protein levels (Inoue et al., 2011). CCT5 was found to interact with Epstein-Barr virus protein EBNA-3 via yeast 2-hybrid assays (Kashuba et al., 1999). Yeast 2-hybrid screening also detected an *in vitro* interaction between CCT3 and the p6 domain of the HIV-1 Gag protein (Hong et al., 2001), and yeast CCT4 was found to interact *in vitro* with HIV-1 integrase (Parissi et al., 2001).

When each of the eight subunits are expressed in *E. coli* individually, only CCT4 and CCT5 form TRiC complex-like double ring structures. These complexes formed by CCT4 and CCT5 alone were assayed for chaperonin activity using a luciferase refolding assay and an Hyd-Crys aggregation suppression assay. The luciferase refolding assay measures the ability of the chaperone to mediate the *in vitro* refolding of denatured luciferase, with luciferase expression as the readout for successful refolding. The Hyd-Crys assay is used to test the ability of the chaperonin to prevent the aggregation and precipitation of a human protein. Partially folded Hyd-Crys forms aggregates and the suppression of these aggregates upon interaction of unfolded Hyd-Crys with the chaperonins is used as a readout for refolding activity. The CCT4 and CCT5

complexes had comparable chaperonin activity to the TRiC complex according to these assays (Sergeeva et al., 2013).

### **Protein targets folded by TRiC/CCT**

The TRiC/CCT complex functions in folding many substrates, notably including actin and tubulin (Frydman et al., 1992; Gao et al., 1992; Gao et al., 1993; Melki et al., 1993; Rommelaere et al., 1993; Sternlicht et al., 1993; Yaffe et al., 1992). Several mutations in TRiC subunits have been associated with cytoskeletal defects. Chen et al. (1994) characterized CCT2 and CCT3 mutations in yeast that produced defects in microtubule assembly *in vivo* after a 16 hour incubation at 14°C. Interactions between the TRiC/CCT complex and other cytoskeletal proteins, such as cofilin, have also been reported (Dunn et al., 2001).

In pulse-chase experiments that labeled newly synthesized proteins, approximately 9-15% of labeled proteins visualized by two dimensional gel electrophoresis coimmunoprecipitated with the TRiC complex. Most of these proteins were of molecular weight between 30 and 60 kDa, though some were between 100 and 120 kDa (Thulasiraman et al., 1999). Over a hundred have been identified, with examples listed in Table 1.3. However, there is disagreement as to whether this is an accurate representation of TRiC/CCT substrates *in vivo* (Willison, 2018).

**Table 1.3: Select Proteins that Interact with the TRiC/CCT Complex**

<b>Functions</b>	<b>Selected Proteins (Yam et al., 2008)</b>
<b>Cytoskeletal</b>	$\alpha$ actin, $\beta$ actin, $\alpha$ tubulin, $\beta$ tubulin, cofilin, kinesin-related protein, myosin, ARP2
<b>Cell cycle</b>	cyclin E1, CDC10, CDC15, CDC20, CDC28, CDC55, CKA1
<b>DNA/chromatin</b>	Histone deacetylase 3, HOS2, DNA helicase Q1, RAD28
<b>Viral</b>	Hepatitis B virus capsid protein, EBNA1, M-PMV p4
<b>Other</b>	GAPDH, luciferase

## **Conclusion**

Both MID1IP1 and CCT2 have the potential to function in HIV-1 infection in microtubule-dependent mechanisms during the early post-fusion stage of the life cycle. Assaying for the effect of these proteins has the potential to identify host factors necessary for infection and to clarify the less understood mechanisms of this stage of the life cycle.

In chapter two, we test the hypothesis that MID1IP1 is necessary for the entry stage of the viral life cycle through a mechanism dependent on an unknown microtubule-related function. As two screens have implicated MID1IP1 in retroviral infection, we assayed for the effects of MID1IP1 depletion on the HIV-1 life cycle. The functions of MID1IP1 with respect to microtubules remain unexplored. We assay for potential effects on microtubule stability in chapter three.

In chapter four, we examine the potential of another gene, CCT2, to serve as a host factor involved in HIV-1 replication. We assay for the effect of CCT2 on the entry stage of the HIV-1 life cycle.

## **Chapter 2: MID1IP1 Does Not Function in the Early Phase of the HIV-1 Viral Life Cycle through Viral Gene Expression**

MID1IP1 is one of hundreds of proteins implicated through large-scale screens as a potential host factor facilitating HIV-1 infection (Brass et al., 2008; König et al., 2008; Zhou et al., 2008; Zhu et al., 2014). Adding strength to the possibility of a potential effect is the fact that the gene was recovered in two independent screens and that one of those screens found an effect in experiments testing for both HIV-1 and MLV infectivity. Brass et al. (2008) conducted an siRNA screen in TZM-bl cells which were infected with replication-competent HIV-III<sub>B</sub>, staining for capsid protein 48 hours post-infection as a readout. MID1IP1 was one of 273 hits that reduced the percentage of cells that stained for capsid by at least two standard deviations and was confirmed to do so in a follow-up experiment. 33% of cells stained for capsid after the knockdown of MID1IP1 relative to controls (set at 100%).

König et al. (2008) conducted an siRNA screen in 293T cells and infected cells with a single-cycle HIV-1 reporter virus. MID1IP1 was one of 295 genes whose knockdown inhibited infection by at least 45%. siRNA knockdowns of MID1IP1 using 2 individual siRNAs produced an average 10 fold reduction in reporter expression relative to the control, and similar results (corresponding to an average 9 fold reduction in reporter expression) were obtained with an MLV reporter virus. The use of a single-cycle reporter virus necessitates that the effect on the viral life cycle must have occurred between the time of initial entry of virus into the cell and translation of viral mRNAs derived from the provirus.

Very little is understood about the functions of MID1IP1. It was identified as an interacting protein for the E3 ligase MID1 and participates in lipogenesis. There is evidence

regarding the intracellular localization of this protein, specifically at the site of microtubules, and MID1 is responsible for this localization. The only functional evidence with respect to the cytoskeleton comes from its homolog in zebrafish, which has cytoskeletal functions during cytokinesis in development.

If MID1IP1 was demonstrated to have both involvement in the HIV-1 life cycle and a microtubule-related function, a reasonable hypothesis would be that MID1IP1 affects viral infection by altering microtubule function, given that microtubules are already known to participate in infection (Sabo et al., 2013). That would place the role of MID1IP1 in the HIV-1 life cycle during the early post-fusion stage, after the capsid is released into the cytoplasm but before nuclear entry. This is one of the least understood stages of the viral life cycle. In this chapter, siRNA and CRISPR/Cas9 experiments are used to determine that MID1IP1 does not function in the entry stage of the HIV-1 life cycle at its endogenous levels of expression in human 293 cells.

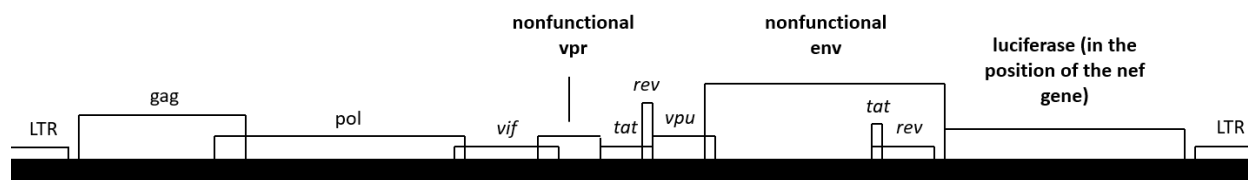
### **siRNA Knockdowns of MID1IP1 in TE671 Cells**

In order to test the results of the large-scale screens implicating MID1IP1 in the entry stages of HIV-1 infection, we conducted several initial RNAi experiments. TE671 cells were transfected with siRNA pools targeting MID1IP1, and were then infected with a single-cycle VSV-G pseudotyped HIV-1 reporter virus. These single-cycle reporter viruses were produced by transfection of 293T cells with plasmids expressing the viral machinery and harvest of the supernatant 48 hours later. These plasmids include a viral vector genome (pNL4-3.Luc.R-.E-, Figure 2.1) in which a reporter gene (in our case, luciferase) is expressed after successful delivery of the viral DNA into an infected cell. The vector does not encode the viral proteins that

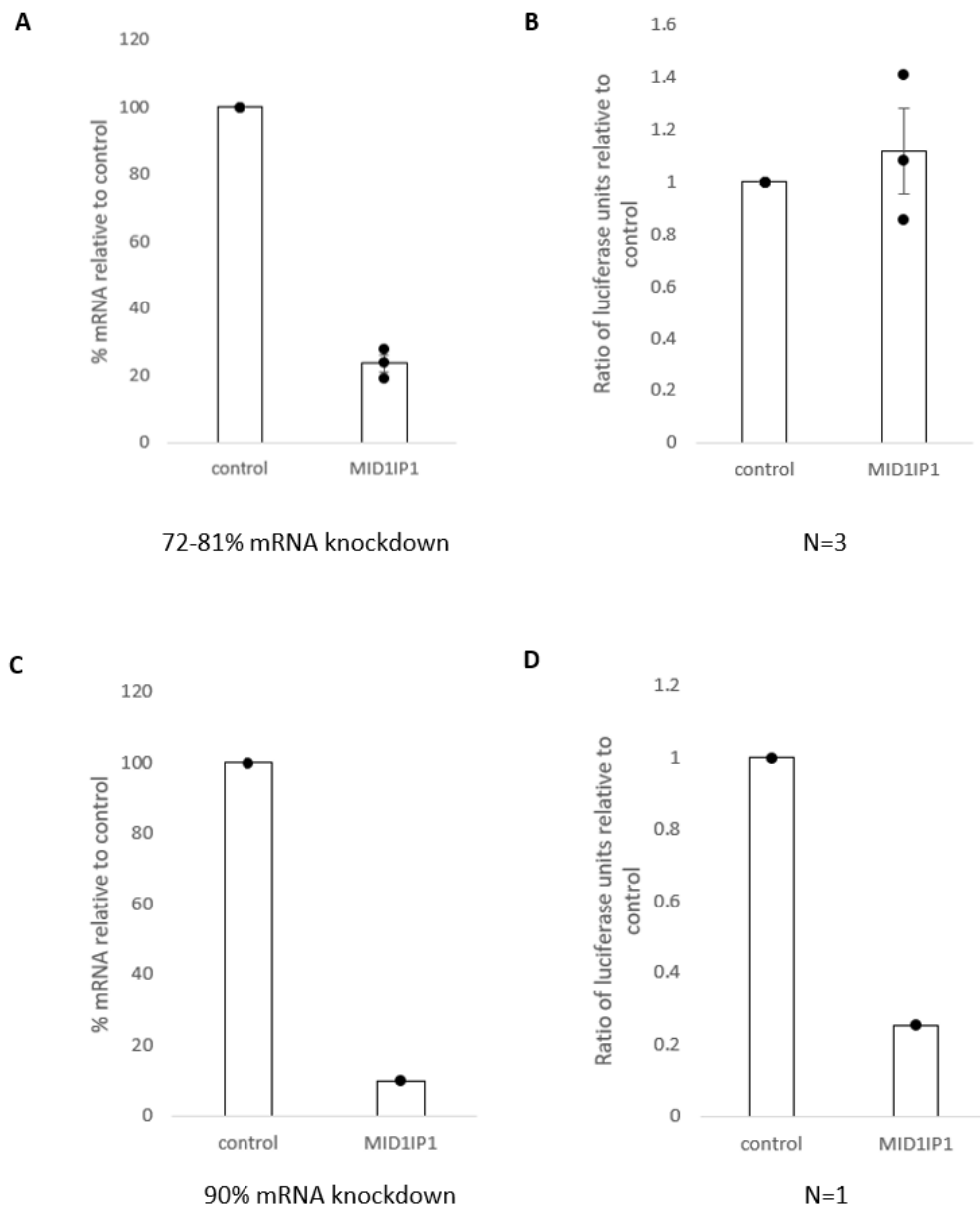
would be needed to produce new progeny infectious virus. VSV-G or other envelope proteins are expressed from a separate plasmid.

The vector is delivered by the viral proteins that are expressed by the producer cell from the transfected DNAs, and cannot replicate after this delivery process. These reporter viruses readout the events of entry, reverse transcription, integration into the host genome, transcription of the provirus, and translation of the viral reporter mRNAs to ultimately induce luciferase expression. HIV-1 envelopes are replaced in these experiments with envelope proteins from other viruses in a process known as pseudotyping. VSV-G is a common choice due to its high stability and high efficiency, and because it expands the tropism of reporter viruses beyond the natural cellular targets for HIV-1 infection, allowing them to infect virtually all mammalian cells.

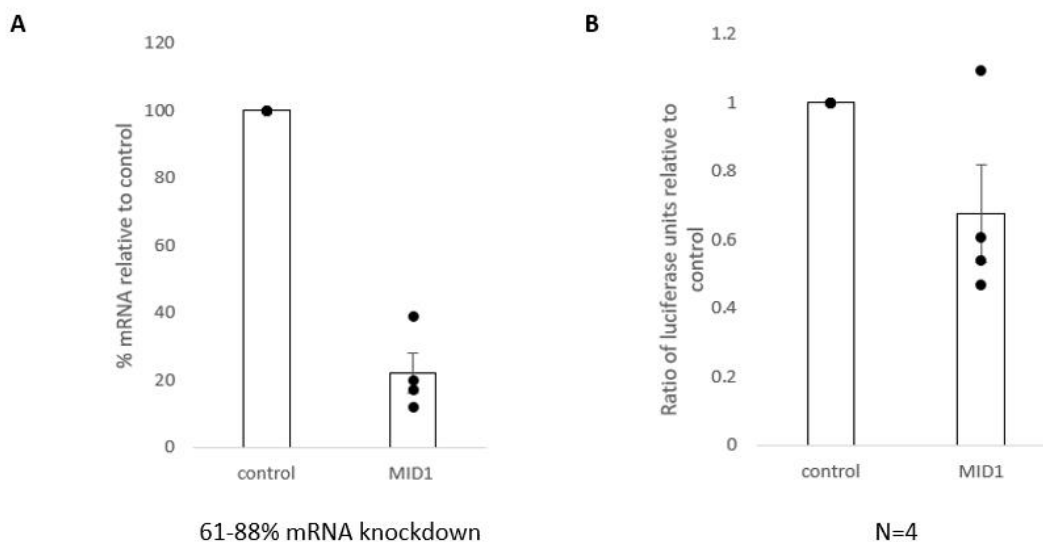
To knockdown expression of MID1IP1, we transfected TE671 cells with 150 pmol of the MID1IP1 siRNA pools, two times, 24 hours apart. We then infected the cells with reporter virus diluted in 200  $\mu$ L media. After 48 hrs, lysates were prepared and luciferase assays were performed by the Luciferase Assay System (Promega) protocol. As endogenous levels of MID1IP1 protein were not detected in Western blots with any of several available MID1IP1 antibodies (though these antibodies were able to detect overexpression levels of MID1IP1), we used mRNA levels to assess the efficiency of the knockdown. RNA was isolated at the time of infection by the RNeasy Mini Kit (Qiagen), and the levels of mRNA were determined by qRT-PCR using SYBR Green to produce florescent signal upon binding to ds DNA. The number of amplification cycles at which the florescent signal overcame the background threshold were measured and normalized to the cellular GAPDH mRNA. It should be noted that mRNA levels do not assay for the effects of the turnover of MID1IP1 proteins made prior to the knockdown.



**Figure 2.1: Representation of the HIV-1 machinery plus luciferase reporter encoded in pNL4-3.Luc.R-E.** Differences from wild-type HIV-1 pNL4-3 viral clone are bolded. Luciferase is cloned in the position of the *nef* gene, while both *vpr* and *env* possess frameshift mutations rendering them nonfunctional. Envelope is required for the production of infectious single-cycle reporter viruses and an envelope protein must be expressed from another plasmid to pseudotype these particles. Vpr and Nef are not required for *in vitro* infection.



**Figure 2.2: siRNA knockdown of MID1IP1 in TE671 cells.** A) The percentage of MID1IP1 mRNA in cells transfected with control and MID1IP1 siRNA pools where dots signify individual experiments. Error bars represent standard error of the mean across three experiments conducted in duplicate. B) The ratio of luciferase units relative to control cells when transfected cells are infected with a single-cycle VSV-G pseudotyped HIV-1 luciferase reporter virus where dots signify individual experiments. Error bars represent standard error of the mean across three experiments conducted in duplicate or triplicate. C) The percentage of MID1IP1 mRNA in cells transfected with control and MID1IP1 siRNA pools in a single experiment which produced a 90% mRNA knockdown. D) The ratio of luciferase units relative to control cells when transfected cells are infected with a single-cycle VSV-G pseudotyped HIV-1 luciferase reporter virus in a single experiment which produced a 90% mRNA knockdown.



**Figure 2.3: siRNA knockdown of MID1 does not produce an effect on luciferase reporter expression in TE671 cells.** A) The percentage of MID1 mRNA in cells transfected with control and MID1 siRNA pools where dots signify individual experiments. Error bars represent standard error of the mean across four experiments conducted in duplicate. B) The ratio of luciferase units relative to control cells when transfected cells are infected with a single-cycle VSV-G pseudotyped HIV-1 luciferase reporter virus where dots signify individual experiments. Error bars represent standard error of the mean across four experiments conducted in duplicate or triplicate.

The knockdown of MID1IP1 in our experiments resulted in no significant change in the susceptibility of TE671 cells to transduction by our reporter virus. We conducted a total of four tests. Three of our experiments produced a 72-81% reduction in mRNA levels of MID1IP1, and the luciferase reporter levels in these experiments exhibited no difference between control and knockdown cells (Figure 2.2A-B). However, one of the four experiments produced a 90% reduction in mRNA levels of MID1IP1, and this stronger knockdown did result in a five-fold decrease in luciferase expression after infection with the reporter virus (Figure 2.2C-D). Multiple dilutions of virus were performed per experiment. The dilution at which one repeat correlated to a potential effect is shown.

As the single repeat of this experiment with the greatest knockdown was the only one which demonstrated an effect on the reporter, we could not conclude whether this result was simply an outlier or whether a strong knockdown of approximately 90% was required to cause an effect. We were unable to achieve a similar knockdown efficiency in any of multiple repeated experiments. Thus these preliminary siRNA knockdown experiments in TE671 cells suggested but did not prove that MID1IP1 might have a modest effect on HIV-1 infection.

### **siRNA Knockdown of MID1 Does Not Affect HIV-1 Infectivity in TE671 Cells**

MID1 is one of the few known interacting partners for MID1IP1 and is responsible for its localization to microtubules. If MID1IP1 were to function in the HIV-1 viral life cycle, it is plausible that the mechanism might be dependent on its localization to microtubules (and thus MID1). There might also be as yet unknown aspects of the interaction between MID1 and MID1IP1, which may or may not involve microtubules or microtubule stability. MID1 has not previously been connected to HIV-1, and none of the screens which implicated hundreds of other

potential host factors found evidence for an effect from MID1. However, there are several reasons why any given host factor might not be picked up in large-scale genetic screening experiments. The knockdown might not be sufficiently efficient to produce an effect; the gene might be excluded from the knockdown library due to an effect on cell viability; or the statistical cutoffs used in the particular screen may have been too stringent to detect a subtle effect. Additionally, screens monitoring physical interactions such as yeast-2-hybrid assays may not identify host proteins which do not directly interact with viral proteins but nevertheless do participate in infection.

We conducted experiments using siRNA pools to knockdown MID1. Cells were transfected with siRNAs and then infected with single-cycle VSV-G pseudotyped HIV-1 luciferase reporter virus. The cells were lysed and the lysates were assayed for luciferase expression 48 hours post-infection. The extent of the knockdown was assessed by extracting RNA at the time of infection and qRT-PCR following the same procedure as for the MID1IP1 siRNA knockdowns. Four experiments each produced between a 61-88% mRNA knockdown, but we measured no significant effects on reporter virus expression – less than two-fold reduction in infectivity (Figure 2.3). While we cannot completely rule out the possibility of a small role for MID1, there is no evidence suggesting MID1 plays a significant role in HIV-1 infection.

**A** MID1IP1 (wt)

**Met Met** QICDTYNQKHSLFNA **Met** NRFIVAVNN **Met** DQTV **Met** VPSLLRDVPLADPGLDNDVGVVEVGGSGGCLER  
 ERTPPVPDSGSANGSFFAPSRD **Met** YSHYVLLKSI RNDIEWGLVHQPPPPAGSEEGSAWKS KDILVDLGHLEG  
 ADAGEEDLEQQFH YHLRGLHTVLSKLTRKANILTNRYKQEIGFGN WGH **Stop**

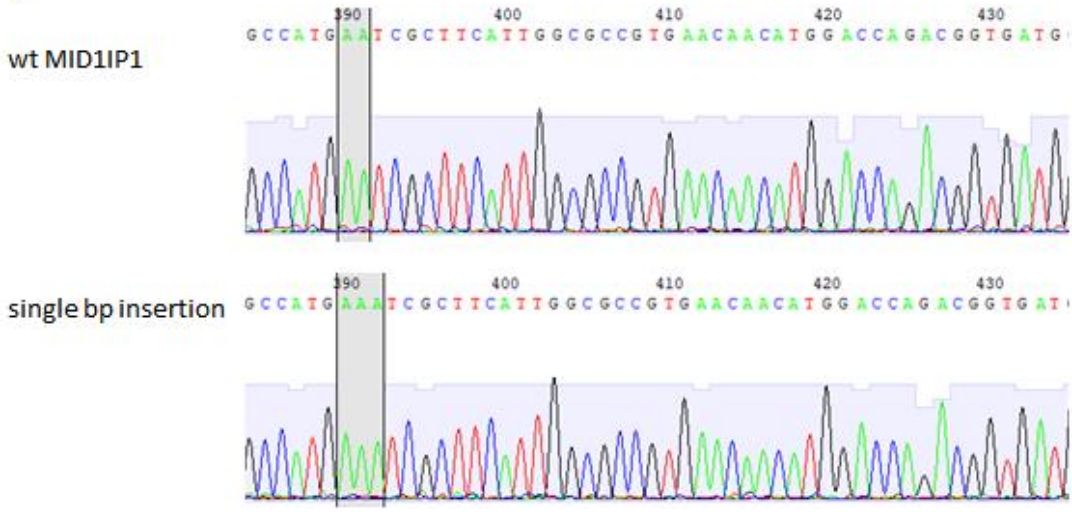
atgatgcaaatctgcgacacctacaaccagaagcactcgctctttaacgccatgaatcgcttcattggcgccgtgaacaacatggaccagcggatggtgccagctt  
 gctgcgacgctgcccctggctgaccccgggttagacaacgatgttggcgtggagtaggcggcagtgccgctgctggaggagcgcacccccagctcccgactcg  
 ggaagcgcaatggcagctttttcgcctctcgggacatgtacagccactacgtcttcaagtcaccccaacacatcagtggggggctctgaccagccgct  
 ccaccgctgggagcaggaggcagtgctggaagtccaaggacatcctggtgacctgggccacttggagggtgaggacccggcgaagaagacctggaacagc  
 agttccactaccactgctcgggctgcactgtgctctgaaactcagcgcgaaagccaacatcctcactaacagatacaagcaggagatcggcttccgaattgggg  
 cactga

**B**

MID1IP1 (wt)	* * * * *	cactcgctctttaacgccatga-atcgcttcattggcgccgtgaacaacatg
CRISPR M1		
	* * * * *	CACTCGCTCTTTAACGCCATGAAATCGCTTCATTGGCGCCGTGAACAACATG
MID1IP1 (wt)	* * * * *	cactcgctctttaacgccatga-a---tcgcttcattggcgccgtgaacaacatg
CRISPR M2		
	* * * * *	CACTCGCTCTTTAACGCCATGAA---TCGCTTCATTGGCGCCGTGAACAACATG
MID1IP1 (wt)	* * * * *	cactcgctctttaacgccatga-atcgcttcattggcgccgtgaacaacatg
CRISPR M3		
	* * * * *	CACTCGCTCTTTAACGCCATGAAATCGCTTCATTGGCGCCGTGAACAACATG

197 bp deletion

**C**



**Figure 2.4: Genomic DNA in 293 CRISPR cells for MID1IP1.** A) The amino acid and DNA coding sequences for MID1IP1, with the DNA sequence targeted by the CRISPR system highlighted in red. The sequence for the single guide RNA was generated using the design tools provided by Shalem et al. (2014) at [tools.genome-engineering.org](http://tools.genome-engineering.org). B) The DNA sequences of mutant alleles in M1, M2, and M3 cells aligned with wild-type *MID1IP1* DNA. The sequence targeted by the guide RNA is boxed, while mutations are highlighted. All sequences from cell line M1 contained the same mutant allele. Sequences from cell line M2 contained two mutant alleles, while sequences from cell line M3 contained three mutant alleles. Alignments were made using the ApE - A Plasmid Editor software. C) Sequences from wild-type and mutant *MID1IP1* containing a single base pair insertion. The two peaks indicating the lack of an insertion are highlighted for wild-type *MID1IP1*, while the three peaks indicating the presence of an insertion are highlighted for mutant *MID1IP1*. Data was visualized using the ApE - A Plasmid Editor software, from which images were obtained.

