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Viral and host determinants for
Crm1-mediated nuclear export of HIV RNA

by

David Scott Booth

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biophysics

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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by

David Scott Booth

DEDICATION

This work is dedicated to my family:

my father, Charles Rex Booth,
whose respect for nature and talent for tinkering influenced me to become a scientist;

my mother, Reina Esperanza Acosta de Booth,
who always advocated for my education and endowed me with lots of mañas;

my sister, Holly Lee Booth,
who inspires me to be understanding and to seek greater perspectives;

and my husband, Stephen Randall Lugar,
who challenges me to think and to feel in more profound ways.

I love you all...a lot.

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I first want to recognize the UCSF community for making this work happen. There are many people who I'll mention specifically but many more I won't. More than the sum of their individual contributions is the gestalt that makes this place one of the best for figuring out how biological processes work and for becoming a scientist. What I've learned here is much greater than what can be communicated in this dissertation.

My bosses, Alan Frankel and Yifan Cheng, were the two most directly responsible for this work. I depended on Alan when I was really struggling, and he would step-in to provide support and direction in ways that I didn't know I needed until much later. He helped me focus my curiosity with a lot of patience and still let me explore different directions while maintaining high standards. From Yifan I learned to appreciate the art of doing science and the willingness to test any good idea. He lightened the blows from negative results and excitedly encouraged me to keep on getting more data when things went well. More than anything, I appreciated both of their tremendous faith in my ability at the times I had the most doubts.

In the Frankel and Cheng Labs, I got to work with a cohort of scientists that I respect and admire a lot. I've been lucky to have Rob Nakamura provide wise perspectives on scientific and life pursuits. I always look forward to coffee breaks with Tyler Faust, who teaches me tons through his carefully executed experiments. Bhargavi has been a good partner in interpreting structures of Rev. Jason Fernandes keeps us honest about the complexities of viruses and does more than his share to keep the lab running. Ivan D'Orso helped me appreciate a holistic approach to science. David Crosby provided the ability for our lab to do experiments with viruses. Alan's lab also benefits from some unusually great neighbors in the Yamamoto and

Andino Labs who I view as extended lab mates. In Yifan's lab, it's been great to go through grad school with Kiyoshi Egami, who is as gruff as he is kind. Maofu Liao, Shenping Wu, Augustin Avila-Sakar, Homin Kim, and Xueming Li all taught me how to collect and process EM data, and I learned even more when the lab grew to include David Bulkley, Jean-Paul Armache, and Gira Bhabha. In both labs I had some great summer students, rotation students, and technicians who all taught me some valuable lessons and new ways of seeing science. They are Brian Linhares, Elle Roberson, Christina Hueschen, Isabel Ribeiro, Naomi Yonis, David Paquette, Beth Adams, Julia Jennings, and Rachy Levin. Both labs were part of a center to study HIV accessory and regulatory proteins that led to a great collaboration with Yun Bai and Jennifer Doudna toward the end of grad school and input from a large group of interested scientists.

My qualifying exam was one of the most important events that helped me refine this work and get me to really consider how I want to do science. My wonderful chair was Carol Gross. I will never understand how one person can care so much for so many different people, but Carol absolutely does. She leveled with me and challenged me to seek out new ways of thinking. After quals, she would always stop me in the hall to ask how I was doing, offer me a word of wisdom, and relay her unwavering support.

So much of the work in this thesis could not have been done without the resources provided by another member of my qualifying exam committee, David Agard. He is incredibly generous, and I always could rely on him to be engaged and to keep me thinking—hard—about what was the most important thing to be done. These qualities are reflected in his lab, where I had a lot of friends. The first was Kyle Eagan who was a summer student in the Agard Lab the same time I was in the Frankel Lab. I've enjoyed his rigor, enthusiasm, intellect, and curiosity ever since we shared that first summer at UCSF. Some of the other Agards who especially helped this work

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I was really lucky to rotate in Peter Walter’s Lab before he served on both my qualifying exam and thesis committees. I consider Peter to be the oracle of UCSF because discussing science with him for five minutes provides six months of thinking. He would always go out of his way to encourage me to put my thoughts and self forward. Most of all, he taught me the importance of asking open-ended biological questions. I’m also grateful for the group of post-docs and grad students I met through his lab, especially Ciara Gallagher who was a connection to the lab throughout graduate school.