### **Mutant *MIDIIP1* in 293 CRISPR Cells**

The lack of consistency in the extent of mRNA knockdowns in RNAi experiments, in situations where an unknown threshold of knockdown must be achieved to produce an effect, makes it difficult to assess the lack of a phenotype. To produce a more consistent effect on *MIDIIP1* levels than could be achieved using siRNA knockdowns, the CRISPR/Cas9 system was used to generate frameshift mutations in *MIDIIP1* in 293 cells. As all known splicing variants of *MIDIIP1* are translated to form the same full-length protein and the amino acid sequence for that protein is encoded by a single exon, we used the design tools provided by Shalem et al. (2014) to create a single guide RNA targeting this exon (Figure 2.4A).

Cells were infected with lentiviral vectors carrying the Cas9 nuclease and a single guide RNA sequence that targets Cas9 to *MIDIIP1*. Control cell lines N1 and N2 were generated by infection with the identical lentiviral particles containing the CRISPR system and antibiotic selection as used for the generation of the *MIDIIP1* targeted cell lines, except the encoded guide RNA did not target any genomic DNA. Integrations of the construct were selected for by antibiotic resistance, and individual surviving colonies were picked and expanded. Endogenous levels of *MIDIIP1* protein in 293 cells could not be detected by Western blots. In order to distinguish which of the cell lines derived from clones have loss-of-function mutations in all *MIDIIP1* alleles, their genomic DNA was assayed for frameshift mutations.

The 293 cell line is an aneuploid line, with more than two copies of many chromosomes. The karyotype is not highly stable, and cells in the population may have varying copy numbers of a given gene (Bylund et al., 2004; Lin et al., 2014). Thus, it is important to probe the putatively mutant clonal cell lines for the collection of alleles that they may contain. The integrated CRISPR/Cas9 system in these cell lines targets *MIDIIP1* for induction of double-

stranded breaks, which may then be repaired with or without mutations. As such, the CRISPR cell lines selected for by antibiotic resistance might have all wild-type copies of MID1IP1, a mixture of wild-type and mutant copies, or multiple mutant copies. In order to distinguish between these, DNA was extracted from the CRISPR cell lines and the CRISPR targeting site was amplified using PCR. The PCR products were ligated into plasmids, and bacterial transformation was used to amplify the plasmids. After the bacteria were plated such that individual colonies should contain plasmids carrying the same amplified PCR product, five or six colonies were picked per cell line and plasmids were extracted for sequencing.

As expected, the DNA sequences revealed some cell lines with no mutations in the target sequence, some with a mixture of wild-type and mutant alleles, and some with multiple alleles with various mutations. Both in frame and out of frame mutations occurred. Three cell lines were identified for which all of the obtained sequence data had frameshift mutations. For one mutant cell line (M1), all six cloned sequences had the same single base pair insertion. The mutant cell line M2 yielded clones with two different frameshift mutations, one of which was a single base pair insertion and the other of which was a four base pair deletion. Mutant cell line M3 yielded three different mutant alleles which produced frameshift mutations. These were a single base pair insertion, a 20 base pair deletion, and a 197 base pair deletion (Figure 2.4B).

**A** MID1IP1 (wt)

**Met Met** QICDTYNQKHSLFNA **Met** NRFIGAVNN **Met** DQTV **Met** VPSLLRDVPLADPGLDNDVGVVEVGGSGGCLE  
ERTPPVPDSSANGSFFAPSRD **Met** YSHYVLLKSIRNDIEWGVLHQPPPPAGSEEGSAWKS KDILVDLGHLEG  
ADAGEEDLEQQFH YHLRGLHTVLSKLTRKANILTNR YKQEIGFGN WGH **Stop**

**B** CRISPR M1

**Met Met** QICDTYNQKHSLFNA **Met** KSLHWRREQHGPDGDGAQLAARRAPG **Stop** P RVRQRCWRGGRRQWRLP  
GGAHAPSPRLGKRQWQLFRALSGHVQPLRASQVHPQRHRVGGPAPAASTGWERGGQCLEVQGHPPGGP  
LGCGRRRRRPGTAVPLPPARAAHCALETHAQSQH PH **Stop Q** IQAGDRLRQLG PL

**C** CRISPR M2

**Met Met** QICDTYNQKHSLFNA **Met** KSLHWRREQHGPDGDGAQLAARRAPG **Stop** P RVRQRCWRGGRRQWRLP  
GGAHAPSPRLGKRQWQLFRALSGHVQPLRASQVHPQRHRVGGPAPAASTGWERGGQCLEVQGHPPGGP  
LGCGRRRRRPGTAVPLPPARAAHCALETHAQSQH PH **Stop Q** IQAGDRLRQLG PL

**Met Met** QICDTYNQKHSLFNA **Met** KSLHWRREQHGPDGDGAQLAARRAPG **Stop** P RVRQRCWRGGRRQWRLP  
P GGAHAPSPRLGKRQWQLFRALSGHVQPLRASQVHPQRHRVGGPAPAASTGWERGGQCLEVQGHPPGGP  
PLGGCGRRRRRPGTAVPLPPARAAHCALETHAQSQH PH **Stop Q** IQAGDRLRQLG PL

**D** CRISPR M3

**Met Met** QICDTYNQKHSLFNA **Met** KSLHWRREQHGPDGDGAQLAARRAPG **Stop** P RVRQRCWRGGRRQWRLP  
GGAHAPSPRLGKRQWQLFRALSGHVQPLRASQVHPQRHRVGGPAPAASTGWERGGQCLEVQGHPPGGP  
LGCGRRRRRPGTAVPLPPARAAHCALETHAQSQH PH **Stop Q** IQAGDRLRQLG PL

**Met Met** QICDTYNQKHSLHWRREQHGPDGDGAQLAARRAPG **Stop** P RVRQRCWRGGRRQWRLP GGAHAPSP  
RLGKRQWQLFRALSGHVQPLRASQVHPQRHRVGGPAPAASTGWERGGQCLEVQGHPPGGP PLGGCGRRR  
RRPGTAVPLPPARAAHCALETHAQSQH PH **Stop Q** IQAGDRLRQLG PL

**Met Met** QICDTYNQKHSLFNALSGHVQPLRASQVHPQRHRVGGPAPAASTGWERGGQCLEVQGHPPGGP  
LGCGRRRRRPGTAVPLPPARAAHCALETHAQSQH PH **Stop Q** IQAGDRLRQLG PL

**Figure 2.5: Coding sequences for MID1IP1 CRISPR mutants produce frameshift mutations and premature stop codons.** A) The wild-type amino acid sequence for MID1IP1. B) The amino acid sequence for the single mutant allele present in cell line M1, which contains a frameshift mutation and premature stop codon. C) The amino acid sequence for the two mutant alleles present in cell line M2, which both contain frameshift mutations and premature stop codons. D) The amino acid sequence for the three mutant alleles present in cell line M3, two of which contain frameshift mutations and premature stop codons. The third contains a large deletion and frameshift mutation. Sequences were translated using ExpASy ([web.expasy.org/translate](http://web.expasy.org/translate)).

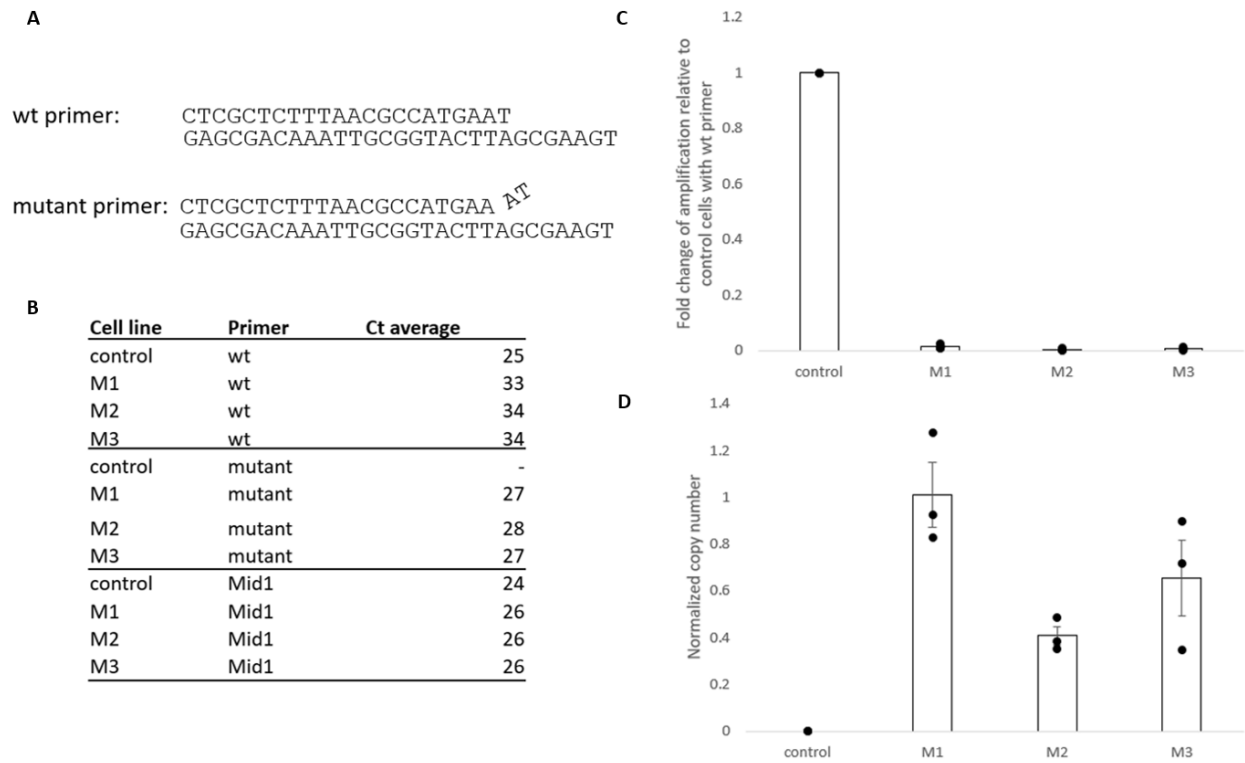
Curiously, the single base pair insertion present in all three cell lines is the exact same mutation--an adenine residue inserted between two other adenine residues. This raised the question of whether this a sequencing error in which the software analyzing the raw sequence data has mistaken two peaks for three. Examining the sequencing data confirms that each incidence of this single base pair mutation is accompanied by three clear peaks, distinct from the two peaks present in the wild-type sequence (Figure 2.4C). The sequence data suggest that this is a legitimate insertion, and potentially a common mistake for the repair machinery to make when repairing this double-stranded break.

The protein predicted by conceptual translation of the mutant alleles with frameshift mutations were compared with the wild-type sequence for *MIDIIP1* (Figure 2.5). The allele with the single base pair insertion which is present in all three mutant cell lines produces a premature stop codon, as do the 4 and 20 bp deletion mutations in the M2 and M3 cell lines. The 197 base pair deletion mutation produces a frameshift mutation following a large deletion towards the beginning of the sequence.

As substitutions and other non-frameshifting mutations might produce a protein still capable of most or all of its functions, we chose only the three CRISPR cell lines in which all of the sequenced target DNA contained frameshift mutations for further investigation. These lines were likely to contain only mutant alleles, but there remained the possibility that they harbored a wild-type allele in one chromosome of their aneuploid genomes. These three cell lines were then assayed for the possible presence of a wild-type copy of *MIDIIP1* in their DNA. qPCR was conducted using primers capable of amplifying the CRISPR targeted region of wild-type *MIDIIP1*. As all three cell lines contain a single base pair insertion, a primer was designed such that it would selectively amplify wild-type *MIDIIP1* and not the mutant sequence. Another

primer was designed to be capable of amplifying the single base pair mutation but not the wild-type sequence (Figure 2.6A). This would allow us to determine whether these primers are capable of distinguishing between the wild-type sequence and the single base pair mutation.

The DNA of each of the mutant cell lines and a control cell line in which the *MID1IP1* gene was not targeted by the CRISPR system were amplified using both sets of primers in qPCR experiments. The readout of the PCR DNA products were followed over the course of the reaction cycles, and the cycle count to exceed the background threshold was determined. The amplification products generated using the wild-type primer was detected almost 10 cycles later in mutant than in the control cells, corresponding to an average fold difference in the levels of amplifiable wild-type sequences of 65, 195, and 141 fold lower for M1, M2, and M3 respectively relative to the control (Figure 2.6B-C). These are far below single-copy levels, essentially background in the assay. These numbers demonstrate that virtually no wild-type *MID1IP1* allele was amplified from the *MID1IP1* CRISPR cell lines in these experiments. This confirms that the wild type primer does distinguish between wild-type DNA and the single base pair insertion, and indicates that no wild-type copy of *MID1IP1* is present in these mutant cell lines. Even a single copy of the wild-type allele in these clonal lines would have been readily detected.



**Figure 2.6: Three 293 CRISPR cell lines do not contain a wild-type copy of *MIDI1P1*.** A) Sequences for the wild-type mutant forward primers designed to distinguish between wild-type *MIDI1P1* and the single base pair insertion mutation aligned to the wild-type *MIDI1P1* sequence. The overlap of the primers with the CRISPR-targeted site are highlighted in red. B) The average Cq values across three qPCR experiments for control and *MIDI1P1*-targeted CRISPR cells that have been amplified with wild-type or mutant primers. Primers amplifying *MIDI1* serve as a control between cell lines. C) The fold change of product amplified using wild-type primers relative to the control cell line. D) The fold change of product amplified using mutant primers relative to the products amplified using wild-type primers in the control cell line. Error bars represent standard error of the mean across three experiments performed in duplicate.

No amplification product was detected by using the mutant primer to amplify DNA from the control cell line, indicating that this primer is specific for the single base pair insertion and does not amplify wild-type MID1IP1 (Figure 2.6B). We could also normalize the detection of the mutant allele with the mutant primer in control and mutant cells to the detection of the wild-type allele with the wild-type primer in control cells. This would provide a measure of the copy number relative to a cell line in which all alleles are amplified by the primers. The amplification by the mutant primer in mutant cells yielded nearly identical levels of product (1 fold, 2.4 fold, and 1.5 fold less product in M1, M2, and M3 cell lines respectively) as the amplification using wild-type primer in control cells (Figure 2.6D). These results were consistent with at least one copy of the mutant allele containing the single base pair insertion being present in each mutant cell line.

### **Reporter Viruses Do Not Indicate an Effect on Infectivity in MID1IP1 Targeted 293 Cells**

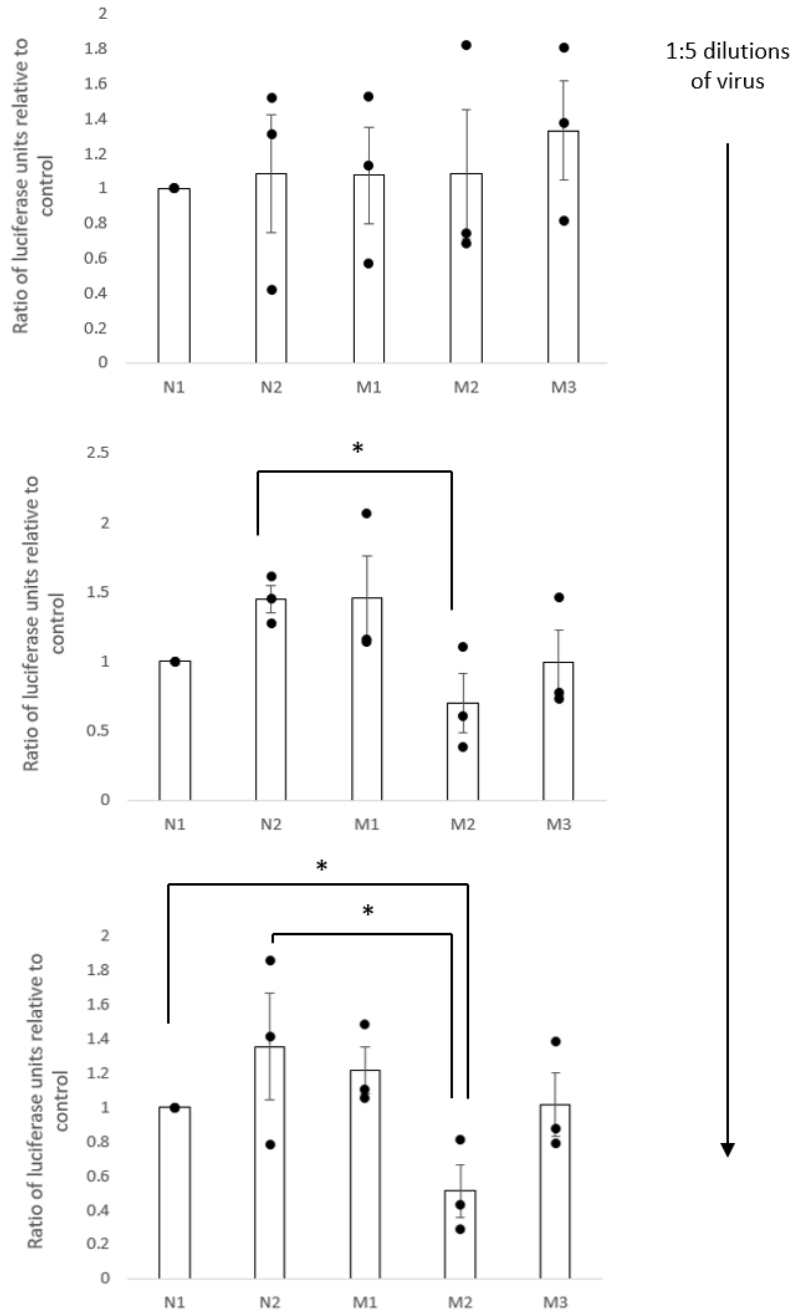
To determine whether the MID1IP1 mutations in our 293 CRISPR KO cell lines affect the entry stages of the retroviral life cycle, cells were infected with single-cycle luciferase reporter viruses. HIV-1 reporter viruses were pseudotyped with VSV-G or amphotropic envelope. We performed infections of the mutant lines, and control lines in parallel.

VSV-G pseudotyped reporter virus infection produced little to no difference in luciferase expression in the three experimental lines relative to the two control cell lines across three dilutions of virus (Figure 2.7). All the lines were readily infected by the vectors. At two different dilutions of virus, the differences in reporter virus expression between M2 and at least one control cell line were found to be barely statistically significant (P values ranging between .03-

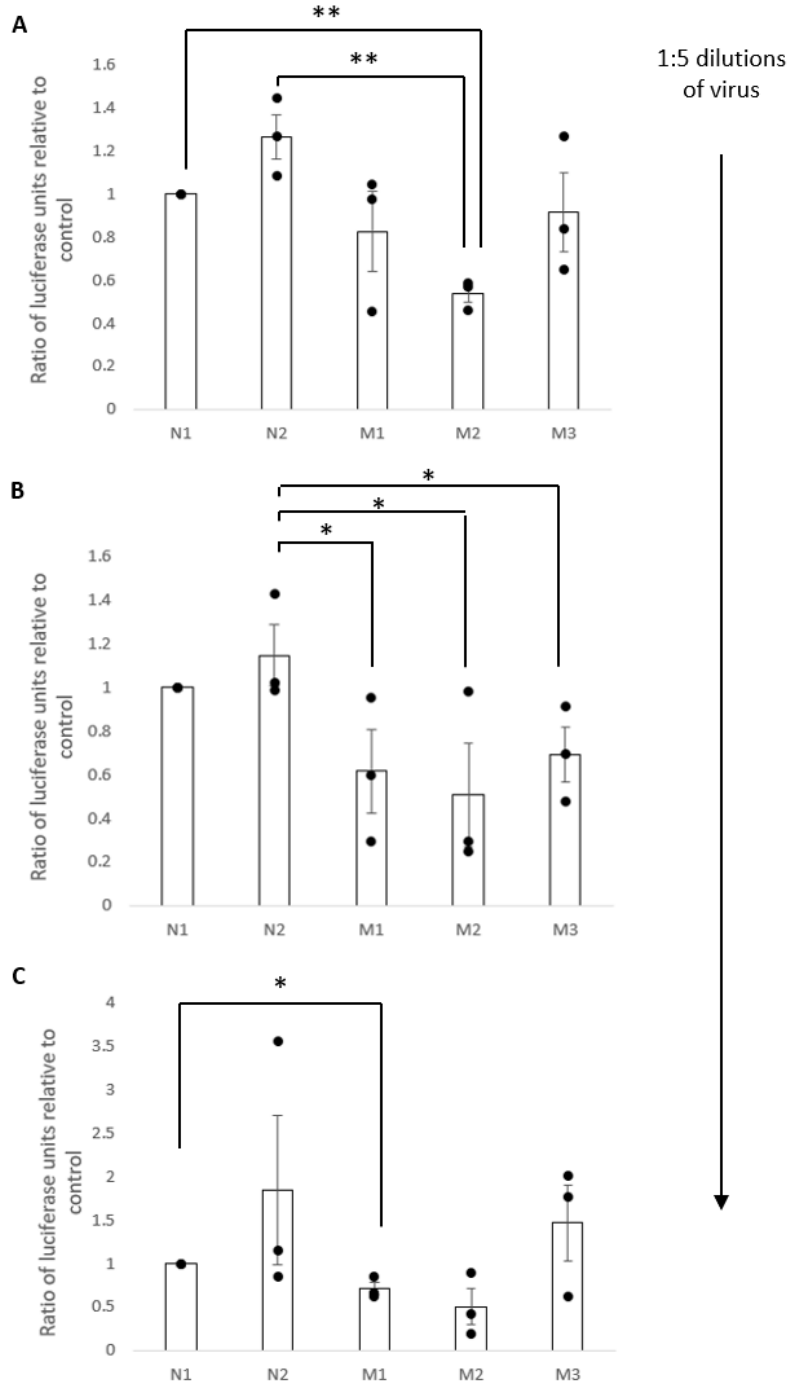
.05). However, the effect was only a two fold change in expression levels (the fold changes of the three statistically significant result ranging from 1.9 to 2.6 fold). Additionally, M2 was the only mutant cell line out of three to exhibit this effect. Between the small size of the effect and its lack of consistency across the three mutant cell lines, we conclude that this result is not due to a lack of MID1IP1 expression. This slight effect may be a real effect that is indicative of a non-MID1IP1 related cause in the M2 cell line.

Phenotypes in a given targeted cell line can be due to off-target effects from the guide RNAs in CRISPR experiments. These CRISPR cell lines were selected for frameshift mutations in *MID1IP1*, but it is possible for the CRISPR/Cas9 system to have targeted other parts of the genome as well. Therefore M2 may have an unknown off-target mutation that has a slight effect on viral infection. Furthermore, the mutant cell lines could have different combinations of distinct mutant alleles that give minor phenotypes. Finally, it is possible that a given clonal line has acquired random mutations anywhere in the genome, and because the lines are aneuploid and not highly stable, a given clone may have a distinctive karyotype due to segregating chromosomes.

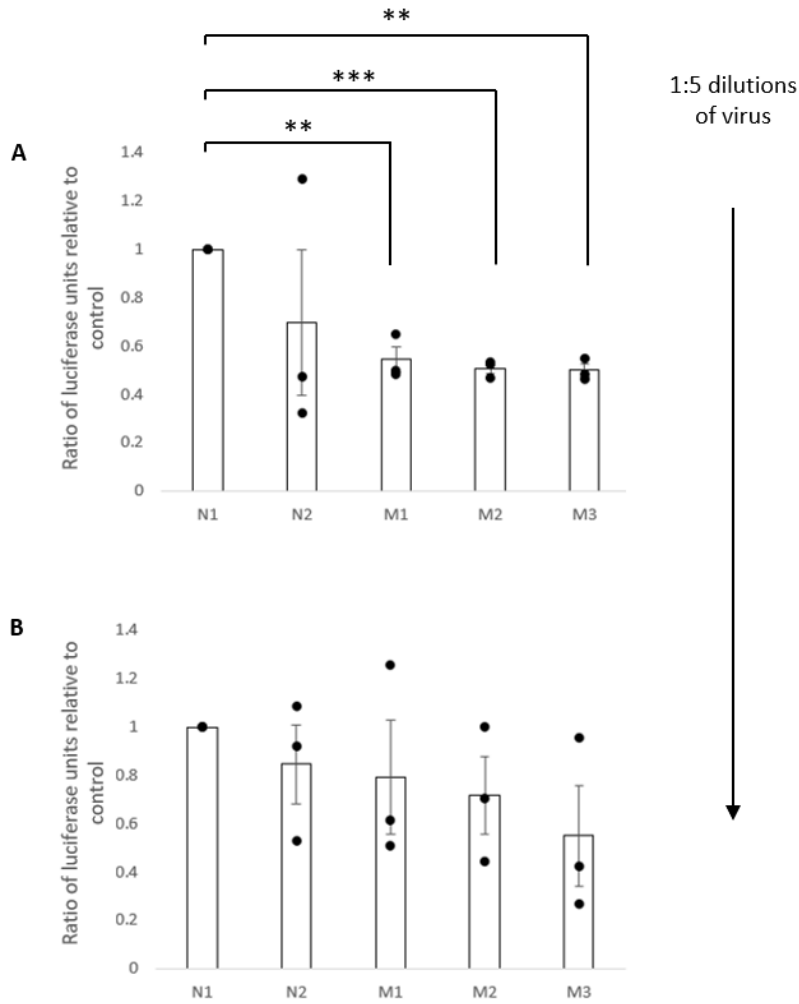
As is true of any engineered lines, the M2 cell line may have general issues of cell physiology or metabolism, rather than any specific effects on the viral life cycle. Because the effect on virus transduction is small, is limited to only one of three mutant cell lines, and is technically significant relative to both controls used in the experiment only at one virus dilution, we do not consider the effect meaningful.



**Figure 2.7: VSV-G pseudotyped HIV-1 reporter viruses do not indicate a change in infectivity between CRISPR and control cells.** A) Ratio of luciferase units relative to N1 control cells when control (N1-N2) and CRISPR (M1-3) cells are infected with a single-cycle VSV-G pseudotyped HIV-1 luciferase reporter virus. B) Ratio of luciferase units relative to N1 control cells when control (N1-N2) and CRISPR (M1-3) cells are infected luciferase reporter virus diluted 1:5 from A. C) Ratio of luciferase units relative to N1 control cells when control (N1-N2) and CRISPR (M1-3) cells are infected luciferase reporter virus diluted 1:5 from B. The dots in the data signify individual experiments. Error bars represent standard error of the mean across three experiments conducted in triplicate. Student's t tests were used to determine p values. \* denotes a P value of < 0.05.



**Figure 2.8: Amphotropic pseudotyped HIV-1 reporter viruses do not indicate a change in infectivity between CRISPR and control cells.** A) Ratio of luciferase units relative to N1 control cells when control (N1-N2) and CRISPR (M1-3) cells are infected with a single-cycle Amphotropic pseudotyped HIV-1 luciferase reporter virus. B) Ratio of luciferase units relative to N1 control cells when control (N1-N2) and CRISPR (M1-3) cells are infected luciferase reporter virus diluted 1:5 from A. C) Ratio of luciferase units relative to N1 control cells when control (N1-N2) and CRISPR (M1-3) cells are infected luciferase reporter virus diluted 1:5 from B. The dots in the data signify individual experiments. Error bars represent standard error of the mean across three experiments conducted in triplicate. Student's t tests were used to determine p values. \* denotes a P value of < 0.05. \*\* denotes a P value of < 0.01.



**Figure 2.9: VSV-G pseudotyped MLV reporter viruses do not indicate a change in infectivity between CRISPR and control cells.** A) Ratio of luciferase units relative to N1 control cells when control (N1-N2) and CRISPR (M1-3) cells are infected with a single-cycle VSV-G pseudotyped MLV luciferase reporter virus. B) Ratio of luciferase units relative to N1 control cells when control (N1-N2) and CRISPR (M1-3) cells are infected luciferase reporter virus diluted 1:5 from A. C) Ratio of luciferase units relative to N1 control cells when control (N1-N2) and CRISPR (M1-3) cells are infected luciferase reporter virus diluted 1:5 from B. The dots in the data signify individual experiments. Error bars represent standard error of the mean across three experiments conducted in triplicate. Student's t tests were used to determine p values. \*\* denotes a P value of < 0.01. \*\*\* denotes a P value of < 0.001.

Infections of the KO cell lines with amphotropic pseudotyped HIV-1 luciferase reporter virus produced similar results to the VSV-G pseudotype infections (Figure 2.8). The M1 and M3 lines were comparably sensitive to transduction by these viruses as the controls. The M2 cell line was the only one of the mutant cell lines to have a statistically significant difference from both control cell lines at the highest dilution of the viral reporter assayed. It had P values of .003 relative to N1 and .006 relative to N2, but as with experiments where cells were infected with VSV-G pseudotyped reporters, the effect was only approximately two fold (1.9 fold relative to N1, 2.4 fold relative to N2). At the second highest dilution, all three mutant cells lines had statistically significant differences relative to one control cell line but not the other. At the lowest dilution of viral reporter, one mutant cell line (M1) had a statistically significant change in reporter expression relative to only one of the two control cell lines, but the effect itself was small enough to discount (1.4 fold).

VSV-G and amphotropic pseudotyped HIV-1 reporters differ in their mechanism of entry into the cytoplasm: entry by VSV-G utilizes pH-dependent clathrin-mediated endocytosis (Aiken, 1997; Marsh and Helenius, 2006), while amphotropic envelope utilizes a pH-independent pathway, possibly fusion at the plasma membrane or macropinocytosis (McClure et al., 1990; Rasmussen and Vilhardt, 2015). After release of the viral core into the cytoplasm, the life cycle is thought to progress in the same manner. As in the VSV-G pseudotyped experiments, the M2 cell line was the only mutant cell line to produce a slight yet statistically significant effect relative to both controls at any dilution of the viral reporter. It was also the only cell line to produce a statistically significant effect at more than one dilution. It remains the only candidate among the mutant cell lines for producing a real effect on luciferase expression. The fact that the other two lines show no defect in virus transduction suggests that KO of MID1IP1 is not

responsible. Therefore our data suggest that MID1IP1 does not play a role in the early stages of the HIV-1 life cycle.

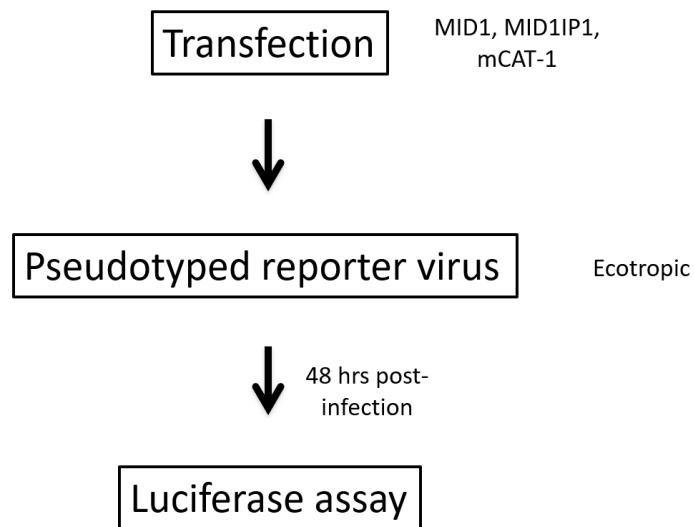
As König et al. (2008) recovered MID1IP1 as a gene affecting transduction in a parallel screen for genes affecting MLV infection as well as HIV-1 infection in 293T cells, we tested the susceptibility of these 293 mutant cell lines to VSV-G pseudotyped MLV luciferase reporter viruses (Figure 2.9). It should be noted that MLV naturally infects mouse cells rather than human cells. Mutant and control cells were infected with various dilutions of the virus preparations, and lysates were assayed for luciferase at 48 h post infection. At the highest dilution of viral reporter, all three mutant cell lines had a slight (approximately two fold) but statistically significant (P values of .007, .0009, and .001 for cell lines M1, M2, and M3 respectively) reduction in luciferase expression relative to one control cell line (N1), but not the other (N2). At a 1:5 dilution of viral reporter, none of the mutant cell lines showed a statistically significant difference from the control lines in susceptibility. The data do not indicate that MID1IP1 participates significantly in the MLV life cycle.

Though MID1IP1 has been implicated as a potential host factor contributing to HIV-1 infection in two screens and MLV infection in one screen, our data does not support such a role for the protein. At least in 293 cells, CRISPR-induced mutations which should produce a loss-of-function phenotype do not lead to any consistent changes in HIV-1 or MLV reporter virus expression upon infection of these mutant cell lines. Thus the hypothesis that MID1IP1 might function in the early post-fusion stage of the HIV-1 life cycle is not supported by the data in this chapter. We conclude that the data does not support a role in 293 cells for MID1IP1 in the HIV-1 or MLV life cycle early after infection.

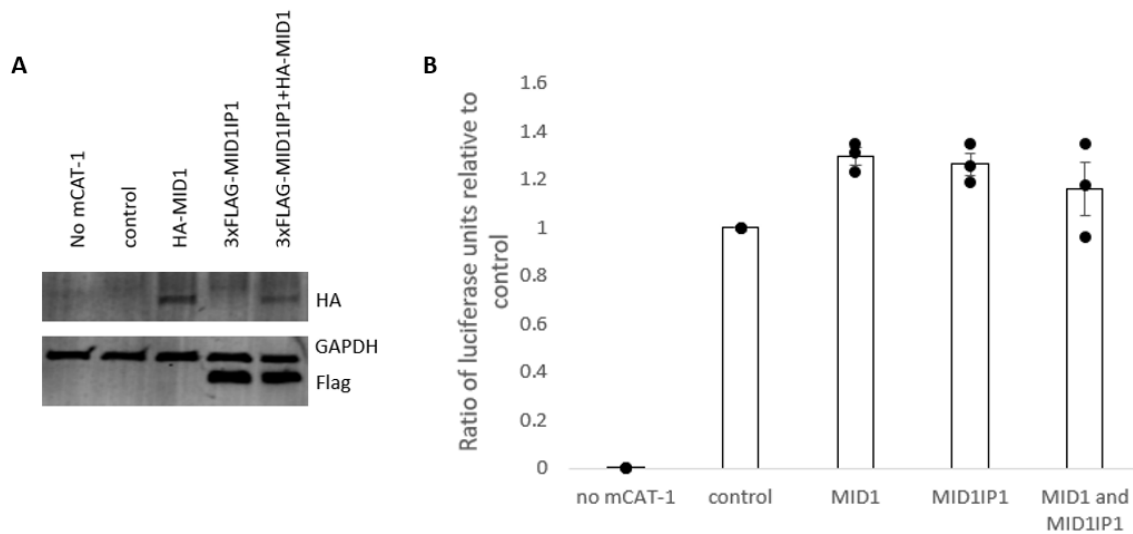
## **Overexpression of MID1IP1 and MID1 does not demonstrate an effect on HIV-1 Infectivity**

Given the interplay between microtubule stability and HIV-1 infection, and the suggestion proposed by Berti et al. (2004) that the overexpression of MID1IP1 and MID1 may affect microtubule stability, we were motivated to test whether overexpression of these proteins impacts HIV-1 infection. Cells were co-transfected with mCAT-1, the receptor for the ecotropic envelope of MLV, and infected with ecotropic pseudotyped HIV-1 reporter virus. This was done so that only the cells that were transfected with the plasmids and therefore expressing mCAT-1 would be capable of being infected by the reporter virus. We tested for luciferase reporter expression in cells transfected with MID1IP1, MID1, or both relative to controls (Figure 2.10).

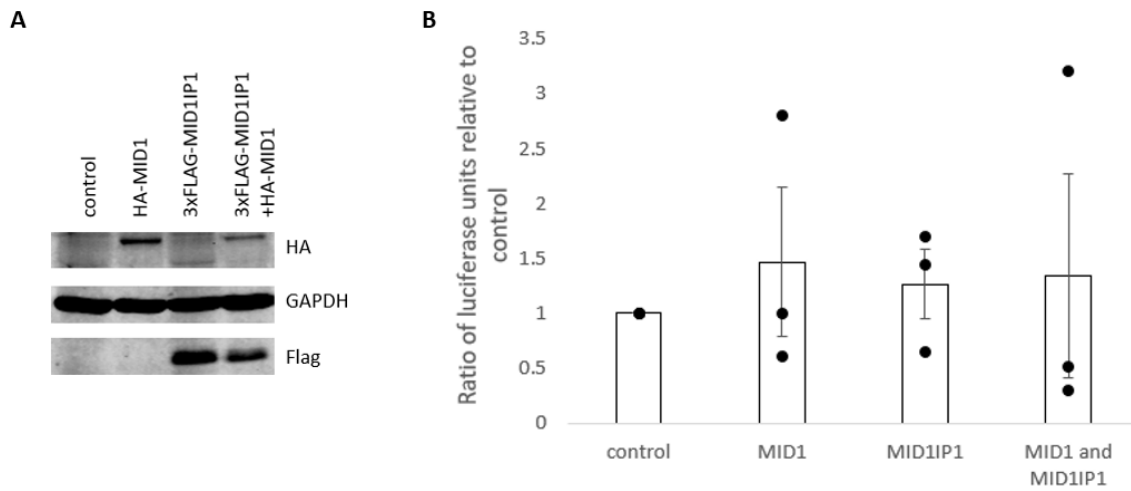
TE671 cells were transfected with combinations of control, MID1, and MID1IP1 expressing plasmids, along with the mCAT-1 receptor. In an additional control for the lack of mCAT-1, cells were transfected with a control empty vector plasmid in place of mCAT-1. Expression of the luciferase reporter was clearly mCAT-1 dependent as expected, but the expression of the reporter did not change with the transfection of plasmids expressing MID1, MID1IP1, or both concurrently (Figure 2.11). Similar results were obtained in HeLa cells (Figure 2.12). Our results indicate that the overexpression of MID1, MID1IP1, or both did not produce an effect on the portion of the viral life cycle from entry through translation in either TE671 or HeLa cells.



**Figure 2.10: Flow chart of experimental procedure for overexpression of MID1 and MID1IP1.** Cells are transfected with either a combination of plasmids expressing MID1 or MID1IP1 and control plasmids. These plasmids are cotransfected with mCAT-1, the receptor used by ecotropic envelope to mediate viral entry into cells. 48 hours post-transfection, cells are infected with ecotropic pseudotyped HIV-1 luciferase reporter virus such that only mCAT-1 expressing cells are capable of being infected by the reporter. 48 hours post-infection, cells are assayed for luciferase expression.



**Figure 2.11: Overexpression of MID1IP1 and MID1 does not produce an effect on HIV-1 infectivity in TE671 cells.** A) Western blot for TE671 cells transfected with a control plasmid (without mCAT-1), a control plasmid (with mCAT-1), HA-MID1 (with mCAT-1), 3xFLAG-MID1IP1 (with mCAT-1), and both HA-MID1 and 3xFLAG-MID1IP1 (with mCAT-1). B) Ratio of luciferase units relative to control cells when transfected cells are infected with a single-cycle ecotropic pseudotyped HIV-1 luciferase reporter virus. Dots represent individual experiments. Error bars represent standard error of the mean across three experiments conducted in triplicate.



**Figure 2.12: Overexpression of MID1IP1 and MID1 does not produce an effect on HIV-1 infectivity in HeLa cells.** A) Western blot for HeLa cells transfected with a control plasmid, HA-MID1, 3xFLAG-MID1IP1, and both HA-MID1 and 3xFLAG-MID1IP1. All cells were cotransfected with mCAT-1. B) Ratio of luciferase units relative to control cells when transfected cells are infected with a single-cycle ecotropic pseudotyped HIV-1 luciferase reporter virus. Dots represent individual experiments. Error bars represent standard error of the mean across three experiments conducted in triplicate.

## Conclusion

The screen data presented by Brass et al. (2008) reported a modest, roughly three fold, decrease in capsid staining 48 hours post-infection in TZM-bl cells after MID1IP1 had been knocked down. The screen data presented by König et al. (2008) reported a 10 fold decrease in reporter expression after infection with a single-cycle reporter virus in 293T cells with a MID1IP1 knockdown. The methodology in our experiments is closest to König et al. (2008), as both sets of experiments involve using single-cycle reporter virus to assay for the entry stages of HIV-1 in similar cell types. The experiments differ in that we used 293 cells rather than 293T cells, mutated the genome of cells with CRISPR instead of transiently knocking down with RNAi, and did not conduct our experiments as part of a high-throughput procedure.

Our results do not align with the ten fold decrease seen by König et al. (2008). If the effect of a transient knockdown was real, it might be expected that a permanent loss-of-function mutation at the DNA level would also produce an effect at least as strong the transient effect, unless the CRISPR cells have compensatory mutations for any essential functions that MID1IP1 might have. One caveat to our experiments is that we were unable to detect endogenous levels of MID1IP1 protein with antibodies in Western blots, and therefore relied on genomic data to conclude that no wild-type copy of the *MID1IP1* gene exists in these CRISPR cells. These data seem compelling.

It should be noted that we cannot preclude the possibility that these mutant alleles may use alternate start sites to produce alternate proteins from these sequences which might retain some functional ability. Such events are always possible. Tuladhar et al. (2019) tested 13 CRISPR cell lines with frameshift mutations for expression of the targeted protein with two different antibodies, finding that in four of these cell lines, at least one of the antibodies detected

proteins of a different size not present in controls. The study found that such new proteins may be produced by the exclusion of the mutated exon in mRNA, alternate initiation sites for translation, or the activation of pseudo-mRNAs that otherwise would have been subjected to nonsense-mediated decay due to a premature stop codon.

Exclusion of the mutated exon is not a viable method of producing a truncated MID1IP1 protein in CRISPR cells, as only one exon in *MID1IP1* contains the coding sequence for the protein. However, translation from alternate initiation sites remains a possibility. MID1IP1 is a small 183 amino acid long protein with six methionine residues. Three of these residues occur within the first 17 base pairs, with all *MID1IP1* mutations in our three CRISPR cell lines either occurring after them or eliminating them altogether. Three occur after the mutation in at least one allele per cell line.

If a truncated MID1IP1 protein were to be produced from these transcripts, they would be the most likely alternate start sites, theoretically capable of producing 97-156 amino acid long truncated proteins (53-85% of the full-length protein). For these reasons, the inability to detect the protein of interest with Western blotting is considered a more stringent verification for loss-of-function than the absence of wild-type genomic DNA, though it should be noted that not all antibodies would be capable of detecting truncated proteins. Nonetheless, between three cell lines with no wild-type copy of *MID1IP1* and the mutation of the only exon in the gene which contains coding DNA, these CRISPR cell lines are likely to suffer the complete loss of MID1IP1 function.