My refuge during grad school was in Christine Guthrie’s lab. I started going to her lab meetings after my qualifying exam when she declared, “You need more biology.” In the first lab meeting I attended, I heard the words phenotype, phenocopy, and tetra-type tetrad more quickly than I could understand them, so I thought I should stay to learn what they meant and put Christine on my thesis committee. I always felt Christine was my champion. She did so much to instill me with confidence and to open my mind to new possibilities. In her lab, I became part of crew who I consider to be some of the most thoughtful and open-minded scientists. I’ve really appreciated my time and friendships with all of them. Among them is John Abelson, who retired from being a professor at Cal Tech by going back to the bench in Christine’s lab. As a biochemist, he helped translate genetics into a language I could understand, and he guided me in the right direction to get my experiments working. A couple more people in the lab who gave me

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There is a large crew of people during my time at UCSF who really went out of their way to help me. Here are some of the standouts that deserve a lot of gratitude: Rebecca Brown, Julia Molla, and Janet Gumbs for making stuff happen and helping me survive; John Bruning for endless empathy and frivolity and fidelity; Zach Newby for always finding out how I was really doing; John Gross for his integrity as a person and scientist; Stephen Floor for exuding brilliance and compassion; Quincey Justman for building a community; Jaline Gerardin and Gabe Rocklin for teaching me the virtues of carefully and slowly practicing an entire piece; Edwin Rodriguez for dreaming about what is yet to be discovered; Ashley Acevedo for stirring trouble and helping me appreciate viruses; Genevieve Erwin and Charles Greenberg for laughing through Bootcamp;

Hana El-Samad for sharpening my quantitative reasoning; Shannon Noonan, Lauren Goins, Juliet Girard, and Thomas Noriega for trying to make a difference; Monica Guo for teaching me a little bacterial genetics; Lance Raynor and Peter Straus for helping me think and feel through good and bad times; and so many more who have made this place and time remarkable.

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The people who know my best and worst sides are my family. They all have been unfailingly supportive, whatever I chose to do, and always try to get me to have a little more fun. This work is dedicated to all of them. I've also enjoyed encouragement from my extended family David and Lola Mendoza and from my newest family Anne, Matt, Julie, Ellis, and Beck Lugar. Stephen especially deserves an extra word of thanks because he is present when I can't contain my excitement about something I've learned and when things are so nonsensical that I can't contain my exasperation. You're my favorite. Thanks for being there.

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In chapter one, figure four is adapted from previously published work on which I am an author: Daugherty MD, Booth DS, Jayaraman B, Cheng Y, Frankel AD. (2010) HIV Rev response element (RRE) directs assembly of the Rev homooligomer into discrete asymmetric complexes. *Proc Natl Acad Sci USA* 107:12481-6, Copyright (2010), National Academy of Sciences, USA. Its use in this dissertation is consistent with policies of the National Academy of Sciences.

In addition to the work presented here, I have been fortunate to be involved with other projects that resulted in authorship on other publications listed in the Appendix. I have not included this work because these publications but were not germane to this dissertation. I thank my colleagues for the opportunity to work with them on those projects.

Viral and host determinants for Crm1-mediated nuclear export of HIV RNA

by David Scott Booth

ABSTRACT

The human immunodeficiency virus (HIV) protein Rev plays a central role in the viral lifecycle by post-transcriptionally regulating the expression of structural and envelope proteins and by providing genomic RNA for new virions. Rev accomplishes these tasks by interfacing with the host nuclear transport machinery to facilitate the nuclear export of unspliced and partially spliced viral RNAs. A cis-acting element within these RNAs, the Rev Response Element (RRE), directs the assembly of a Rev homooligomer that recruits the nuclear export adaptors Crm1 and Ran. In this dissertation, I share my efforts to understand this critical host-virus interaction. By providing the first structure of the entire complex with supporting biochemistry, this work reveals evolutionary changes in both the host and viral components that determine how HIV adapts the Rev-RRE complex to its human host. Specifically, these results highlight a novel attribute conserved in simian primates for Crm1 to form an ordered dimer that facilitates the cooperative recognition of the Rev-RRE complex and correlates to increased nuclear export of viral RNAs. Mutations in the RRE that augment nuclear export can change how Rev-RRE encounters this Crm1 dimer. These findings lay the groundwork for future studies to potentially develop novel therapeutics that antagonize Crm1 dimerization, to determine a high-resolution structure of the Rev-RRE complex bound to its host cofactors, and to investigate the fate of the virus-host complex during subsequent steps of virion biogenesis.