There remains the possibility that the ten fold effect seen in the initial Konig screen was due to off-target effects from the siRNAs, which were not induced in the CRISPR knockout cells. (The CRISPR methodology could also produce off-target effects, but the non-target

sequences affected by the CRISPR guide RNAs and the siRNAs would not be the same targets.) Off-target effects and false positives are common in siRNA screens, making this a likely explanation for the discrepancy between our findings and those of König et al. (2008) with respect to MID1IP1.

Notably, though Brass et al. (2008) used a fully infectious virus in their screen, they also distinguished early events through viral gene expression from exit events of the HIV-1 life cycle by assaying for capsid staining 48 hours post-infection. Due to the timing of their assay, their readout should have detected most of their capsid staining from the first round of infection. Their results from this experiment, as with König et al. (2008) and our own experiments, were intended to pick up the early events rather than the exit events of the viral life cycle. The discrepancy between our findings and those of Brass et al. (2008) may be explained by either off-target effects or false positives in their siRNA screen or by cell-type differences between 293 cells and TZM-bl cells.

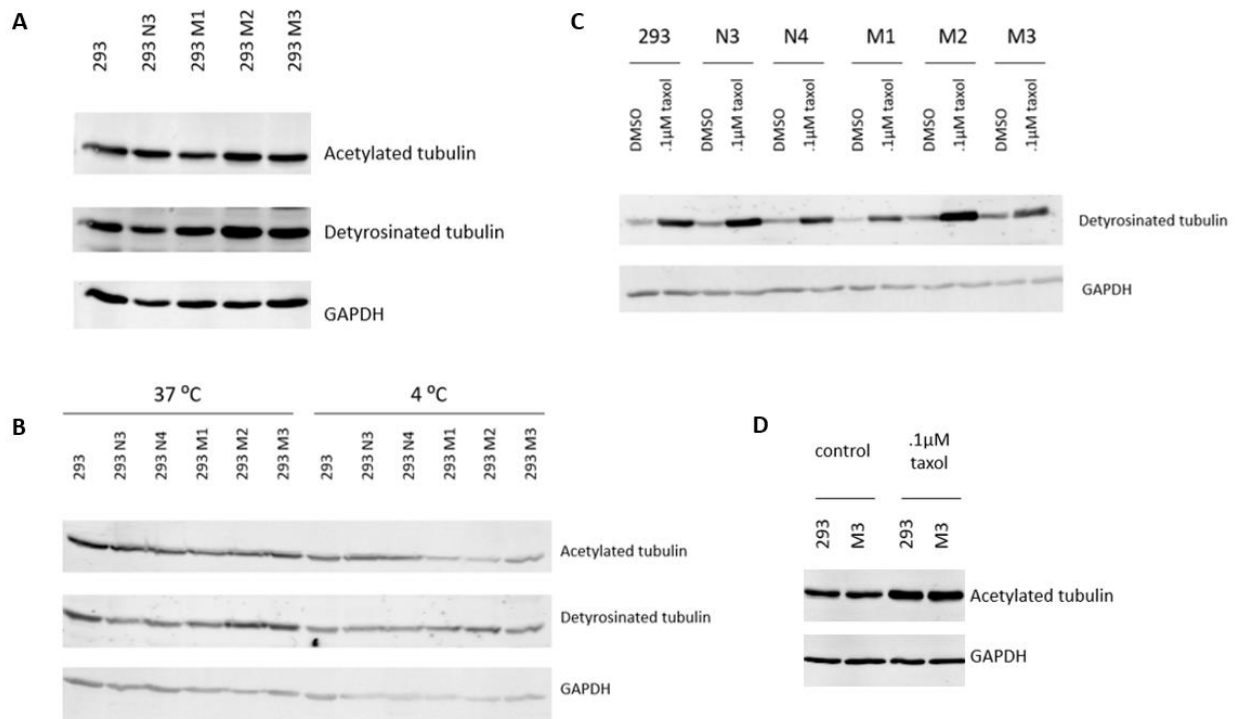
Finally, it should be noted that our experiments only tested for the stages of the life cycle capable of being assayed for with a single-cycle reporter, and would not have detected effects on the exit stages of the HIV-1 life cycle after translation. As such, our conclusions are limited to the entry stages of the HIV-1 life cycle through gene expression. A potential future experiment to determine whether MID1IP1 might have an effect on the exit stages of the viral life cycle would consist of assaying for those stages in our 293 CRISPR cells. This may be done by transfecting cells with viral DNAs encoding Gag protein and assaying for viral proteins present in the virion-like particles released into the supernatant.

## **Chapter 3: Markers of Microtubule Stability Do Not Reveal Functions of MID1IP1 at Endogenous Levels in 293 CRISPR Cells**

In chapter two, we describe the generation of three 293 cell lines in which the *MID1IP1* gene contains CRISPR-induced frameshift mutations, and experiments testing those cell lines for reporter virus expression. In this section, we examine those cell lines further in an attempt to elucidate the endogenous functions of the MID1IP1 protein. Berti *et. al.* (2004) previously proposed a connection between the overexpression of MID1IP1 (and MID1) and microtubule stability. While this effect remains to be confirmed, the same study also found that MID1IP1 localizes to microtubules. Given the shortage of information on the functions of MID1IP1, it is a plausible theory that the protein may have some role pertaining to microtubule stability. We conducted experiments to determine whether mutant cell lines exhibit a difference in the expression of markers of microtubule stability relative to controls.

### **MID1IP1 Targeted 293 CRISPR Cells Retain Similar Levels of Staining for Acetylated and Detyrosinated Tubulin as Control Cells**

Western blots were conducted for these 293 CRISPR cells using antibodies for two markers for microtubule stability: acetylated and detyrosinated tubulin. This is a simple way to assay for potential differences in stable microtubule between *MID1IP1* targeted- and control cell lines. We detected no strong differences in the intensities of the bands detected with antibodies to either modified tubulins, between control and CRISPR cells (Figure 3.1A).



**Figure 3.1: Markers of microtubule stability in 293 CRISPR cells.** A) Western blot of acetylated and detyrosinated tubulin in control cells (293 and N3) and MID1IP1 CRISPR cells (M1-3). Results were obtained from at least three independent experiments. B) Western blot for control (293, N3, N4) and MID1IP1 CRISPR cells (M1-3) incubated at 4°C for 1 hour. Results were obtained from at least three independent experiments. C) Western blot of detyrosinated tubulin for control (293, N3, N4) and MID1IP1 CRISPR cells (M1-3) treated with .1µM taxol for 1 hour. Results were obtained across 2-3 independent experiments. D) Western blot of acetylated tubulin for a control (293) and MID1IP1 CRISPR cell line (M3) treated with .1µM taxol for 1 hour. Results were obtained from three independent experiments.

Cells were then subjected to several conditions which affect microtubule dynamics to determine whether MID1IP1 could affect the rate at which markers of microtubule stability are accumulated or lost. Lower temperatures have previously been found to prompt the depolymerization of microtubules, with only a small subset remaining stable (Wallin and Stromberg, 1995). Cells were incubated at 4°C for 1 hour to determine whether differences in markers for microtubule stability may be detected after exposure to destabilizing conditions. There were no consistent differences in acetylated and detyrosinated tubulin levels between control and CRISPR cells (Figure 3.1B). There was a small amount of fluctuation between experiments where the mutant cell lines appeared to have more or less acetylated tubulin after exposure to cold relative to a control, but these observations were not made consistently across multiple experiments.

Cells were also treated with taxol, which induces microtubule stability, to determine whether differences in staining for these markers could be detected after treatment (Figure 3.1C-D). Consistent increases in detyrosinated tubulin were detected after taxol treatment, as would be expected, but the increases detected in CRISPR cells were not consistently different from control cells. In figure 3.1C, cell line M2 appears to have greater staining for detyrosinated tubulin than controls upon taxol treatment, while cell line M1 has less and cell line M3 is in line with control levels. Independent experiments showed some modest fluctuations in detyrosination and acetylation levels, but as with cold stability experiments, these differences were not seen consistently in the same cell lines upon repeated experiments. Thus we were unable to find evidence for a function related to microtubule stability for MID1IP1 with the experiments we conducted in 293 CRISPR cells.

The experiments testing for markers of microtubule stability in CRISPR cells indicated that the levels of acetylated and detyrosinated tubulin in 293 cells is not dependent on MID1IP1. As these markers serve as a readout for microtubule stability, this implies that MID1IP1 does not affect the amount of stable microtubules in cells or the proportions of microtubules which accumulate these specific post-translational modifications. It should be noted that these Western blot experiments can only provide a readout for the total amount of stable microtubules in the cells, and that immunofluorescence experiments would be capable of more thoroughly investigating potential effects on the distribution of microtubule stability throughout the cell. It remains possible that MID1IP1 may have cell-type specific effects in other cell lines, or that MID1IP1 might affect microtubules in 293 cells in some other way.

## **Chapter 4: Chaperonin Component CCT2 May Function in the Early Phase of the HIV-1 Viral Life Cycle through Viral Gene Expression**

Chapters two and three addressed the potential role of host factor MID1IP1 in HIV-1 infection and investigated its potential functions in microtubule stability, concluding that MID1IP1 is not a promising candidate for study with respect to effects on HIV-1 infection. Of the other candidate genes implicated as host factors participating in the HIV-1 life cycle by siRNA screens, we also chose the chaperonin subunit CCT2 for further study. One such screen conducted by Zhou et al. (2008) recovered CCT2 as a candidate gene. The data indicated CCT2 might function in entry, exit, or both stages of the viral life cycle. We tested the effect a CCT2 knockdown might have on viral replication using a single-cycle reporter virus.

### **siRNA Knockdown of CCT2 Demonstrates a Slight Effect on HIV-1 Infectivity in TE671 Cells**

TE671 cells were transfected with siRNA pools of 4 siRNAs targeting CCT2 twice over two days, split into 24 well plates 24 hours after the last transfection, and then infected with a VSV-G pseudotyped HIV-1 luciferase reporter virus 48 hours after the last transfection. Luciferase expression was assayed for 48 hours post-infection as a readout for the completion of the entry stages of the viral life cycle through gene expression. These experiments revealed a modest impact of the knockdown on the efficiency of virus transduction (Figure 4.1B-C). We observed an average of a 2.2 fold decrease of luciferase expression in knockdown cells relative

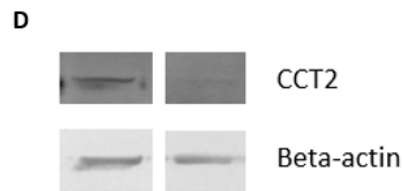
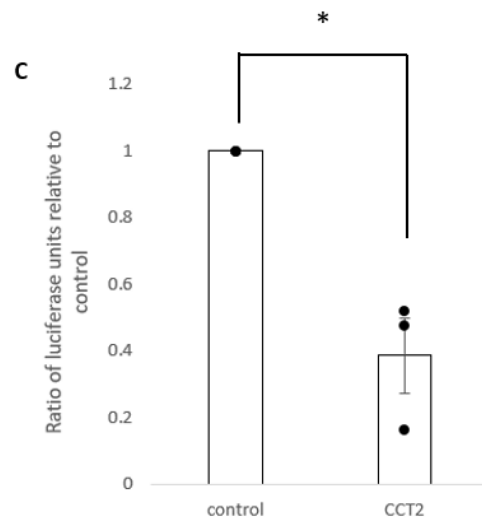
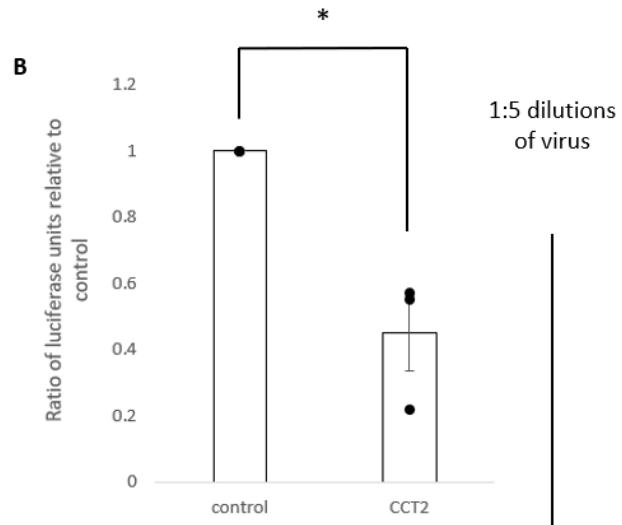
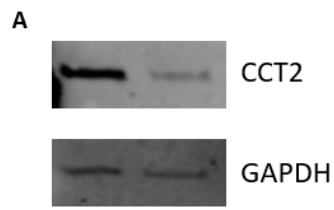
to the control cells, at one dilution of the transducing virus preparation ( $P = .02$ ), and an average of a 2.6 fold decrease of luciferase expression in the knockdown cells relative to the control, at a 1:5 lower dilution of virus ( $P = .02$ ). A repeat of the experiment that produced the strongest knockdown visible by Western blotting (Figure 4.1D) also correlated with the strongest effect on luciferase expression (4.5 fold and 6.2 fold at each dilution respectively).

These data suggest that CCT2 may have a slight effect on HIV-1 infection, with the potential for a larger effect in the event of a particularly strong knockdown. This result falls in line with the evidence produced in the screen conducted by Zhou et al. (2008). However, this does not rule out the possibility that a CCT2 knockdown creates an unhealthy overall environment in the cell that has a nonspecific consequence of affecting the efficiency of the viral life cycle.

A caveat of these experiments is that the reporter used in them, firefly luciferase, is also folded by the TRiC complex. Human TRiC contributes to luciferase folding *in vitro* in assays where luciferase was unfolded, incubated with purified human TRiC, and assayed for expression (Knee et al., 2013). In rabbit reticulocyte lysate, three molecular chaperones (Hsp70, Hsp40, and TRiC) were found associated with nascent luciferase polypeptides and immunodepletion of each of the three without affecting the other two produced decreases in luciferase expression. (A 70% depletion of TRiC corresponded to a 65% reduction in luciferase expression) (Frydman et al., 1994). These experiments do not necessarily mean that luciferase requires TRiC activity to fold *in vivo* in human cells. However, as luciferase is a potential target of TRiC activity, an experiment should be conducted where we express luciferase from a plasmid in TE671 cells with a CCT2 knockdown. Comparing the effect on luciferase expression from a plasmid to expression from a provirus delivered through a reporter virus should establish whether the effects seen in

CCT2 knockdown cells are due to a potential effect of CCT2 and TRiC on luciferase folding *in vivo*.

The two fold effect on luciferase expression present in our data is insufficient to progress to experiments that could define the stages of the viral life cycle affected by CCT2. These would involve assaying for reverse transcription and nuclear entry products, but these assays have more than two-fold variation across experiments. In order to obtain consistent and strong depletions of CCT2 in cells, we attempted to create stable knockdowns using shRNA and loss-of-function mutations using CRISPR/Cas9. CRISPR cell lines for CCT2 were made in parallel with CRISPR cell lines for LEDGF (as well as other components of the TRiC/CCT complex). Loss of LEDGF expression in several 293 cell lines produced by CRISPR was readily detected. No such loss of protein expression was detected in attempted KO of CCT2 (or any other TRiC/CCT subunit). Similarly, we attempted to make stable knockdowns for CCT2 in both 293 and TE671 cells, but none of the cell lines demonstrated a reduction in CCT2 levels by Western blotting relative to the control.



**Figure 4.1: siRNA knockdown of CCT2 in TE671 cells.** A) Western blot staining for CCT2 in cells transfected with control and CCT2 siRNA pools. B) The ratio of luciferase units relative to control cells when transfected cells are infected with a single-cycle VSV-G pseudotyped HIV-1 luciferase reporter virus. Dots represent the three individual experiments. C) The ratio of luciferase units relative to control cells when transfected cells are infected with a single-cycle VSV-G pseudotyped HIV-1 luciferase reporter virus at a 1:5 dilution from B. The ratio of luciferase units relative to control cells when transfected cells are infected with a single-cycle VSV-G pseudotyped HIV-1 luciferase reporter virus. Error bars represent standard error of the mean across three experiments conducted in triplicate. Error bars represent standard error of the mean across three experiments conducted in triplicate. D) Western blot staining for CCT2 in cells transfected with control and CCT2 siRNA pools, representing the single repeat of the experiment with the strongest knockdown for CCT2.

Our inability to consistently reduce CCT2 levels supports the likely possibility that the protein is essential and that cells may only be capable of withstanding transient knockdowns of CCT2 rather than a sustained depletion. This also relates to the question of whether the effects on reporter expression detected in HIV-1 infection experiments might be a result of CCT2 knockdowns adversely affecting the health of the cell rather than participating directly in the viral life cycle. Further experimentation would be required to deduce whether the effect of CCT2 on the viral life cycle is a byproduct of its potential effect on the cellular environment by using assays for cell viability. From our experiments with siRNA and our attempts to deplete CCT2 with stable knockdowns or CRISPR induced loss-of-function mutations, CCT2 appears to be a challenging target for such research.

## Chapter 5: Discussion

### MID1IP1 and MID1 in Retroviral Infection

The early post-fusion stage of the HIV-1 life cycle in particular remains poorly understood. There is evidence for an interlinkage between reverse transcription, capsid uncoating, and microtubule transport. However, how all of these steps are connected and in what order they occur remains to be determined. The identification of more host proteins involved in this stage of the life cycle--and subsequent experimentation to determine the effects these proteins have on readouts for reverse transcription and uncoating--may clarify the mechanism. Our experiments do not support a role for MID1IP1 and MID1 in the early post-fusion stage of HIV-1 infection or at any step in infection from entry to viral gene expression.

Some clues about the role of various host genes in controlling virus replication can be gleaned from the regulation of expression of those genes. For example, those genes that are activated by the interferon pathway – the interferon stimulated genes (ISGs) – often have antiviral activity. In this context the transcriptional promoter of the MID1 is notable. Expression of the gene is driven by a human endogenous retroviral element. This element acts as a tissue-specific promoter and enhancer for MID1 expression, upregulating MID1 expression in certain cells (such as 293 cells) (Landry et al., 2002). This raises the possibility that there might be a selective advantage for host cells to upregulating MID1 during viral infection. However, given that this promoter is responsible for tissue-specific upregulation of MID1 in the absence of infection, it may simply have been selected for this functionality. Our experiments did not find an effect of overexpression of MID1 on the entry stages of the HIV-1 life cycle in HeLa or TE671 cells, suggesting that any potential selective advantage either applies only to other

viruses, or is only active in other cell or tissue types. Uchil et al. (2008) conducted similar experiments in 293 cells, which are known to be upregulated for MID1 expression by the retroelement, and found no effect of MID1 overexpression on HIV-1 or MLV using single-cycle reporter viruses.

Our experiments testing the functions of MID1IP1 and MID1 only assay for effects on the life cycle between the time of viral entry into the cell and the time of viral gene expression. As such, further experimentation may determine whether these proteins have effects on the exit stage of the viral life cycle. Viral DNAs encoding Gag protein can be introduced into cells with altered expression of MID1IP1 or MID1, resulting in the production and release of virion-like particles into the supernatant. The production of these virus-like particles depends on the exit stage of the viral life cycle and they may be assayed for by collecting the supernatant, purifying the particles by pelleting through a sucrose cushion, and using Western blots to detect viral protein staining. This, in combination with assays for the early stage of viral infection, will indicate whether MID1IP1 functions in HIV-1 infection at any stage of the life cycle.

### **Potential Microtubule-Related Functions for MID1IP1**

The functions of MID1IP1 remain mostly uncharacterized. Its localization and its association with MID1 are the only proven connections it has with microtubules, but neither of these pieces of evidence provide any direct information about its functional role. Presumably, MID1IP1 might have an effect on either microtubules or the environment around them. This encompasses several possibilities. MID1IP1 may have a direct effect on tubulin monomers or polymers. It might have an indirect effect on microtubules by interacting with other proteins that

directly affect the microtubule network. Additionally, it could enhance or inhibit the activity of any protein that itself has a direct or indirect effect on microtubules.

MID1IP1 might play many different microtubule-related roles in cells. MID1IP1 could affect microtubule stability, but also could modulate microtubule dynamics, cargo transport, or post-translational modifications. Microtubule stability is only one possible microtubule-related function MID1IP1 might participate in. We chose to assay for it because of the screens implicating MID1IP1 in HIV-1 or MLV infection at a step prior to translation from the provirus. As Berti et al. (2004) had already discovered the localization of the protein and suggested a role for it in microtubule stability, and as there is evidence of a necessary role for microtubule stability in HIV-1 infection, it was a plausible theory. However, if MID1IP1 does not play a role in the entry stage of the HIV-1 life cycle (as our data suggests), there is less reason to think MID1IP1 might function in microtubule stability rather than any other microtubule related function. In fact, our data suggests that MID1IP1 is dispensable for microtubule stability in 293 cells.

There is considerable evidence that stable microtubules play a supportive role in the HIV-1 life cycle. Several proteins which affect microtubule stability have an impact on the infectivity of HIV-1 reporter viruses. The depletion of +TIP binding factor EB1 both decreases acetylated tubulin and strongly inhibits infection (Sabo et al., 2013). Stable microtubules, which are resistant to the mechanisms of several depolymerizing drugs that have only slight effects on HIV-1 infectivity, play an important role in the entry stage of HIV-1 infection that can compensate for the loss of dynamic microtubules. Perhaps the strongest evidence is that fact that HIV-1 infection itself increases microtubule stability in cells.

Due to the importance of microtubule stability in the HIV-1 life cycle, any condition that inhibits microtubule stabilization in cells would be expected to have an effect on HIV-1 infection. That our MID1IP1 mutant CRISPR cell lines do not have any such effect suggests that MID1IP1 is not required for microtubule stability in these particular cells. This does not necessarily mean that MID1IP1 has no function related to microtubule stability. It is possible that MID1IP1 functions in a redundant pathway, and that parallel mechanisms act in its absence. If HIV-1 can induce microtubule stability upon infection in a pathway independent of MID1IP1, while MID1IP1 functions in a different but dispensable pathway mediating microtubule stabilization, then a loss-of-function for this protein may have no impact on infection.

Any potential effect of MID1IP1 on microtubule stability may also be cell-type dependent. The different post-translational modifications that may be enriched on tubulin vary in different cell types or subcellular locations, which may coincide with different levels or types of stable microtubules (Bulinski et al., 1988; Song and Brady, 2015; Yu et al., 2015). Our data might not support a role for MID1IP1 in either HIV-1 infection or microtubule stability in 293 cells, but the case might be different in the cell types that HIV-1 naturally infects. It might in particular be different in TE671 cells, for which our siRNA experiments were inconclusive but did provide some preliminary results raising the possibility of an effect.

We also experimented with overexpression levels of MID1IP1 and MID1. The overexpression of proteins may or may not produce phenotypes in general, or on virus replication in particular. The +TIP binding protein EB1 does induce a significant phenotype upon overexpression, as CHME3 cells stably overexpressing EB1 produced both an increase in acetylated microtubules and luciferase expression after infection with a reporter virus (Sabo et al., 2013). In fact, many of the known regulators of microtubule stability that participate in HIV-

1 infection (HDAC6, moesin, ezrin, Dia1, Dia2) have produced effects on the HIV-1 life cycle when overexpressed. However, our experiments overexpressing MID1IP1 and MID1 did not induce any consistent effects on HIV-1 infection in two cell lines (TE671 and HeLa). Our data do support the conclusion that MID1IP1 does not provide a function that is indispensable for microtubule stability in 293 cells. This leaves the possibilities that MID1IP1 may have no role in microtubule stabilization, that it may have a redundant role, or that it might be required for microtubule stabilization only in certain cell types.

### **Potential Interactions Between MID1 and MID1IP1**

The question remains as to whether the interaction of MID1IP1 and MID1 has any function beyond the role of MID1 in the localization of MID1IP1 to microtubules. It is possible that this is the extent of their interactions and that MID1IP1 engages in whatever functional activity it has at the site of microtubules independent of MID1. It is also possible that MID1IP1 is a target for ubiquitinylation by MID1's E3 ligase activity. To determine whether this is the case, future experiments could test whether MID1 is capable of ubiquitinating MID1IP1. Conversely, MID1IP1 could affect MID1 activity in a manner unrelated to microtubule stability (such as physically blocking another potential binding partner, for example).

If any functional activity for MID1IP1 were identified, it could then be determined whether that function was retained with mutant MID1 which only expresses the microtubule- and MID1IP1 binding domains (coiled-coiled and COS box), or one with a mutation inactivating the E3 ligase activity. Such constructs should still be capable of recruiting MID1IP1 to microtubules, but would lack E3 ligase activity or any functions attributable to other MID1 domains. This

would experimentally distinguish between potential MID1IP1 functions which rely on MID1 for localization versus functions for which other MID1 domains are also required.

It would also be of interest to determine whether MID2 can substitute for any of the effects MID1 might have on MID1IP1, as the two TRIM proteins share 83% similarity and 76% identity. MID1 and MID2 both localize to microtubules and a high-throughput proteomics experiment identified MID2 and MID1IP1 as binding partners using yeast 2-hybrid assays (Rolland et al., 2014). MID1 is known to be able to localize MID1IP1 to microtubules, and it would be possible to test whether MID2 could potentially have this same effect.

Experiments in which cells transfected with MID1 mutants that no longer retain the ability to bind microtubules (but do still bind MID1IP1) show MID1IP1 following the distribution pattern of MID1 rather than localizing to microtubules as it does in the presence of wild-type MID1 (Berti et al., 2004). As this experiment was conducted at overexpression levels, it provides no indication of whether endogenous levels of MID2 or any other protein might also be capable of recruiting MID1IP1 to microtubules. Furthermore, even if experimental evidence that MID1 was primarily responsible for MID1IP1 localization were obtained in one cell line, MID1 and MID2 can have variable expression patterns in different tissues (Buchner et al., 1999), which may cause discrepancies in the extent to which each of them is needed for the localization of MID1IP1 in different cell lines. An experiment similar to the one performed with MID1 mutants could be performed with MID2 mutants. This would demonstrate whether or not MID2 also has the potential to recruit MID1IP1 to microtubules.

## CCT2 in Retroviral Infection

CCT2 remains a candidate for a regulator of the early post-fusion stage of the HIV-1 life cycle. Our experiments indicate the potential for small effects on virus infection as a result of siRNA knockdowns. This may be confirmed by adding CCT2 back into the system and determining whether this rescues the effect. These effects may be further explored by determining whether they are observed in multiple cell lines and whether those experiments can produce effects greater than 2 fold in at least one cell line. A consistently larger effect may make it possible to assay for reverse transcription and nuclear entry products, which would allow a determination of the stage of the life cycle affected by CCT2. There are multiple steps that might be affected. One plausible mechanism would be a role in the folding of tubulin and actin, which may place its effect during microtubule transport or uncoating. However, perturbing the actin network in cells might also affect the actin-dependent clustering of receptors at the cell surface that enables initial entry into cells.

CCT2 has also been implicated in the exit stage of HIV-1 infection in a previous screen (Zhou et al., 2008). Our experiments did not test for the exit stage of infection post-gene expression, as we infected with a single-cycle luciferase reporter and used luciferase expression as the readout. Further experimentation is required to determine if this potential effect on the exit stage recurs in cell lines outside of the HeLa P4/R5 cells used in the screen. The possibility of a role for CCT2 in the exit stage of the viral life cycle creates the potential that the TRiC complex might be involved in the folding of HIV-1 proteins, similar to its role in folding proteins from other viruses (Table 1.3). Should any viral proteins require TRiC complex activity to reach their native confirmation *in vivo*, this would occur independently of the luciferase expression used as a readout in these experiments. Direct effects of TRiC complex folding activity on viral proteins

may be detected by assaying for noninfectious virus-like particles produced in the supernatant or, if the particular viral protein is packaged into the virus-like particle and functions during the entry stage of infection, by producing single-cycle reporter viruses in CCT2 deficient cells and infecting fresh cells to determine the infectivity of the viral particles.

There are eight different subunits that make up the TRiC/CCT complex. If the knockdown of CCT2 produces an effect on HIV-1 infection in a TRiC dependent mechanism, we might expect that the knockdown of any single one of these subunits should have a similar effect on HIV-1 infection. Conversely, if only a CCT2 knockdown produces an effect on viral infection, then this would suggest a special role of CCT2 in the life cycle. Such a role might reflect the ability of CCT2 to bind a target protein and either sequester it or induce conformational changes independently of the TRiC/CCT complex. Such substrate specificities have been observed for other CCT subunits.

In addition to their functions as part of the TRiC complex, there is some evidence that components of the complex might have functions as monomers as well. Depleting each of the CCT subunits individually by RNAi in BE cells (human colon carcinoma) reveals that the knockdown of CCT5 produces a different effect on cell shape than the knockdown of the other CCTs, leading to narrow and elongated cells (Brackley and Grantham, 2010). When GFP-tagged versions of each of the mouse CCT subunits were overexpressed individually in BALB 3T3 cells, only the transfection of CCT4 resulted in cell surface protrusions (Spiess et al., 2015). The potential for CCT2 monomeric function, rather than the effect of CCT2 depletion on the TRiC complex, to explain potential effects on the HIV-1 life cycle can be tested for by depleting various CCT subunits individually and assaying for effects on viral expression.

The roles of the TRiC complex and other TRiC/CCT components identified in various viral life cycles implies this complex may function in a mechanism general to several viruses rather than specific to HIV-1. In order for the complex to play a role in any particular viral life cycle, that virus must be dependent on at least one protein that requires TRiC activity to fold into its native confirmation. If the TRiC complex folds a host protein involved in mechanisms important for multiple viral life cycles, then it could be that the complex is generally important for viral infections that share the use of certain host pathways. These host pathways would most likely pertain to the cytoskeleton, given the known function of the TRiC complex in the folding of both tubulin and actin. Aside from HIV-1, the depletion of a TRiC complex component has a known functional effect on the life cycles of three different viruses. The cytoskeleton plays a role in the life cycle of each of those viruses: influenza A (Bedi and Ono, 2019), rabies virus (Zan et al., 2017), and hepatitis C virus (Bost et al., 2003). This provides a possible common mechanism for TRiC activity in all four viral life cycles for which there is evidence of an effect on infection.

However, if the TRiC complex functions in viral life cycles through the folding of viral proteins rather than through the cytoskeleton, then the importance of TRiC in any viral life cycle may vary on a case-by-case basis. Potential physical interactions of TRiC components have been identified with several viral proteins, including HIV-1 p6, HIV-1 integrase, influenza A PB2, and M-PMV p4. This suggests that these viral proteins are potential targets for TRiC/CCT protein folding activity.

It is possible that the TRiC complex does both of these things: fold proteins important in several viral life cycles (such as actin and tubulin), as well as folding various viral proteins directly. This means the complex may affect the infection of a single virus through more than one pathway. Alternatively, it means that the complex may affect the infection of one individual

viruses through a single pathway (such as its effects on the cytoskeleton), but use a different mechanism (such as a direct role in viral protein folding) to affect the life cycle of another virus. In HIV-1 there is enough evidence to warrant further investigation into the roles of CCT2 in both entry and exit stages of infection, implying the potential for effects on multiple steps within the life cycle.

## **Conclusion**

Our data suggests that MID1IP1 does not play a role in the early steps of HIV-1 or MLV infection in 293 cells. These results contradict the data previously reported in screens by Brass et al. (2008) and König et al. (2008). The latter screen in particular was conducted with a similar methodology, using a similar cell type (293T versus 293 cells) and readout (single-cycle reporter virus). It is possible that MID1IP1 was a false positive in these screens or has a cell-type dependent effect. Additionally, the lack of an effect of MID1IP1 depletion in 293 cells on the HIV-1 life cycle suggests that MID1IP1 is dispensable for microtubule stability in this cell line.

CCT2 remains a protein of interest to study with respect to HIV-1 infection. Further experimentation must be done to confirm that the slight effect that CCT2 depletion produces on HIV-1 infection is due to a role in the life cycle rather than a general effect on viability. If the effect on infection is confirmed, there are several avenues for further study. It may be determined what pathway in the viral life cycle CCT2 affects, whether the effect is dependent on the TRiC complex or CCT2 alone, or if the effect is due to the role of TRiC/CCT in the folding of tubulin or actin. Whether CCT2 or the TRiC complex affect the life cycle indirectly through the folding of a necessary host protein, directly through the folding of viral proteins, or both, may form an interesting avenue of study.

## Chapter 6: Materials and Methods

### Cell Culture

HEK293, HEK293T, TE671, and HeLa cells (ATCC) were cultured in DMEM media with 10% FBS, penicillin, streptomycin, and L-glutamate at 37°C and 5% CO<sub>2</sub>. Cells were passaged 1 to 5 with the addition of trypsin every two days. Cells were checked routinely for mycoplasma.

### Production of Viral Reporters

1 x 10<sup>9</sup> HEK293T cells in a 10 cm plate were transfected with plasmids using 30 µL lipofectamine 2000 (Life Technologies) as the transfection reagent in 500 µL Opti-MEM. Cells were incubated for 6 hours, then washed and new media was added. For the production of HIV-1 single-cycle reporter viruses, 9 µg of a plasmid containing the HIV-1 machinery plus luciferase reporter (pNL4-3.Luc.R-.E-, originally contributed by Dr. Nathaniel Landau, NYU) and 3 µg of a plasmid expressing the envelope protein used for pseudotyping, either pMD.G for VSV-G (Wang and Goff, 2015), pHit456 for MLV Amphotropic derived from amphotropic MLV clone 4070A (Cannon et al., 1996; Page et al., 1990), or pHit123 for MLV ecotropic (Soneoka et al., 1995) were transfected to pseudotype the virus-like particles. For the production of MLV single-cycle reporter viruses, 4.5 µg of a plasmid containing the gag-pol machinery for NB-tropic MLV (pCMVintron) (Wang and Goff, 2015), 4.5 µg of a plasmid containing firefly luciferase with packaging sequence (pNCA-Luc), and 3 µg of a plasmid expressing VSV-G were transfected. Lentiviral delivery vectors were made by transfecting 7.5 µg of the cloned plentiCRISPR plasmid (from Feng Zhang, Addgene 52961) along with 6 µg of the packaging plasmid pCMVΔR8.2 and 1.5 µg of a VSV-G expressing plasmid using 45 µL lipofectamine 2000.

The culture supernatant medium was collected 48 hours post-transfection, centrifuged for 5 min at 1000 rpm, filtered through a 0.45µm filter, treated with 100mM HEPES (LifeTechnologies), aliquoted, and frozen at -80°C.

### **Luciferase Reporter Assay**

Cells seeded at  $.1 \times 10^6$  cells per well into 24 well plates the previous day in 1 mL media were infected at several dilutions of virus in 200 µL media with 8 µg/ml polybrene (Sigma). At 48 hours post-infection, cells were lysed with passive lysis buffer and quantified according to the Luciferase Assay System (Promega) protocol. Total protein levels were quantified using the Pierce BCA Protein Assay (Thermo Scientific) and used to normalize luciferase units between wells.

### **RNAi**

150 pmol of SMARTpool: ON-TARGETplus siRNA pools for MID1IP1, MID1, and CCT2 or ON-TARGETplus Non-targeting Control Pool (Dharmacon) were transfected into cells using 4 µL lipofectamine RNAiMax (Life Technologies) in Opti-MEM. Two such transfections were conducted successively over the course of two days. On the day following the second transfection, cells were split into five wells in 24 well plates at  $.1 \times 10^6$  cells per well in 1 mL media. At 24 hours after replating, cells were infected with luciferase reporter virus or collected for RNA extraction/Western blotting. Infected cells were assayed for reporter expression 48 hours post-infection.

## **RNA Extraction and qPCR**

RNA was extracted using the RNeasy Mini Kit (Qiagen) from each well in 24 well plates at confluence. 350  $\mu$ L Buffer RLT was used for lysis. 2  $\mu$ g RNA was treated with 2  $\mu$ L DNase I recombinant, RNase-free (Roche) in 20  $\mu$ L 1x incubation buffer for 20 min at 37°C, then 2  $\mu$ L 25 mM EDTA was added and the enzyme was heat inactivated at 75°C for 10 min. Reverse transcription was carried out using the High-Capacity cDNA Reverse Transcription kit (ThermoFisher). qPCR using 5  $\mu$ L cDNA and FastStart universal SYBR Green Master (Roche) was carried out with 7500 Fast Real-Time PCR System (Applied Biosystems).

## **Cloning**

A CRISPR guide sequence targeting MID1IP1 was designed and cloned into the lentiCRISPR plasmid according to Shalem et al. (2014). The following primers were used for cloning:

Nontargeting (forward) – caccggagccggaccgccacgtaa

Nontargeting (reverse) – aaacttacgtggcgggtccggctcc

MID1IP1 (forward) – caccggggcgccaatgaagcgattca

MID1IP1 (reverse) – aaactgaatcgcttcattggcgccc

The nontargeting guide sequence (gagccggaccgccacgtaa) used in the design of these primers was obtained from Michael Metzger through personal correspondence (CUMC, at the time).

MID1IP1 and MID1 human cDNA sequences were obtained from the Mammalian Gene Collection (MGC) (Dharmacon) and cloned into pcDNA3.1 3xFlag. Primers were designed with

restriction sites and PCR with KOD Hot Start DNA Polymerase (EMD Millipore) was used to produce cDNA sequences which could be cut and ligated into cut pcDNA3.1 3xFlag. For MID1, the HA tag was included in the forward primer and restriction sites were used which cut out the 3xFlag tag. The following primers were used:

EcoRI + MID1IP1 (forward) –

5' gaattcgatgcaaactctgacacactaca 3'

pOTB7 (MID1IP1 containing plasmid) + XhoI (reverse) –

5' GCACCCGACATAGATGCCTCGAG 3'

NheI + HA + MID1 (forward) –

5' ggaaaGCTAGCatgTACCCATACGATGTTCCAGATTACGCTgaaacactggagtcaga 3'

MID1 + XhoI (reverse) –

5' ttccCTCGAGtcacggcagctgctctgt 3'

These constructs express N-terminal tagged MID1IP1 and MID1. Cloned plasmids were sequenced using GENEWIZ.

### **Generation of CRISPR Cells**

1 mL VSV-G pseudotyped lentiviral delivery vectors containing the CRISPR machinery and guide RNA sequence were used to infect 293 cells seeded at  $1 \times 10^9$  cells in 10 cm plates in 10

mL media. After 48 hours, cells were placed in media containing 1 µg/mL puromycin (Sigma) for antibiotic selection and grown for two weeks. Surviving colonies were picked and expanded.

### **DNA Sequencing and qPCR of CRISPR Cell Lines**

DNA was extracted from the CRISPR cell lines that were selected for by antibiotic resistance using the DNeasy Blood & Tissue Kit (Qiagen). The CRISPR targeting site in MID1IP1 was amplified with PCR using the following primers:

Forward – 5' TGAGCTCAGGCTTTTCGGAG 3'

Reverse – 5' TGTTCAGGTCTTCTTCGCC 3'

Amplified PCR products obtained from the CRISPR targeted cell lines were ligated into the pJet1.2/blunt plasmid DNA from the CloneJET PCR Cloning kit (ThermoFisher) according to the manufacturer's protocol. The ligation mixtures were used to transform DH5α cells by heat shock (15 min incubation on ice, 30 sec heat shock at 42°C, 1 min on ice; then 750 µL LB media was added and the solution was shaken for 45 min at 37°C). The bacteria were plated on medium with 100µ/mL ampicillin. Six colonies were picked per CRISPR cell line and plasmids were extracted from them using the QIAquick Gel Extraction Kit (Qiagen). DNA sequencing of the plasmids was performed by GENEWIZ.

To detect the presence of wild type or mutant alleles for MID1IP1, qPCR using DNA extracted from CRISPR cells and FastStart universal SYBR Green Master (Roche) was carried out with

LightCycler 96 (Roche) (40 cycles of 95°C for 10 sec, 60°C for 10 sec, 72°C for 20 sec) using the following primers:

Wild-type MID1IP1 (forward) – 5' CTCGCTCTTTAACGCCATGAAT 3'

Mutant MID1IP1 (forward) – 5' CTCGCTCTTTAACGCCATGAAAT 3'

Reverse – 5' GTGAACAACATGGACCAGACG 3'

### **Microtubule Polymerization/Depolymerization**

For microtubule stabilization experiments, cells were treated with 0.1 μM taxol (Sigma) for 1 hour, then lysed for Western blotting. For cold stability experiments, cells were incubated at 4°C for 1 hour before being lysed for Western blotting.

### **Overexpression**

Cells seeded the previous day at  $1 \times 10^6$  cells per well in 6 well plates with 2 mL media were transfected with control plasmid (empty vector pcDNA3.1 3xFlag), or 1.5 μg each of plasmids expressing HA-MID1, 3xFLAG-MID1IP1, or both HA-MID1 and 3xFLAG-MID1IP1 at. All cells were co-transfected with 0.75 μg HA-mCAT-1 (Wang and Goff, 2015) to induce expression of the ecotropic virus receptor. A total of 3.75 μg plasmid DNAs were transfected into each well in 100 μL OPTI-MEM, adding control pcDNA3.1 3xFlag to the wells where the aforementioned plasmids did not reach that total. In TE671 cells an additional well of cells was transfected with only control plasmid and no HA-mCAT-1. Transfections were conducted using 6 μL lipofectamine 2000 in 100 μL OPTI-MEM. 4 hrs later, cells were washed and media was changed. At 24 hours post-transfection, cells were split into multiple wells or collected for

Western blotting (for experiments in TE671 cells). On the following day, cells were infected with several dilutions of reporter virus or collected for Western blotting (for experiments in HeLa cells).

### **Cell Lysis and Western Blotting**

Cells were lysed after trypsinization with 50  $\mu$ L CelLytic M Cell Lysis Reagent (Sigma) 24 hours after being seeded at  $.1 \times 10^6$  cells per well in 24 well plates containing 1 mL media. After the addition of lysis buffer, tubes were incubated on ice for 30 min and vortexed every ten minutes, then centrifuged at 13,000 rpm for 15 min at 4°C. Protein loading buffer (5x) (National Diagnostics) was diluted in the supernatants and the mixture was boiled for 3 min. SDS-PAGE electrophoresis using 4-20% acrylamide Mini-PROTEAN® TGX™ Precast Gels (Bio-Rad) (approximately 1 hr run time at 140 V) was conducted, transfers were completed using PVDF membranes (1.5 hr run time at 100 V), and fluorescent antibodies were used for probing according to the protocol stated in the Antibodies section. Images were generated using an Odyssey imaging system (LI-COR).

### **Antibodies**

The following primary antibodies were used at the indicated dilutions in Blocking Buffer for Fluorescent Western Blotting (Rockland): GAPDH (sc-25778; Santa Cruz) at 1:20,000; Flag (F7425; Sigma) at 1:1000; acetylated tubulin (T7451; Sigma) at 1:20,000; detyrosinated tubulin (AB3201; EMD Millipore) at 1:1000; HA (901501; Biolegend) at 1:5000; CCT2/TCP1beta (ab92746; Abcam) at 1:10,000;  $\beta$ -actin (A1978; Sigma) at 1:1000. Filters were incubated in

(buffer) at temp for 1 hr at room temperature or overnight at 4°C, washed 4x with TBST, and then exposed to secondary antibodies.

The following secondary antibodies were used at the indicated dilutions: IRDye® 680RD Goat anti-Rabbit IgG (LI-COR) at 1:20,000; IRDye® 800CW Goat anti-Mouse IgG (LI-COR) at 1:80,000. Filters were incubated at room temperature for 1 h, washed 4x with TBST, and then imaged by an Odyssey imaging system (LI-COR).

### **shRNA**

The human pLKO1 lentiviral shRNA set for TRC Lentiviral Human CCT2 shRNA was obtained from Dharmacon. Lentiviral particles for delivery were made by transfecting 7.5 µg of an shRNA plasmid, 6 µg pCMVΔR8.2, and 1.5 µg VSV-G into a 10 cm dish of 293T cells seeded at  $1 \times 10^9$  the previous day with 45 µg lipofectamine 2000. Virus particles were harvested after 48 hours.

TE cells seeded at  $1 \times 10^6$  in 6 well plates were infected with 100 µL harvested particles in 2 mL media and were placed in media containing 2 µg/mL puromycin 48 hours later. Antibiotic selection continued for two weeks, after which the surviving cells were pooled.