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CHAPTER 1: Introduction

HIV remains a global plague

Viruses have had a major role in shaping human history. Our DNA is replete with the vestiges of ancient viruses that hint at the roles that viruses may have played in building our genomes¹. The positive selection of genes that ward off viruses further indicates that viruses apply a consistent selective pressure for our evolution². Beyond our genetic information, viruses have defined epochs in modern history³. Currently, Ebola, perhaps the most virulent virus, is gripping the world with the largest known outbreak occurring in West Africa⁴, reminding us that a particle with a genome 0.001% the size of our own can wreak havoc on human populations. Yet, the worst scourge since the 1980s has been human immunodeficiency virus (HIV). Almost 75 million people have contracted HIV and approximately 36 million people have died from acquired immune deficiency syndrome (AIDS), the disease caused by HIV, since the beginning of the pandemic. The most recent available statistics for 2012 report that 35.3 million people are currently living with the disease and 1.6 million people die annually from AIDS⁵.

HIV is an especially pernicious retrovirus because it initially infects immune cells⁶ and can ‘hide’ in latent reservoirs that are refractory to treatment⁷. Hopes for cures have been tempered when HIV was detected in individuals who were considered rid of the virus⁸. In developed nations, drugs can effectively manage and prevent HIV^{9, 10}, but mutations arising from replication allow HIV to evolve resistance to drugs¹¹⁻¹³, necessitating ongoing investigations into new therapeutic targets. Hampering those efforts, however, is the lack of good animal models to study infection^{14, 15}, although some progress has been made in monkeys¹⁶. Even at the most basic level of research, viral proteins have notoriously difficult biochemical properties that can impede

research¹⁷. Thus, work spanning these levels of inquiry can still provide novel insights into HIV biology and treatment.

In this dissertation, I share my efforts to understand how the HIV protein Rev works with its human cofactor Crm1 to facilitate nuclear export of HIV RNA in order to express viral proteins and assemble new virions. This critical step in the viral lifecycle is currently untargeted by any therapeutics¹⁸, so finding specific interactions between HIV and Crm1 could potentially lead to the development of new drugs to fight the virus. More interesting to me, this part of the HIV lifecycle is central for understanding how HIV initiates biogenesis, and studying Rev could potentially reveal important principles about nuclear transport. Working from a biophysical and biochemical perspective, I try to relate the assembly of Rev with Crm1 to nuclear transport activity. This approach has revealed new principles about Crm1 recognizing its viral substrate that could broadly apply to cell biology and has provided insight into adaptations that HIV evolves in order to stimulate nuclear export for increased virion production. This introduction provides a narrative for the development of this project, highlighting important results from the following chapters in order to provide the context for the significance of these findings.

The HIV protein Rev promotes the nuclear export of viral RNAs

HIV biogenesis depends on the proper localization of viral RNAs. Once the virus has integrated into the host genome, a full-length viral RNA is rarely made, and what is made is spliced and exported to produce mRNAs for translating the viral proteins Tat, Rev, and Nef¹⁹ (Figure 1-Early). Tat and Rev are regulatory proteins that act at stages of transcription and nuclear export, respectively, to boost HIV gene expression (Figure 1-Late). Tat stimulates transcriptional elongation to synthesize more RNA²⁰, but intron-containing RNAs that provide