## References

- Aggarwal, A., Hitchen, T.L., Ootes, L., McAllery, S., Wong, A., Nguyen, K., McCluskey, A., Robinson, P.J., and Turville, S.G. (2017). HIV infection is influenced by dynamin at 3 independent points in the viral life cycle. *Traffic* 18, 392-410.
- Aiken, C. (1997). Pseudotyping human immunodeficiency virus type 1 (HIV-1) by the glycoprotein of vesicular stomatitis virus targets HIV-1 entry to an endocytic pathway and suppresses both the requirement for Nef and the sensitivity to cyclosporin A. *J Virol* 71, 5871-5877.
- Ambrose, Z., and Aiken, C. (2014). HIV-1 uncoating: connection to nuclear entry and regulation by host proteins. *Virology* 454-455, 371-379.
- Anand, A.R., Zhao, H., Nagaraja, T., Robinson, L.A., and Ganju, R.K. (2013). N-terminal Slit2 inhibits HIV-1 replication by regulating the actin cytoskeleton. *Retrovirology* 10, 2.
- Aranda-Orgilles, B., Aigner, J., Kunath, M., Lurz, R., Schneider, R., and Schweiger, S. (2008a). Active transport of the ubiquitin ligase MID1 along the microtubules is regulated by protein phosphatase 2A. *PLoS One* 3, e3507.
- Aranda-Orgilles, B., Rutschow, D., Zeller, R., Karagiannidis, A.I., Kohler, A., Chen, C., Wilson, T., Krause, S., Roepcke, S., Lilley, D., *et al.* (2011). Protein phosphatase 2A (PP2A)-specific ubiquitin ligase MID1 is a sequence-dependent regulator of translation efficiency controlling 3-phosphoinositide-dependent protein kinase-1 (PDK-1). *J Biol Chem* 286, 39945-39957.
- Aranda-Orgilles, B., Trockenbacher, A., Winter, J., Aigner, J., Kohler, A., Jastrzebska, E., Stahl, J., Muller, E.C., Otto, A., Wanker, E.E., *et al.* (2008b). The Opitz syndrome gene product MID1 assembles a microtubule-associated ribonucleoprotein complex. *Hum Genet* 123, 163-176.
- Arhel, N. (2010). Revisiting HIV-1 uncoating. *Retrovirology* 7, 96.
- Arriagada, G. (2017). Retroviruses and microtubule-associated motor proteins. *Cell Microbiol* 19.
- Banerjee, A. (1997). Differential effects of colchicine and its B-ring modified analog MTPT on the assembly-independent GTPase activity of purified beta-tubulin isoforms from bovine brain. *Biochem Biophys Res Commun* 231, 698-700.
- Barr, S.D., Smiley, J.R., and Bushman, F.D. (2008). The interferon response inhibits HIV particle production by induction of TRIM22. *PLoS Pathog* 4, e1000007.
- Bartolini, F., and Gundersen, G.G. (2010). Formins and microtubules. *Biochim Biophys Acta* 1803, 164-173.

- Baskaran, R., and Velmurugan, B.K. (2018). Protein phosphatase 2A as therapeutic targets in various disease models. *Life Sci* 210, 40-46.
- Bastos, R.N., Cundell, M.J., and Barr, F.A. (2014). KIF4A and PP2A-B56 form a spatially restricted feedback loop opposing Aurora B at the anaphase central spindle. *J Cell Biol* 207, 683-693.
- Bedi, S., and Ono, A. (2019). Friend or Foe: The Role of the Cytoskeleton in Influenza A Virus Assembly. *Viruses* 11.
- Berger, E.A., Murphy, P.M., and Farber, J.M. (1999). Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. *Annu Rev Immunol* 17, 657-700.
- Berka, U., Hamann, M.V., and Lindemann, D. (2013). Early events in foamy virus-host interaction and intracellular trafficking. *Viruses* 5, 1055-1074.
- Berti, C., Fontanella, B., Ferrentino, R., and Meroni, G. (2004). Mig12, a novel Opitz syndrome gene product partner, is expressed in the embryonic ventral midline and co-operates with Mid1 to bundle and stabilize microtubules. *BMC Cell Biol* 5, 9.
- Bhoj, E.J., Haye, D., Toutain, A., Bonneau, D., Nielsen, I.K., Lund, I.B., Bogaard, P., Leenskold, S., Karaer, K., Wild, K.T., *et al.* (2018). Phenotypic spectrum associated with SPECC1L pathogenic variants: new families and critical review of the nosology of Teebi, Opitz GBBB, and Baraitser-Winter syndromes. *Eur J Med Genet*, 103588.
- Bost, A.G., Venable, D., Liu, L., and Heinz, B.A. (2003). Cytoskeletal requirements for hepatitis C virus (HCV) RNA synthesis in the HCV replicon cell culture system. *J Virol* 77, 4401-4408.
- Bouhouche, A., Benomar, A., Bouslam, N., Chkili, T., and Yahyaoui, M. (2006). Mutation in the epsilon subunit of the cytosolic chaperonin-containing t-complex peptide-1 (Cct5) gene causes autosomal recessive mutilating sensory neuropathy with spastic paraplegia. *J Med Genet* 43, 441-443.
- Brackley, K.I., and Grantham, J. (2010). Subunits of the chaperonin CCT interact with F-actin and influence cell shape and cytoskeletal assembly. *Exp Cell Res* 316, 543-553.
- Braoudaki, M., and Tzortzatou-Stathopoulou, F. (2011). Tumorigenesis related to retroviral infections. *J Infect Dev Ctries* 5, 751-758.
- Brass, A.L., Dykxhoorn, D.M., Benita, Y., Yan, N., Engelman, A., Xavier, R.J., Lieberman, J., and Elledge, S.J. (2008). Identification of host proteins required for HIV infection through a functional genomic screen. *Science* 319, 921-926.
- Briggs, J.A., and Krausslich, H.G. (2011). The molecular architecture of HIV. *J Mol Biol* 410, 491-500.
- Brugmann, S.A., Allen, N.C., James, A.W., Mekonnen, Z., Madan, E., and Helms, J.A. (2010). A primary cilia-dependent etiology for midline facial disorders. *Hum Mol Genet* 19, 1577-1592.

- Buchner, G., Montini, E., Andolfi, G., Quaderi, N., Cainarca, S., Messali, S., Bassi, M.T., Ballabio, A., Meroni, G., and Franco, B. (1999). MID2, a homologue of the Opitz syndrome gene MID1: similarities in subcellular localization and differences in expression during development. *Hum Mol Genet* 8, 1397-1407.
- Bukrinskaya, A., Brichacek, B., Mann, A., and Stevenson, M. (1998). Establishment of a functional human immunodeficiency virus type 1 (HIV-1) reverse transcription complex involves the cytoskeleton. *J Exp Med* 188, 2113-2125.
- Bulinski, J.C., Richards, J.E., and Piperno, G. (1988). Posttranslational modifications of alpha tubulin: detyrosination and acetylation differentiate populations of interphase microtubules in cultured cells. *J Cell Biol* 106, 1213-1220.
- Bushman, F.D., Malani, N., Fernandes, J., D'Orso, I., Cagney, G., Diamond, T.L., Zhou, H., Hazuda, D.J., Espeseth, A.S., Konig, R., *et al.* (2009). Host cell factors in HIV replication: meta-analysis of genome-wide studies. *PLoS Pathog* 5, e1000437.
- Butler, S.L., Hansen, M.S., and Bushman, F.D. (2001). A quantitative assay for HIV DNA integration in vivo. *Nat Med* 7, 631-634.
- Bylund, L., Kytola, S., Lui, W.O., Larsson, C., and Weber, G. (2004). Analysis of the cytogenetic stability of the human embryonal kidney cell line 293 by cytogenetic and STR profiling approaches. *Cytogenet Genome Res* 106, 28-32.
- Cai, D., McEwen, D.P., Martens, J.R., Meyhofer, E., and Verhey, K.J. (2009). Single molecule imaging reveals differences in microtubule track selection between Kinesin motors. *PLoS Biol* 7, e1000216.
- Cainarca, S., Messali, S., Ballabio, A., and Meroni, G. (1999). Functional characterization of the Opitz syndrome gene product (midin): evidence for homodimerization and association with microtubules throughout the cell cycle. *Hum Mol Genet* 8, 1387-1396.
- Caly, L., Kassouf, V.T., Moseley, G.W., Diefenbach, R.J., Cunningham, A.L., and Jans, D.A. (2016). Fast track, dynein-dependent nuclear targeting of human immunodeficiency virus Vpr protein; impaired trafficking in a clinical isolate. *Biochem Biophys Res Commun* 470, 735-740.
- Campbell, E.M., Nunez, R., and Hope, T.J. (2004). Disruption of the actin cytoskeleton can complement the ability of Nef to enhance human immunodeficiency virus type 1 infectivity. *J Virol* 78, 5745-5755.
- Campbell, E.M., Perez, O., Melar, M., and Hope, T.J. (2007). Labeling HIV-1 virions with two fluorescent proteins allows identification of virions that have productively entered the target cell. *Virology* 360, 286-293.
- Campbell, I.D., and Spitzfaden, C. (1994). Building proteins with fibronectin type III modules. *Structure* 2, 333-337.

- Cannon, P.M., Kim, N., Kingsman, S.M., and Kingsman, A.J. (1996). Murine leukemia virus-based Tat-inducible long terminal repeat replacement vectors: a new system for anti-human immunodeficiency virus gene therapy. *J Virol* 70, 8234-8240.
- Capalbo, G., Mueller-Kuller, T., Markovic, S., Klein, S.A., Dietrich, U., Hoelzer, D., Ottmann, O.G., and Scheuring, U.J. (2011). Knockdown of ERM family member moesin in host cells increases HIV type 1 replication. *AIDS Res Hum Retroviruses* 27, 1317-1322.
- Carlton, J.G., and Martin-Serrano, J. (2007). Parallels between cytokinesis and retroviral budding: a role for the ESCRT machinery. *Science* 316, 1908-1912.
- Carnes, S.K., Zhou, J., and Aiken, C. (2018). HIV-1 Engages a Dynein-Dynactin-BICD2 Complex for Infection and Transport to the Nucleus. *J Virol* 92.
- Chen, R., Le Rouzic, E., Kearney, J.A., Mansky, L.M., and Benichou, S. (2004). Vpr-mediated incorporation of UNG2 into HIV-1 particles is required to modulate the virus mutation rate and for replication in macrophages. *J Biol Chem* 279, 28419-28425.
- Chen, X., Sullivan, D.S., and Huffaker, T.C. (1994). Two yeast genes with similarity to TCP-1 are required for microtubule and actin function in vivo. *Proc Natl Acad Sci U S A* 91, 9111-9115.
- Chertova, E., Chertov, O., Coren, L.V., Roser, J.D., Trubey, C.M., Bess, J.W., Jr., Sowder, R.C., 2nd, Barsov, E., Hood, B.L., Fisher, R.J., *et al.* (2006). Proteomic and biochemical analysis of purified human immunodeficiency virus type 1 produced from infected monocyte-derived macrophages. *J Virol* 80, 9039-9052.
- Clark, J., Grznarova, P., Stansell, E., Diehl, W., Lipov, J., Spearman, P., Ruml, T., and Hunter, E. (2013). A Mason-Pfizer Monkey virus Gag-GFP fusion vector allows visualization of capsid transport in live cells and demonstrates a role for microtubules. *PLoS One* 8, e83863.
- Colomer-Lluch, M., Ruiz, A., Moris, A., and Prado, J.G. (2018). Restriction Factors: From Intrinsic Viral Restriction to Shaping Cellular Immunity Against HIV-1. *Front Immunol* 9, 2876.
- Cong, Y., Baker, M.L., Jakana, J., Woolford, D., Miller, E.J., Reissmann, S., Kumar, R.N., Redding-Johanson, A.M., Bath, T.S., Mukhopadhyay, A., *et al.* (2010). 4.0-Å resolution cryo-EM structure of the mammalian chaperonin TRiC/CCT reveals its unique subunit arrangement. *Proc Natl Acad Sci U S A* 107, 4967-4972.
- Conticello, S.G., Harris, R.S., and Neuberger, M.S. (2003). The Vif protein of HIV triggers degradation of the human antiretroviral DNA deaminase APOBEC3G. *Curr Biol* 13, 2009-2013.
- Cordero, D.R., Brugmann, S., Chu, Y., Bajpai, R., Jame, M., and Helms, J.A. (2011). Cranial neural crest cells on the move: their roles in craniofacial development. *Am J Med Genet A* 155a, 270-279.

- Cox, T.C., Allen, L.R., Cox, L.L., Hopwood, B., Goodwin, B., Haan, E., and Suthers, G.K. (2000). New mutations in MID1 provide support for loss of function as the cause of X-linked Opitz syndrome. *Hum Mol Genet* 9, 2553-2562.
- Cundell, M.J., Bastos, R.N., Zhang, T., Holder, J., Gruneberg, U., Novak, B., and Barr, F.A. (2013). The BEG (PP2A-B55/ENSA/Greatwall) pathway ensures cytokinesis follows chromosome separation. *Mol Cell* 52, 393-405.
- D'Cruz, A.A., Babon, J.J., Norton, R.S., Nicola, N.A., and Nicholson, S.E. (2013). Structure and function of the SPRY/B30.2 domain proteins involved in innate immunity. *Protein Sci* 22, 1-10.
- De Rijck, J., de Kogel, C., Demeulemeester, J., Vets, S., El Ashkar, S., Malani, N., Bushman, F.D., Landuyt, B., Husson, S.J., Busschots, K., *et al.* (2013). The BET family of proteins targets moloney murine leukemia virus integration near transcription start sites. *Cell Rep* 5, 886-894.
- Debyser, Z., Christ, F., De Rijck, J., and Gijssbers, R. (2015). Host factors for retroviral integration site selection. *Trends Biochem Sci* 40, 108-116.
- Dehmelt, L., and Halpain, S. (2005). The MAP2/Tau family of microtubule-associated proteins. *Genome Biol* 6, 204.
- Dekker, C., Roe, S.M., McCormack, E.A., Beuron, F., Pearl, L.H., and Willison, K.R. (2011). The crystal structure of yeast CCT reveals intrinsic asymmetry of eukaryotic cytosolic chaperonins. *Embo j* 30, 3078-3090.
- Dekker, C., Stirling, P.C., McCormack, E.A., Filmore, H., Paul, A., Brost, R.L., Costanzo, M., Boone, C., Leroux, M.R., and Willison, K.R. (2008). The interaction network of the chaperonin CCT. *Embo j* 27, 1827-1839.
- Delaney, M.K., Malikov, V., Chai, Q., Zhao, G., and Naghavi, M.H. (2017). Distinct functions of diaphanous-related formins regulate HIV-1 uncoating and transport. *Proc Natl Acad Sci U S A* 114, E6932-e6941.
- Desfarges, S., Salin, B., Calmels, C., Andreola, M.L., Parissi, V., and Fournier, M. (2009). HIV-1 integrase trafficking in *S. cerevisiae*: a useful model to dissect the microtubule network involvement of viral protein nuclear import. *Yeast* 26, 39-54.
- Dharan, A., and Campbell, E.M. (2018). Role of Microtubules and Microtubule-Associated Proteins in HIV-1 Infection. *J Virol* 92.
- Dharan, A., Opp, S., Abdel-Rahim, O., Keceli, S.K., Imam, S., Diaz-Griffero, F., and Campbell, E.M. (2017). Bicaudal D2 facilitates the cytoplasmic trafficking and nuclear import of HIV-1 genomes during infection. *Proc Natl Acad Sci U S A* 114, E10707-e10716.
- Dharan, A., Talley, S., Tripathi, A., Mamede, J.I., Majetschak, M., Hope, T.J., and Campbell, E.M. (2016). KIF5B and Nup358 Cooperatively Mediate the Nuclear Import of HIV-1 during Infection. *PLoS Pathog* 12, e1005700.

- Dodding, M.P., and Way, M. (2011). Coupling viruses to dynein and kinesin-1. *Embo j* 30, 3527-3539.
- Du, H., Huang, Y., Zaghlula, M., Walters, E., Cox, T.C., and Massiah, M.A. (2013). The MID1 E3 ligase catalyzes the polyubiquitination of Alpha4 (alpha4), a regulatory subunit of protein phosphatase 2A (PP2A): novel insights into MID1-mediated regulation of PP2A. *J Biol Chem* 288, 21341-21350.
- Du, H., Wu, K., Didoronkute, A., Levy, M.V., Todi, N., Shchelokova, A., and Massiah, M.A. (2014). MID1 catalyzes the ubiquitination of protein phosphatase 2A and mutations within its Bbox1 domain disrupt polyubiquitination of alpha4 but not of PP2Ac. *PLoS One* 9, e107428.
- Dube, M., Bego, M.G., Paquay, C., and Cohen, E.A. (2010). Modulation of HIV-1-host interaction: role of the Vpu accessory protein. *Retrovirology* 7, 114.
- Dunn, A.Y., Melville, M.W., and Frydman, J. (2001). Review: cellular substrates of the eukaryotic chaperonin TRiC/CCT. *J Struct Biol* 135, 176-184.
- Emery, A., Zhou, S., Pollom, E., and Swanstrom, R. (2017). Characterizing HIV-1 Splicing by Using Next-Generation Sequencing. *J Virol* 91.
- Engelman, A., and Cherepanov, P. (2008). The lentiviral integrase binding protein LEDGF/p75 and HIV-1 replication. *PLoS Pathog* 4, e1000046.
- Eno, C., and Pelegri, F. (2018). Modulation of F-actin dynamics by maternal Mid1ip1L controls germ plasm aggregation and furrow recruitment in the zebrafish embryo. *Development* 145.
- Eno, C., Solanki, B., and Pelegri, F. (2016). aura (mid1ip1l) regulates the cytoskeleton at the zebrafish egg-to-embryo transition. *Development* 143, 1585-1599.
- Fackler, O.T., and Krausslich, H.G. (2006). Interactions of human retroviruses with the host cell cytoskeleton. *Curr Opin Microbiol* 9, 409-415.
- Fauci, A.S., and Desrosiers, R.C. (1997). Pathogenesis of HIV and SIV. In *Retroviruses*, J.M. Coffin, S.H. Hughes, and H.E. Varmus, eds. (Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press).
- Faust, T.B., Binning, J.M., Gross, J.D., and Frankel, A.D. (2017). Making Sense of Multifunctional Proteins: Human Immunodeficiency Virus Type 1 Accessory and Regulatory Proteins and Connections to Transcription. *Annu Rev Virol* 4, 241-260.
- Fellay, J., Shianna, K.V., Ge, D., Colombo, S., Ledergerber, B., Weale, M., Zhang, K., Gumbs, C., Castagna, A., Cossarizza, A., *et al.* (2007). A whole-genome association study of major determinants for host control of HIV-1. *Science* 317, 944-947.
- Feng, Y., Baig, T.T., Love, R.P., and Chelico, L. (2014). Suppression of APOBEC3-mediated restriction of HIV-1 by Vif. *Front Microbiol* 5, 450.

- Fernandez, J., Portilho, D.M., Danckaert, A., Munier, S., Becker, A., Roux, P., Zambo, A., Shorte, S., Jacob, Y., Vidalain, P.O., *et al.* (2015). Microtubule-associated proteins 1 (MAP1) promote human immunodeficiency virus type I (HIV-1) intracytoplasmic routing to the nucleus. *J Biol Chem* *290*, 4631-4646.
- Fislova, T., Thomas, B., Graef, K.M., and Fodor, E. (2010). Association of the influenza virus RNA polymerase subunit PB2 with the host chaperonin CCT. *J Virol* *84*, 8691-8699.
- Fontanella, B., Russolillo, G., and Meroni, G. (2008). MID1 mutations in patients with X-linked Opitz G/BBB syndrome. *Hum Mutat* *29*, 584-594.
- Forshey, B.M., von Schwedler, U., Sundquist, W.I., and Aiken, C. (2002). Formation of a human immunodeficiency virus type 1 core of optimal stability is crucial for viral replication. *J Virol* *76*, 5667-5677.
- Frydman, J., Nimmesgern, E., Erdjument-Bromage, H., Wall, J.S., Tempst, P., and Hartl, F.U. (1992). Function in protein folding of TRiC, a cytosolic ring complex containing TCP-1 and structurally related subunits. *Embo j* *11*, 4767-4778.
- Frydman, J., Nimmesgern, E., Ohtsuka, K., and Hartl, F.U. (1994). Folding of nascent polypeptide chains in a high molecular mass assembly with molecular chaperones. *Nature* *370*, 111-117.
- Gallo, D.E., and Hope, T.J. (2012). Knockdown of MAP4 and DNAL1 produces a post-fusion and pre-nuclear translocation impairment in HIV-1 replication. *Virology* *422*, 13-21.
- Gao, Y., Thomas, J.O., Chow, R.L., Lee, G.H., and Cowan, N.J. (1992). A cytoplasmic chaperonin that catalyzes beta-actin folding. *Cell* *69*, 1043-1050.
- Gao, Y., Vainberg, I.E., Chow, R.L., and Cowan, N.J. (1993). Two cofactors and cytoplasmic chaperonin are required for the folding of alpha- and beta-tubulin. *Mol Cell Biol* *13*, 2478-2485.
- Gaudin, R., de Alencar, B.C., Arhel, N., and Benaroch, P. (2013). HIV trafficking in host cells: motors wanted! *Trends Cell Biol* *23*, 652-662.
- Gaudin, R., de Alencar, B.C., Jouve, M., Berre, S., Le Boudier, E., Schindler, M., Varthaman, A., Gobert, F.X., and Benaroch, P. (2012). Critical role for the kinesin KIF3A in the HIV life cycle in primary human macrophages. *J Cell Biol* *199*, 467-479.
- Gholkar, A.A., Senese, S., Lo, Y.C., Vides, E., Contreras, E., Hodara, E., Capri, J., Whitelegge, J.P., and Torres, J.Z. (2016). The X-Linked-Intellectual-Disability-Associated Ubiquitin Ligase Mid2 Interacts with Astrin and Regulates Astrin Levels to Promote Cell Division. *Cell Rep* *14*, 180-188.
- Gladue, D.P., Holinka, L.G., Fernandez-Sainz, I.J., Prarat, M.V., O'Donnell, V., Vepkhvadze, N.G., Lu, Z., Risatti, G.R., and Borca, M.V. (2011). Interaction between Core protein of classical swine fever virus with cellular IQGAP1 protein appears essential for virulence in swine. *Virology* *412*, 68-74.

Goedert, M., Jakes, R., Qi, Z., Wang, J.H., and Cohen, P. (1995). Protein phosphatase 2A is the major enzyme in brain that dephosphorylates tau protein phosphorylated by proline-directed protein kinases or cyclic AMP-dependent protein kinase. *J Neurochem* 65, 2804-2807.

Goff, S.P. (2007). Host factors exploited by retroviruses. *Nat Rev Microbiol* 5, 253-263.

Goff, S.P. (2018). Cellular Factors That Regulate Retrovirus Uncoating and Reverse Transcription. In *Retrovirus-Cell Interactions*, L.J. Parent, ed. (London: Academic Press), pp. 51-112.

Guardia, C.M., Farias, G.G., Jia, R., Pu, J., and Bonifacino, J.S. (2016). BORC Functions Upstream of Kinesins 1 and 3 to Coordinate Regional Movement of Lysosomes along Different Microtubule Tracks. *Cell Rep* 17, 1950-1961.

Guest, S.T., Kratche, Z.R., Bollig-Fischer, A., Haddad, R., and Ethier, S.P. (2015). Two members of the TRiC chaperonin complex, CCT2 and TCP1 are essential for survival of breast cancer cells and are linked to driving oncogenes. *Exp Cell Res* 332, 223-235.

Gundersen, G.G., Khawaja, S., and Bulinski, J.C. (1987). Postpolymerization detyrosination of alpha-tubulin: a mechanism for subcellular differentiation of microtubules. *J Cell Biol* 105, 251-264.

Gupta, S.S., Maetzig, T., Maertens, G.N., Sharif, A., Rothe, M., Weidner-Glunde, M., Galla, M., Schambach, A., Cherepanov, P., and Schulz, T.F. (2013). Bromo- and extraterminal domain chromatin regulators serve as cofactors for murine leukemia virus integration. *J Virol* 87, 12721-12736.

Haedicke, J., de Los Santos, K., Goff, S.P., and Naghavi, M.H. (2008). The Ezrin-radixin-moesin family member ezrin regulates stable microtubule formation and retroviral infection. *J Virol* 82, 4665-4670.

Han, K., Lou, D.I., and Sawyer, S.L. (2011a). Identification of a genomic reservoir for new TRIM genes in primate genomes. *PLoS Genet* 7, e1002388.

Han, X., Du, H., and Massiah, M.A. (2011b). Detection and characterization of the in vitro e3 ligase activity of the human MID1 protein. *J Mol Biol* 407, 505-520.

Hancock, W.O. (2014). Bidirectional cargo transport: moving beyond tug of war. *Nat Rev Mol Cell Biol* 15, 615-628.

Harris, R.S., Bishop, K.N., Sheehy, A.M., Craig, H.M., Petersen-Mahrt, S.K., Watt, I.N., Neuberger, M.S., and Malim, M.H. (2003). DNA deamination mediates innate immunity to retroviral infection. *Cell* 113, 803-809.

Hartl, F.U., and Hayer-Hartl, M. (2002). Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science* 295, 1852-1858.

- Herold, N., Anders-Osswein, M., Glass, B., Eckhardt, M., Muller, B., and Krausslich, H.G. (2014). HIV-1 entry in SupT1-R5, CEM-ss, and primary CD4+ T cells occurs at the plasma membrane and does not require endocytosis. *J Virol* 88, 13956-13970.
- Hong, S., Choi, G., Park, S., Chung, A.S., Hunter, E., and Rhee, S.S. (2001). Type D retrovirus Gag polyprotein interacts with the cytosolic chaperonin TRiC. *J Virol* 75, 2526-2534.
- Hook, P., and Vallee, R. (2012). Dynein dynamics. *Nat Struct Mol Biol* 19, 467-469.
- Hook, P., and Vallee, R.B. (2006). The dynein family at a glance. *J Cell Sci* 119, 4369-4371.
- Horwich, A.L., Fenton, W.A., Chapman, E., and Farr, G.W. (2007). Two families of chaperonin: physiology and mechanism. *Annu Rev Cell Dev Biol* 23, 115-145.
- Hu, W.S., and Hughes, S.H. (2012). HIV-1 reverse transcription. *Cold Spring Harb Perspect Med* 2.
- Hulme, A.E., and Hope, T.J. (2014). The cyclosporin A washout assay to detect HIV-1 uncoating in infected cells. *Methods Mol Biol* 1087, 37-46.
- Hulme, A.E., Kelley, Z., Okocha, E.A., and Hope, T.J. (2015). Identification of capsid mutations that alter the rate of HIV-1 uncoating in infected cells. *J Virol* 89, 643-651.
- Hulme, A.E., Perez, O., and Hope, T.J. (2011). Complementary assays reveal a relationship between HIV-1 uncoating and reverse transcription. *Proc Natl Acad Sci U S A* 108, 9975-9980.
- Hurley, J.H. (2010). The ESCRT complexes. *Crit Rev Biochem Mol Biol* 45, 463-487.
- Hurley, J.H., and Hanson, P.I. (2010). Membrane budding and scission by the ESCRT machinery: it's all in the neck. *Nat Rev Mol Cell Biol* 11, 556-566.
- Inoue, Y., Aizaki, H., Hara, H., Matsuda, M., Ando, T., Shimoji, T., Murakami, K., Masaki, T., Shoji, I., Homma, S., *et al.* (2011). Chaperonin TRiC/CCT participates in replication of hepatitis C virus genome via interaction with the viral NS5B protein. *Virology* 410, 38-47.
- Iwase, S.C., Miyazato, P., Katsuya, H., Islam, S., Yang, B.T.J., Ito, J., Matsuo, M., Takeuchi, H., Ishida, T., Matsuda, K., *et al.* (2019). HIV-1 DNA-capture-seq is a useful tool for the comprehensive characterization of HIV-1 provirus. *Sci Rep* 9, 12326.
- Janke, C., and Bulinski, J.C. (2011). Post-translational regulation of the microtubule cytoskeleton: mechanisms and functions. *Nat Rev Mol Cell Biol* 12, 773-786.
- Javadpour, P., Dargahi, L., Ahmadiani, A., and Ghasemi, R. (2019). To be or not to be: PP2A as a dual player in CNS functions, its role in neurodegeneration, and its interaction with brain insulin signaling. *Cell Mol Life Sci* 76, 2277-2297.

- Jayappa, K.D., Ao, Z., Wang, X., Mouland, A.J., Shekhar, S., Yang, X., and Yao, X. (2015). Human immunodeficiency virus type 1 employs the cellular dynein light chain 1 protein for reverse transcription through interaction with its integrase protein. *J Virol* *89*, 3497-3511.
- Jeong, J., Mao, J., Tenzen, T., Kottmann, A.H., and McMahon, A.P. (2004). Hedgehog signaling in the neural crest cells regulates the patterning and growth of facial primordia. *Genes Dev* *18*, 937-951.
- Kalisman, N., Adams, C.M., and Levitt, M. (2012). Subunit order of eukaryotic TRiC/CCT chaperonin by cross-linking, mass spectrometry, and combinatorial homology modeling. *Proc Natl Acad Sci U S A* *109*, 2884-2889.
- Kalpana, G.V., Marmon, S., Wang, W., Crabtree, G.R., and Goff, S.P. (1994). Binding and stimulation of HIV-1 integrase by a human homolog of yeast transcription factor SNF5. *Science* *266*, 2002-2006.
- Kasembeli, M., Lau, W.C., Roh, S.H., Eckols, T.K., Frydman, J., Chiu, W., and Twardy, D.J. (2014). Modulation of STAT3 folding and function by TRiC/CCT chaperonin. *PLoS Biol* *12*, e1001844.
- Kashuba, E., Pokrovskaja, K., Klein, G., and Szekely, L. (1999). Epstein-Barr virus-encoded nuclear protein EBNA-3 interacts with the epsilon-subunit of the T-complex protein 1 chaperonin complex. *J Hum Virol* *2*, 33-37.
- Katsuya, H., Islam, S., Tan, B.J.Y., Ito, J., Miyazato, P., Matsuo, M., Inada, Y., Iwase, S.C., Uchiyama, Y., Hata, H., *et al.* (2019). The Nature of the HTLV-1 Provirus in Naturally Infected Individuals Analyzed by the Viral DNA-Capture-Seq Approach. *Cell Rep* *29*, 724-735.e724.
- Khan, A.S., Bodem, J., Buseyne, F., Gessain, A., Johnson, W., Kuhn, J.H., Kuzmak, J., Lindemann, D., Linial, M.L., Lochelt, M., *et al.* (2018). Spumaretroviruses: Updated taxonomy and nomenclature. *Virology* *516*, 158-164.
- Khawaja, S., Gundersen, G.G., and Bulinski, J.C. (1988). Enhanced stability of microtubules enriched in deetyrosinated tubulin is not a direct function of deetyrosination level. *J Cell Biol* *106*, 141-149.
- Kim, C.W., Moon, Y.A., Park, S.W., Cheng, D., Kwon, H.J., and Horton, J.D. (2010). Induced polymerization of mammalian acetyl-CoA carboxylase by MIG12 provides a tertiary level of regulation of fatty acid synthesis. *Proc Natl Acad Sci U S A* *107*, 9626-9631.
- Kim, K., Dauphin, A., Komurlu, S., McCauley, S.M., Yurkovetskiy, L., Carbone, C., Diehl, W.E., Strambio-De-Castillia, C., Campbell, E.M., and Luban, J. (2019). Cyclophilin A protects HIV-1 from restriction by human TRIM5alpha. *Nat Microbiol* *4*, 2044-2051.
- Kim, S., Willison, K.R., and Horwich, A.L. (1994). Cytosolic chaperonin subunits have a conserved ATPase domain but diverged polypeptide-binding domains. *Trends Biochem Sci* *19*, 543-548.

- King, S.J., and Schroer, T.A. (2000). Dynactin increases the processivity of the cytoplasmic dynein motor. *Nat Cell Biol* 2, 20-24.
- Knee, K.M., Sergeeva, O.A., and King, J.A. (2013). Human TRiC complex purified from HeLa cells contains all eight CCT subunits and is active in vitro. *Cell Stress Chaperones* 18, 137-144.
- Kong, M., Ditsworth, D., Lindsten, T., and Thompson, C.B. (2009). Alpha4 is an essential regulator of PP2A phosphatase activity. *Mol Cell* 36, 51-60.
- Konig, R., Zhou, Y., Elleder, D., Diamond, T.L., Bonamy, G.M., Irelan, J.T., Chiang, C.Y., Tu, B.P., De Jesus, P.D., Lilley, C.E., *et al.* (2008). Global analysis of host-pathogen interactions that regulate early-stage HIV-1 replication. *Cell* 135, 49-60.
- Konishi, Y., and Setou, M. (2009). Tubulin tyrosination navigates the kinesin-1 motor domain to axons. *Nat Neurosci* 12, 559-567.
- Krauss, S., Foerster, J., Schneider, R., and Schweiger, S. (2008). Protein phosphatase 2A and rapamycin regulate the nuclear localization and activity of the transcription factor GLI3. *Cancer Res* 68, 4658-4665.
- Krupovic, M., Blomberg, J., Coffin, J.M., Dasgupta, I., Fan, H., Geering, A.D., Gifford, R., Harrach, B., Hull, R., Johnson, W., *et al.* (2018). Ortervirales: New Virus Order Unifying Five Families of Reverse-Transcribing Viruses. *J Virol* 92.
- Kruszka, P., Li, D., Harr, M.H., Wilson, N.R., Swarr, D., McCormick, E.M., Chiavacci, R.M., Li, M., Martinez, A.F., Hart, R.A., *et al.* (2015). Mutations in SPECC1L, encoding sperm antigen with calponin homology and coiled-coil domains 1-like, are found in some cases of autosomal dominant Opitz G/BBB syndrome. *J Med Genet* 52, 104-110.
- Kubota, H., Hynes, G., Carne, A., Ashworth, A., and Willison, K. (1994). Identification of six Tcp-1-related genes encoding divergent subunits of the TCP-1-containing chaperonin. *Curr Biol* 4, 89-99.
- Kubota, H., Hynes, G., and Willison, K. (1995). The eighth Cct gene, Cctq, encoding the theta subunit of the cytosolic chaperonin containing TCP-1. *Gene* 154, 231-236.
- Landry, J.R., Rouhi, A., Medstrand, P., and Mager, D.L. (2002). The Opitz syndrome gene Mid1 is transcribed from a human endogenous retroviral promoter. *Mol Biol Evol* 19, 1934-1942.
- Le Rouzic, E., and Benichou, S. (2005). The Vpr protein from HIV-1: distinct roles along the viral life cycle. *Retrovirology* 2, 11.
- Lee, M.J., Stephenson, D.A., Groves, M.J., Sweeney, M.G., Davis, M.B., An, S.F., Houlden, H., Salih, M.A., Timmerman, V., de Jonghe, P., *et al.* (2003). Hereditary sensory neuropathy is caused by a mutation in the delta subunit of the cytosolic chaperonin-containing t-complex peptide-1 (Cct4) gene. *Hum Mol Genet* 12, 1917-1925.

- Lee, Y.M., and Kim, W. (2003). Association of human kinesin superfamily protein member 4 with BRCA2-associated factor 35. *Biochem J* 374, 497-503.
- Lehmann, M., Nikolic, D.S., and Piguet, V. (2011). How HIV-1 takes advantage of the cytoskeleton during replication and cell-to-cell transmission. *Viruses* 3, 1757-1776.
- Leitner, A., Joachimiak, L.A., Bracher, A., Monkemeyer, L., Walzthoeni, T., Chen, B., Pechmann, S., Holmes, S., Cong, Y., Ma, B., *et al.* (2012). The molecular architecture of the eukaryotic chaperonin TRiC/CCT. *Structure* 20, 814-825.
- Lemay, J., Maidou-Peindara, P., Bader, T., Ennifar, E., Rain, J.C., Benarous, R., and Liu, L.X. (2008). HuR interacts with human immunodeficiency virus type 1 reverse transcriptase, and modulates reverse transcription in infected cells. *Retrovirology* 5, 47.
- LeNoue-Newton, M., Watkins, G.R., Zou, P., Germane, K.L., McCorvey, L.R., Wadzinski, B.E., and Spiller, B.W. (2011). The E3 ubiquitin ligase- and protein phosphatase 2A (PP2A)-binding domains of the Alpha4 protein are both required for Alpha4 to inhibit PP2A degradation. *J Biol Chem* 286, 17665-17671.
- Lewis, P., Hensel, M., and Emerman, M. (1992). Human immunodeficiency virus infection of cells arrested in the cell cycle. *Embo j* 11, 3053-3058.
- Lewis, P.F., and Emerman, M. (1994). Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. *J Virol* 68, 510-516.
- Li, B., Zhou, T., and Zou, Y. (2016). Mid1/Mid2 expression in craniofacial development and a literature review of X-linked opitz syndrome. *Mol Genet Genomic Med* 4, 95-105.
- Li, R., and Gundersen, G.G. (2008). Beyond polymer polarity: how the cytoskeleton builds a polarized cell. *Nat Rev Mol Cell Biol* 9, 860-873.
- Lin, T.C., Neuner, A., and Schiebel, E. (2015). Targeting of gamma-tubulin complexes to microtubule organizing centers: conservation and divergence. *Trends Cell Biol* 25, 296-307.
- Lin, Y.C., Boone, M., Meuris, L., Lemmens, I., Van Roy, N., Soete, A., Reumers, J., Moisse, M., Plaisance, S., Drmanac, R., *et al.* (2014). Genome dynamics of the human embryonic kidney 293 lineage in response to cell biology manipulations. *Nature communications* 5, 4767.
- Lindemann, D., and Rethwilm, A. (2011). Foamy virus biology and its application for vector development. *Viruses* 3, 561-585.
- Linial, M.L. (1999). Foamy viruses are unconventional retroviruses. *J Virol* 73, 1747-1755.
- Liou, A.K., and Willison, K.R. (1997). Elucidation of the subunit orientation in CCT (chaperonin containing TCP1) from the subunit composition of CCT micro-complexes. *Embo j* 16, 4311-4316.

- Liu, E., Knutzen, C.A., Krauss, S., Schweiger, S., and Chiang, G.G. (2011). Control of mTORC1 signaling by the Opitz syndrome protein MID1. *Proc Natl Acad Sci U S A* 108, 8680-8685.
- Liu, F., Grundke-Iqbal, I., Iqbal, K., and Gong, C.X. (2005). Contributions of protein phosphatases PP1, PP2A, PP2B and PP5 to the regulation of tau phosphorylation. *Eur J Neurosci* 22, 1942-1950.
- Liu, J., Prickett, T.D., Elliott, E., Meroni, G., and Brautigan, D.L. (2001). Phosphorylation and microtubule association of the Opitz syndrome protein mid-1 is regulated by protein phosphatase 2A via binding to the regulatory subunit alpha 4. *Proc Natl Acad Sci U S A* 98, 6650-6655.
- Luban, J., Bossolt, K.L., Franke, E.K., Kalpana, G.V., and Goff, S.P. (1993). Human immunodeficiency virus type 1 Gag protein binds to cyclophilins A and B. *Cell* 73, 1067-1078.
- Luduena, R.F., and Roach, M.C. (1991). Tubulin sulfhydryl groups as probes and targets for antimitotic and antimicrotubule agents. *Pharmacol Ther* 49, 133-152.
- Lukic, Z., Dharan, A., Fricke, T., Diaz-Griffero, F., and Campbell, E.M. (2014). HIV-1 uncoating is facilitated by dynein and kinesin 1. *J Virol* 88, 13613-13625.
- Mabit, H., Nakano, M.Y., Prank, U., Saam, B., Dohner, K., Sodeik, B., and Greber, U.F. (2002). Intact microtubules support adenovirus and herpes simplex virus infections. *J Virol* 76, 9962-9971.
- Maia, N., Nabais Sa, M.J., Tkachenko, N., Soares, G., Marques, I., Rodrigues, B., Fortuna, A.M., Santos, R., de Brouwer, A.P.M., and Jorge, P. (2017). Two Novel Pathogenic MID1 Variants and Genotype-Phenotype Correlation Reanalysis in X-Linked Opitz G/BBB Syndrome. *Mol Syndromol* 9, 45-51.
- Malikov, V., da Silva, E.S., Jovasevic, V., Bennett, G., de Souza Aranha Vieira, D.A., Schulte, B., Diaz-Griffero, F., Walsh, D., and Naghavi, M.H. (2015). HIV-1 capsids bind and exploit the kinesin-1 adaptor FEZ1 for inward movement to the nucleus. *Nature communications* 6, 6660.
- Malikov, V., and Naghavi, M.H. (2017). Localized Phosphorylation of a Kinesin-1 Adaptor by a Capsid-Associated Kinase Regulates HIV-1 Motility and Uncoating. *Cell Rep* 20, 2792-2799.
- Malim, M.H., Hauber, J., Le, S.Y., Maizel, J.V., and Cullen, B.R. (1989). The HIV-1 rev trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. *Nature* 338, 254-257.
- Marmorstein, L.Y., Kinev, A.V., Chan, G.K., Bochar, D.A., Beniya, H., Epstein, J.A., Yen, T.J., and Shiekhattar, R. (2001). A human BRCA2 complex containing a structural DNA binding component influences cell cycle progression. *Cell* 104, 247-257.
- Marsh, M., and Helenius, A. (2006). Virus entry: open sesame. *Cell* 124, 729-740.
- McClure, M.O., Sommerfelt, M.A., Marsh, M., and Weiss, R.A. (1990). The pH independence of mammalian retrovirus infection. *J Gen Virol* 71 ( Pt 4), 767-773.

- McConnell, J.L., Watkins, G.R., Soss, S.E., Franz, H.S., McCorvey, L.R., Spiller, B.W., Chazin, W.J., and Wadzinski, B.E. (2010). Alpha4 is a ubiquitin-binding protein that regulates protein serine/threonine phosphatase 2A ubiquitination. *Biochemistry* 49, 1713-1718.
- McDonald, D., Vodicka, M.A., Lucero, G., Svitkina, T.M., Borisy, G.G., Emerman, M., and Hope, T.J. (2002). Visualization of the intracellular behavior of HIV in living cells. *J Cell Biol* 159, 441-452.
- McNab, F.W., Rajsbaum, R., Stoye, J.P., and O'Garra, A. (2011). Tripartite-motif proteins and innate immune regulation. *Curr Opin Immunol* 23, 46-56.
- Melki, R., Vainberg, I.E., Chow, R.L., and Cowan, N.J. (1993). Chaperonin-mediated folding of vertebrate actin-related protein and gamma-tubulin. *J Cell Biol* 122, 1301-1310.
- Morris, E.J., Nader, G.P., Ramalingam, N., Bartolini, F., and Gundersen, G.G. (2014). Kif4 interacts with EB1 and stabilizes microtubules downstream of Rho-mDia in migrating fibroblasts. *PLoS One* 9, e91568.
- Naghavi, M.H. (2014). Stable microtubule subsets facilitate early HIV-1 infection. *AIDS Res Hum Retroviruses* 30, 211-212.
- Naghavi, M.H., Valente, S., Hatzioannou, T., de Los Santos, K., Wen, Y., Mott, C., Gundersen, G.G., and Goff, S.P. (2007). Moesin regulates stable microtubule formation and limits retroviral infection in cultured cells. *EMBO J* 26, 41-52.
- Narayan, O., and Clements, J.E. (1989). Biology and pathogenesis of lentiviruses. *J Gen Virol* 70 ( Pt 7), 1617-1639.
- Opazo, T., Garces, A., Tapia, D., Barraza, F., Bravo, A., Schwenke, T., Cancino, J., and Arriagada, G. (2017). Functional Evidence of the Involvement of the Dynein Light Chain DYNLRB2 in Murine Leukemia Virus Infection. *J Virol* 91.
- Ospina Stella, A., and Turville, S. (2018). All-Round Manipulation of the Actin Cytoskeleton by HIV. *Viruses* 10.
- Page, K.A., Landau, N.R., and Littman, D.R. (1990). Construction and use of a human immunodeficiency virus vector for analysis of virus infectivity. *J Virol* 64, 5270-5276.
- Parissi, V., Calmels, C., De Soultrait, V.R., Caumont, A., Fournier, M., Chaignepain, S., and Litvak, S. (2001). Functional interactions of human immunodeficiency virus type 1 integrase with human and yeast HSP60. *J Virol* 75, 11344-11353.
- Pawlica, P., and Berthoux, L. (2014). Cytoplasmic dynein promotes HIV-1 uncoating. *Viruses* 6, 4195-4211.
- Pawlica, P., Le Sage, V., Poccardi, N., Tremblay, M.J., Mouland, A.J., and Berthoux, L. (2014). Functional evidence for the involvement of microtubules and dynein motor complexes in TRIM5alpha-mediated restriction of retroviruses. *J Virol* 88, 5661-5676.

- Pereira, E.A., and daSilva, L.L. (2016). HIV-1 Nef: Taking Control of Protein Trafficking. *Traffic* 17, 976-996.
- Perry, J., Short, K.M., Romer, J.T., Swift, S., Cox, T.C., and Ashworth, A. (1999). FXY2/MID2, a gene related to the X-linked Opitz syndrome gene FXY/MID1, maps to Xq22 and encodes a FNIII domain-containing protein that associates with microtubules. *Genomics* 62, 385-394.
- Popov, S., Popova, E., Inoue, M., Wu, Y., and Gottlinger, H. (2018). HIV-1 gag recruits PACSIN2 to promote virus spreading. *Proc Natl Acad Sci U S A* 115, 7093-7098.
- Portran, D., Schaedel, L., Xu, Z., Thery, M., and Nachury, M.V. (2017). Tubulin acetylation protects long-lived microtubules against mechanical ageing. *Nat Cell Biol* 19, 391-398.
- Quaderi, N.A., Schweiger, S., Gaudenz, K., Franco, B., Rugarli, E.I., Berger, W., Feldman, G.J., Volta, M., Andolfi, G., Gilgenkrantz, S., *et al.* (1997). Opitz G/BBB syndrome, a defect of midline development, is due to mutations in a new RING finger gene on Xp22. *Nat Genet* 17, 285-291.
- Rasmussen, I., and Vilhardt, F. (2015). Macropinocytosis is the entry mechanism of amphotropic murine leukemia virus. *J Virol* 89, 1851-1866.
- Robin, N.H., Feldman, G.J., Aronson, A.L., Mitchell, H.F., Weksberg, R., Leonard, C.O., Burton, B.K., Josephson, K.D., Laxova, R., Aleck, K.A., *et al.* (1995). Opitz syndrome is genetically heterogeneous, with one locus on Xp22, and a second locus on 22q11.2. *Nat Genet* 11, 459-461.
- Rolland, T., Tasan, M., Charlotheaux, B., Pevzner, S.J., Zhong, Q., Sahni, N., Yi, S., Lemmens, I., Fontanillo, C., Mosca, R., *et al.* (2014). A proteome-scale map of the human interactome network. *Cell* 159, 1212-1226.
- Rommelaere, H., Van Troys, M., Gao, Y., Melki, R., Cowan, N.J., Vandekerckhove, J., and Ampe, C. (1993). Eukaryotic cytosolic chaperonin contains t-complex polypeptide 1 and seven related subunits. *Proc Natl Acad Sci U S A* 90, 11975-11979.
- Saadi, I., Alkuraya, F.S., Gisselbrecht, S.S., Goessling, W., Cavallesco, R., Turbe-Doan, A., Petrin, A.L., Harris, J., Siddiqui, U., Grix, A.W., Jr., *et al.* (2011). Deficiency of the cytoskeletal protein SPECC1L leads to oblique facial clefting. *Am J Hum Genet* 89, 44-55.
- Sabo, Y., Walsh, D., Barry, D.S., Tinaztepe, S., de Los Santos, K., Goff, S.P., Gundersen, G.G., and Naghavi, M.H. (2013). HIV-1 induces the formation of stable microtubules to enhance early infection. *Cell Host Microbe* 14, 535-546.
- Sanchez, C., Tompa, P., Szucs, K., Friedrich, P., and Avila, J. (1996). Phosphorylation and dephosphorylation in the proline-rich C-terminal domain of microtubule-associated protein 2. *Eur J Biochem* 241, 765-771.
- Sastri, J., and Campbell, E.M. (2011). Recent insights into the mechanism and consequences of TRIM5alpha retroviral restriction. *AIDS Res Hum Retroviruses* 27, 231-238.

- Sattentau, Q.J., and Weiss, R.A. (1988). The CD4 antigen: physiological ligand and HIV receptor. *Cell* 52, 631-633.
- Schaeffer, E., Geleziunas, R., and Greene, W.C. (2001). Human immunodeficiency virus type 1 Nef functions at the level of virus entry by enhancing cytoplasmic delivery of virions. *J Virol* 75, 2993-3000.
- Schaller, T., Bulli, L., Pollpeter, D., Betancor, G., Kutzner, J., Apolonia, L., Herold, N., Burk, R., and Malim, M.H. (2017). Effects of Inner Nuclear Membrane Proteins SUN1/UNC-84A and SUN2/UNC-84B on the Early Steps of HIV-1 Infection. *J Virol* 91.
- Schiff, P.B., Fant, J., and Horwitz, S.B. (1979). Promotion of microtubule assembly in vitro by taxol. *Nature* 277, 665-667.
- Schweiger, S., Foerster, J., Lehmann, T., Suckow, V., Muller, Y.A., Walter, G., Davies, T., Porter, H., van Bokhoven, H., Lunt, P.W., *et al.* (1999). The Opitz syndrome gene product, MID1, associates with microtubules. *Proc Natl Acad Sci U S A* 96, 2794-2799.
- Schweiger, S., Matthes, F., Posey, K., Kickstein, E., Weber, S., Hettich, M.M., Pfurtscheller, S., Ehninger, D., Schneider, R., and Krauss, S. (2017). Resveratrol induces dephosphorylation of Tau by interfering with the MID1-PP2A complex. *Sci Rep* 7, 13753.
- Sebastian, S., and Luban, J. (2005). TRIM5alpha selectively binds a restriction-sensitive retroviral capsid. *Retrovirology* 2, 40.
- Sergeeva, O.A., Chen, B., Haase-Pettingell, C., Ludtke, S.J., Chiu, W., and King, J.A. (2013). Human CCT4 and CCT5 chaperonin subunits expressed in *Escherichia coli* form biologically active homo-oligomers. *J Biol Chem* 288, 17734-17744.
- Serquina, A.K., Das, S.R., Popova, E., Ojelabi, O.A., Roy, C.K., and Gottlinger, H.G. (2013). UPF1 is crucial for the infectivity of human immunodeficiency virus type 1 progeny virions. *J Virol* 87, 8853-8861.
- Shalem, O., Sanjana, N.E., Hartenian, E., Shi, X., Scott, D.A., Mikkelsen, T., Heckl, D., Ebert, B.L., Root, D.E., Doench, J.G., *et al.* (2014). Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science* 343, 84-87.
- Sharma, A., Larue, R.C., Plumb, M.R., Malani, N., Male, F., Slaughter, A., Kessler, J.J., Shkriabai, N., Coward, E., Aiyer, S.S., *et al.* (2013). BET proteins promote efficient murine leukemia virus integration at transcription start sites. *Proc Natl Acad Sci U S A* 110, 12036-12041.
- Short, K.M., and Cox, T.C. (2006). Subclassification of the RBCC/TRIM superfamily reveals a novel motif necessary for microtubule binding. *J Biol Chem* 281, 8970-8980.
- Short, K.M., Hopwood, B., Yi, Z., and Cox, T.C. (2002). MID1 and MID2 homo- and heterodimerise to tether the rapamycin-sensitive PP2A regulatory subunit, alpha 4, to microtubules: implications for the clinical variability of X-linked Opitz GBBB syndrome and other developmental disorders. *BMC Cell Biol* 3, 1.

Sirajuddin, M., Rice, L.M., and Vale, R.D. (2014). Regulation of microtubule motors by tubulin isotypes and post-translational modifications. *Nat Cell Biol* 16, 335-344.

Smith, G.A., Gross, S.P., and Enquist, L.W. (2001). Herpesviruses use bidirectional fast-axonal transport to spread in sensory neurons. *Proc Natl Acad Sci U S A* 98, 3466-3470.

Snider, T.N., and Mishina, Y. (2014). Cranial neural crest cell contribution to craniofacial formation, pathology, and future directions in tissue engineering. *Birth Defects Res C Embryo Today* 102, 324-332.

Soneoka, Y., Cannon, P.M., Ramsdale, E.E., Griffiths, J.C., Romano, G., Kingsman, S.M., and Kingsman, A.J. (1995). A transient three-plasmid expression system for the production of high titer retroviral vectors. *Nucleic Acids Res* 23, 628-633.

Song, Y., and Brady, S.T. (2015). Post-translational modifications of tubulin: pathways to functional diversity of microtubules. *Trends Cell Biol* 25, 125-136.

Sontag, J.M., Nunbhakdi-Craig, V., White, C.L., 3rd, Halpain, S., and Sontag, E. (2012). The protein phosphatase PP2A/B $\alpha$  binds to the microtubule-associated proteins Tau and MAP2 at a motif also recognized by the kinase Fyn: implications for tauopathies. *J Biol Chem* 287, 14984-14993.

Spear, M., Guo, J., Turner, A., Yu, D., Wang, W., Meltzer, B., He, S., Hu, X., Shang, H., Kuhn, J., *et al.* (2014). HIV-1 triggers WAVE2 phosphorylation in primary CD4 T cells and macrophages, mediating Arp2/3-dependent nuclear migration. *J Biol Chem* 289, 6949-6959.

Spieß, C., Meyer, A.S., Reissmann, S., and Frydman, J. (2004). Mechanism of the eukaryotic chaperonin: protein folding in the chamber of secrets. *Trends Cell Biol* 14, 598-604.

Spieß, M., Echbarthi, M., Svanstrom, A., Karlsson, R., and Grantham, J. (2015). Over-Expression Analysis of All Eight Subunits of the Molecular Chaperone CCT in Mammalian Cells Reveals a Novel Function for CCT $\delta$ . *J Mol Biol* 427, 2757-2764.

Steffen, D. (1984). Proviruses are adjacent to c-myc in some murine leukemia virus-induced lymphomas. *Proc Natl Acad Sci U S A* 81, 2097-2101.

Sternlicht, H., Farr, G.W., Sternlicht, M.L., Driscoll, J.K., Willison, K., and Yaffe, M.B. (1993). The t-complex polypeptide 1 complex is a chaperonin for tubulin and actin in vivo. *Proc Natl Acad Sci U S A* 90, 9422-9426.

Stoldt, V., Rademacher, F., Kehren, V., Ernst, J.F., Pearce, D.A., and Sherman, F. (1996). Review: the Cct eukaryotic chaperonin subunits of *Saccharomyces cerevisiae* and other yeasts. *Yeast* 12, 523-529.

Stolp, B., and Fackler, O.T. (2011). How HIV takes advantage of the cytoskeleton in entry and replication. *Viruses* 3, 293-311.

- Stoye, J.P., Blomberg, J., Coffin, J.M., Fan, H., Hahn, B.H., Neil, J., Quackenbush, S., Rethwilm, A., and Tristem, M. (2012). Family Retroviridae. In *Virus Taxonomy—Ninth Report of the International Committee on Taxonomy of Viruses*, M.J.A. A.M.Q. King, E.B. Carstens, E.J.Lefkowitz ed. (London, U.K: Elsevier/Academic Press), pp. 477-494.
- Stremlau, M., Owens, C.M., Perron, M.J., Kiessling, M., Autissier, P., and Sodroski, J. (2004). The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. *Nature* 427, 848-853.
- Su, Y., Qiao, W., Guo, T., Tan, J., Li, Z., Chen, Y., Li, X., Li, Y., Zhou, J., and Chen, Q. (2010). Microtubule-dependent retrograde transport of bovine immunodeficiency virus. *Cell Microbiol* 12, 1098-1107.
- Sundquist, W.I., and Krausslich, H.G. (2012). HIV-1 assembly, budding, and maturation. *Cold Spring Harb Perspect Med* 2, a006924.
- Swanstrom, R., and Wills, J.W. (1997). Synthesis, Assembly, and Processing of Viral Proteins. In *Retroviruses*, J.M. Coffin, S.H. Hughes, and H.E. Varmus, eds. (Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press).
- Tabah, A.A., Tardif, K., and Mansky, L.M. (2014). Anti-HIV-1 activity of Trim 37. *J Gen Virol* 95, 960-967.
- Takata, M.A., Soll, S.J., Emery, A., Blanco-Melo, D., Swanstrom, R., and Bieniasz, P.D. (2018). Global synonymous mutagenesis identifies cis-acting RNA elements that regulate HIV-1 splicing and replication. *PLoS Pathog* 14, e1006824.
- Tam, S., Geller, R., Spiess, C., and Frydman, J. (2006). The chaperonin TRiC controls polyglutamine aggregation and toxicity through subunit-specific interactions. *Nat Cell Biol* 8, 1155-1162.
- Tang, S., Murakami, T., Agresta, B.E., Campbell, S., Freed, E.O., and Levin, J.G. (2001). Human immunodeficiency virus type 1 N-terminal capsid mutants that exhibit aberrant core morphology and are blocked in initiation of reverse transcription in infected cells. *J Virol* 75, 9357-9366.
- Tang, Y., Winkler, U., Freed, E.O., Torrey, T.A., Kim, W., Li, H., Goff, S.P., and Morse, H.C., 3rd (1999). Cellular motor protein KIF-4 associates with retroviral Gag. *J Virol* 73, 10508-10513.
- Tas, R.P., Chazeau, A., Cloin, B.M.C., Lambers, M.L.A., Hoogenraad, C.C., and Kapitein, L.C. (2017). Differentiation between Oppositely Oriented Microtubules Controls Polarized Neuronal Transport. *Neuron* 96, 1264-1271.e1265.
- Taylor, M.P., Koyuncu, O.O., and Enquist, L.W. (2011). Subversion of the actin cytoskeleton during viral infection. *Nat Rev Microbiol* 9, 427-439.
- Telesnitsky, A., and Wolin, S.L. (2016). The Host RNAs in Retroviral Particles. *Viruses* 8.

- Thulasiraman, V., Yang, C.F., and Frydman, J. (1999). In vivo newly translated polypeptides are sequestered in a protected folding environment. *Embo j* 18, 85-95.
- Trockenbacher, A., Suckow, V., Foerster, J., Winter, J., Krauss, S., Ropers, H.H., Schneider, R., and Schweiger, S. (2001). MID1, mutated in Opitz syndrome, encodes an ubiquitin ligase that targets phosphatase 2A for degradation. *Nat Genet* 29, 287-294.
- Tuladhar, R., Yeu, Y., Tyler Piazza, J., Tan, Z., Rene Clemenceau, J., Wu, X., Barrett, Q., Herbert, J., Mathews, D.H., Kim, J., *et al.* (2019). CRISPR-Cas9-based mutagenesis frequently provokes on-target mRNA misregulation. *Nature communications* 10, 4056.
- Uchil, P.D., Quinlan, B.D., Chan, W.T., Luna, J.M., and Mothes, W. (2008). TRIM E3 ligases interfere with early and late stages of the retroviral life cycle. *PLoS Pathog* 4, e16.
- Usmani, S.M., Murooka, T.T., Deruaz, M., Koh, W.H., Sharaf, R.R., Di Pilato, M., Power, K.A., Lopez, P., Hnatiuk, R., Vrbanac, V.D., *et al.* (2019). HIV-1 Balances the Fitness Costs and Benefits of Disrupting the Host Cell Actin Cytoskeleton Early after Mucosal Transmission. *Cell Host Microbe* 25, 73-86.e75.
- Valenzuela-Fernandez, A., Alvarez, S., Gordon-Alonso, M., Barrero, M., Ursa, A., Cabrero, J.R., Fernandez, G., Naranjo-Suarez, S., Yanez-Mo, M., Serrador, J.M., *et al.* (2005). Histone deacetylase 6 regulates human immunodeficiency virus type 1 infection. *Mol Biol Cell* 16, 5445-5454.
- Vallee, R.B., McKenney, R.J., and Ori-McKenney, K.M. (2012). Multiple modes of cytoplasmic dynein regulation. *Nat Cell Biol* 14, 224-230.
- Vallin, J., and Grantham, J. (2019). The role of the molecular chaperone CCT in protein folding and mediation of cytoskeleton-associated processes: implications for cancer cell biology. *Cell Stress Chaperones* 24, 17-27.
- Verhey, K.J., and Hammond, J.W. (2009). Traffic control: regulation of kinesin motors. *Nat Rev Mol Cell Biol* 10, 765-777.
- Vietri, M., Radulovic, M., and Stenmark, H. (2020). The many functions of ESCRTs. *Nat Rev Mol Cell Biol* 21, 25-42.
- Vogt, P.K. (1997a). Historical Introduction to the General Properties of Retroviruses. In *Retroviruses*, J.M. Coffin, S.H. Hughes, and H.E. Varmus, eds. (Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press).
- Vogt, V.M. (1997b). Retroviral Virions and Genomes. In *Retroviruses*, J.M. Coffin, S.H. Hughes, and H.E. Varmus, eds. (Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press).
- Votteler, J., and Sundquist, W.I. (2013). Virus budding and the ESCRT pathway. *Cell Host Microbe* 14, 232-241.

- Wallin, M., and Stromberg, E. (1995). Cold-stable and cold-adapted microtubules. *Int Rev Cytol* 157, 1-31.
- Wang, G.Z., and Goff, S.P. (2015). Postentry restriction of Mason-Pfizer monkey virus in mouse cells. *J Virol* 89, 2813-2819.
- Watkins, G.R., Wang, N., Mazalouskas, M.D., Gomez, R.J., Guthrie, C.R., Kraemer, B.C., Schweiger, S., Spiller, B.W., and Wadzinski, B.E. (2012). Monoubiquitination promotes calpain cleavage of the protein phosphatase 2A (PP2A) regulatory subunit alpha4, altering PP2A stability and microtubule-associated protein phosphorylation. *J Biol Chem* 287, 24207-24215.
- Willison, K.R. (2018). The substrate specificity of eukaryotic cytosolic chaperonin CCT. *Philos Trans R Soc Lond B Biol Sci* 373.
- Wilson, L., Jordan, M.A., Morse, A., and Margolis, R.L. (1982). Interaction of vinblastine with steady-state microtubules in vitro. *J Mol Biol* 159, 125-149.
- Wilson, N.R., Olm-Shipman, A.J., Acevedo, D.S., Palaniyandi, K., Hall, E.G., Kosa, E., Stumpff, K.M., Smith, G.J., Pitstick, L., Liao, E.C., *et al.* (2016). SPECC1L deficiency results in increased adherens junction stability and reduced cranial neural crest cell delamination. *Sci Rep* 6, 17735.
- Winter, J., Basilicata, M.F., Stemmler, M.P., and Krauss, S. (2016). The MID1 protein is a central player during development and in disease. *Front Biosci (Landmark Ed)* 21, 664-682.
- Wlodarchak, N., and Xing, Y. (2016). PP2A as a master regulator of the cell cycle. *Crit Rev Biochem Mol Biol* 51, 162-184.
- Wright, K.M., Du, H., Dagnachew, M., and Massiah, M.A. (2016). Solution structure of the microtubule-targeting COS domain of MID1. *FEBS J* 283, 3089-3102.
- Wright, K.M., Du, H., and Massiah, M.A. (2017). Structural and functional observations of the P151L MID1 mutation reveal alpha4 plays a significant role in X-linked Opitz Syndrome. *Febs j* 284, 2183-2193.
- Wu, G., Zhou, L., Khidr, L., Guo, X.E., Kim, W., Lee, Y.M., Krasieva, T., and Chen, P.L. (2008). A novel role of the chromokinesin Kif4A in DNA damage response. *Cell Cycle* 7, 2013-2020.
- Wu, X., Anderson, J.L., Campbell, E.M., Joseph, A.M., and Hope, T.J. (2006). Proteasome inhibitors uncouple rhesus TRIM5alpha restriction of HIV-1 reverse transcription and infection. *Proc Natl Acad Sci U S A* 103, 7465-7470.
- Xu, Z., Schaedel, L., Portran, D., Aguilar, A., Gaillard, J., Marinkovich, M.P., Thery, M., and Nachury, M.V. (2017). Microtubules acquire resistance from mechanical breakage through intraluminal acetylation. *Science* 356, 328-332.

- Yaffe, M.B., Farr, G.W., Miklos, D., Horwich, A.L., Sternlicht, M.L., and Sternlicht, H. (1992). TCP1 complex is a molecular chaperone in tubulin biogenesis. *Nature* 358, 245-248.
- Yam, A.Y., Xia, Y., Lin, H.T., Burlingame, A., Gerstein, M., and Frydman, J. (2008). Defining the TRiC/CCT interactome links chaperonin function to stabilization of newly made proteins with complex topologies. *Nat Struct Mol Biol* 15, 1255-1262.
- Yang, Y., Fricke, T., and Diaz-Griffero, F. (2013). Inhibition of reverse transcriptase activity increases stability of the HIV-1 core. *J Virol* 87, 683-687.
- Yap, M.W., Nisole, S., Lynch, C., and Stoye, J.P. (2004). Trim5alpha protein restricts both HIV-1 and murine leukemia virus. *Proc Natl Acad Sci U S A* 101, 10786-10791.
- Yoder, A., Guo, J., Yu, D., Cui, Z., Zhang, X.E., and Wu, Y. (2011). Effects of microtubule modulators on HIV-1 infection of transformed and resting CD4 T cells. *J Virol* 85, 3020-3024.
- Young, J.C., Agashe, V.R., Siegers, K., and Hartl, F.U. (2004). Pathways of chaperone-mediated protein folding in the cytosol. *Nat Rev Mol Cell Biol* 5, 781-791.
- Yu, I., Garnham, C.P., and Roll-Mecak, A. (2015). Writing and Reading the Tubulin Code. *J Biol Chem* 290, 17163-17172.
- Yuan, T., Yao, W., Tokunaga, K., Yang, R., and Sun, B. (2016). An HIV-1 capsid binding protein TRIM11 accelerates viral uncoating. *Retrovirology* 13, 72.
- Zan, J., Liu, S., Sun, D.N., Mo, K.K., Yan, Y., Liu, J., Hu, B.L., Gu, J.Y., Liao, M., and Zhou, J.Y. (2017). Rabies Virus Infection Induces Microtubule Depolymerization to Facilitate Viral RNA Synthesis by Upregulating HDAC6. *Front Cell Infect Microbiol* 7, 146.
- Zanchetta, M.E., Napolitano, L.M.R., Maddalo, D., and Meroni, G. (2017). The E3 ubiquitin ligase MID1/TRIM18 promotes atypical ubiquitination of the BRCA2-associated factor 35, BRAF35. *Biochim Biophys Acta Mol Cell Res* 1864, 1844-1854.
- Zhang, J., Wu, X., Zan, J., Wu, Y., Ye, C., Ruan, X., and Zhou, J. (2013). Cellular chaperonin CCTgamma contributes to rabies virus replication during infection. *J Virol* 87, 7608-7621.
- Zheng, Y., Wong, M.L., Alberts, B., and Mitchison, T. (1995). Nucleation of microtubule assembly by a gamma-tubulin-containing ring complex. *Nature* 378, 578-583.
- Zhou, H., Xu, M., Huang, Q., Gates, A.T., Zhang, X.D., Castle, J.C., Stec, E., Ferrer, M., Strulovici, B., Hazuda, D.J., *et al.* (2008). Genome-scale RNAi screen for host factors required for HIV replication. *Cell Host Microbe* 4, 495-504.
- Zhou, J., Scherer, J., Yi, J., and Vallee, R.B. (2018). Role of kinesins in directed adenovirus transport and cytoplasmic exploration. *PLoS Pathog* 14, e1007055.

Zhu, J., Davoli, T., Perriera, J.M., Chin, C.R., Gaiha, G.D., John, S.P., Sigiollot, F.D., Gao, G., Xu, Q., Qu, H., *et al.* (2014). Comprehensive identification of host modulators of HIV-1 replication using multiple orthologous RNAi reagents. *Cell Rep* 9, 752-766.