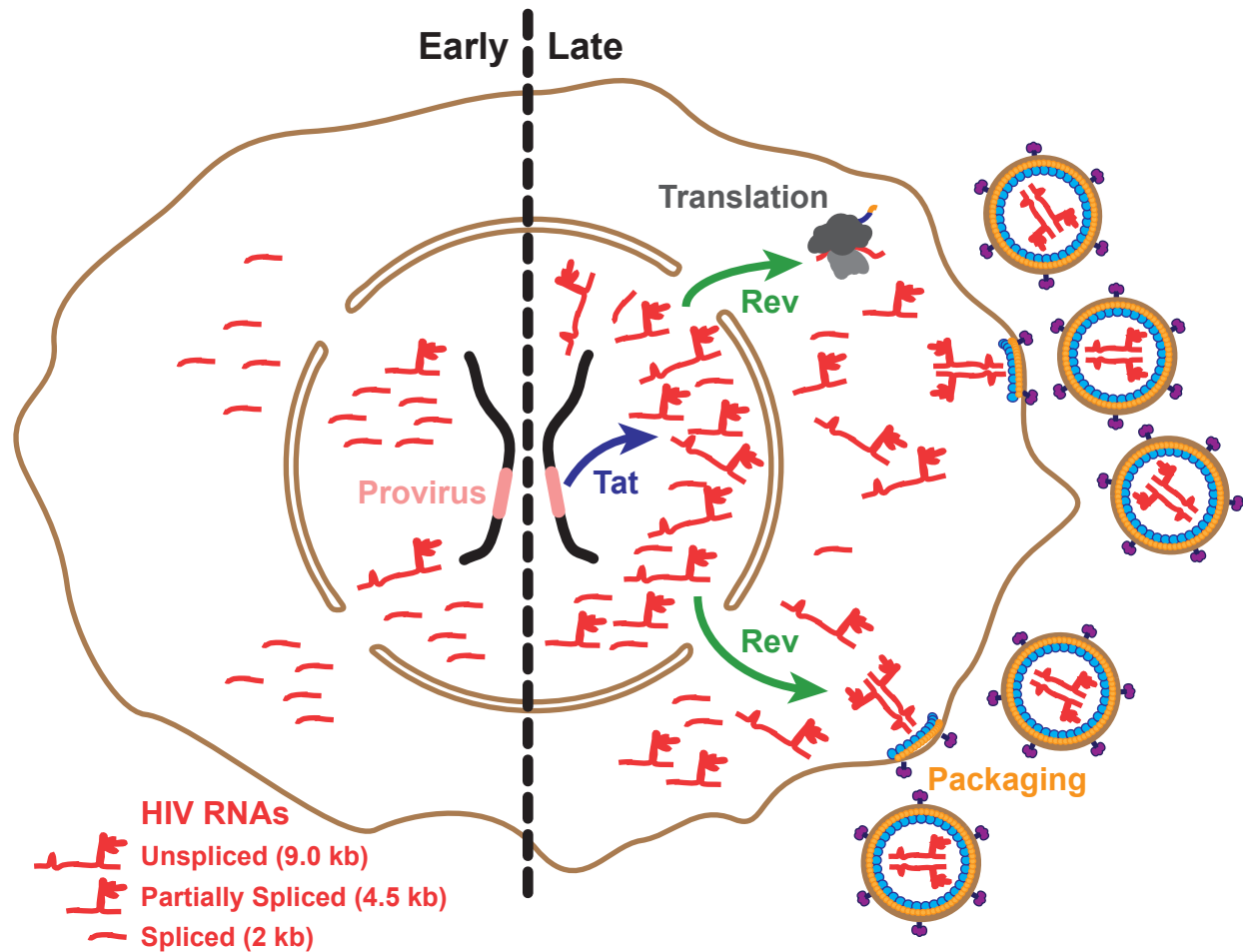


Figure 1: Rev advances the HIV lifecycle by post-transcriptionally enhancing gene expression from partially spliced and unspliced HIV RNAs.

(Early) After HIV has integrated into the host genome as a provirus, only fully spliced HIV RNAs that encode HIV regulatory proteins Tat, Rev, and Nef are exported from the nucleus through the Tap-dependent pathway for expression in the cytoplasm. (Late) After Tat has boosted the transcription of HIV RNA, Rev facilitates the nuclear export of partially spliced and unspliced HIV RNAs for translating structural proteins and for packaging the viral genome into new virions.

the genomes for new virions and encode structural and envelope proteins are retained in the nucleus^{21, 22}. Rev overcomes this nuclear retention by tethering unspliced and singly spliced RNAs to the nuclear export machinery in order to release viral RNAs into the cytoplasm^{23, 24}, where these viral RNAs can be translated or be packaged into new virions emerging from the plasma membrane.

Rev elicits nuclear export by recognizing the Rev Response Element, a cis-acting sequence within in the Env gene of intron-containing RNAs²⁵, and recruiting the Crm1 nuclear export receptor through a nuclear export sequence (NES) found in the disordered carboxy-terminus of Rev^{23, 26} (Figure 2). By helping demonstrate that the nuclear export of mRNAs is an essential and regulated step in gene expression, the discovery of Rev provided a pivotal insight for eukaryotic cell biology²⁷. While the recruitment of Crm1 to mRNAs through an RNA binding protein was thought to be a general mode for nuclear export, studies showed that inhibiting this pathway mainly affected the transport of ribosomal subunits, snRNAs, proteins, and a few mRNAs but did not affect the export of most cellular mRNAs^{23, 28}. Simpler retroviruses helped identify the bulk mRNA transport pathway mediated by Tap^{29, 30}, also known as Mex67, and further work showed that Tap and other nuclear export proteins typically load onto messages during stages of pre-mRNA processing³¹. In contrast, HIV RNA needs to assemble a nuclear export particle apart from RNA processing events so that partially spliced and unspliced isoforms of the viral RNA can move from the nucleus to the cytoplasm³². Some simple retroviruses have a constitutive transport element that also bypasses RNA processing by directly recruiting Tap³³, but intriguing work from the Malim lab has shown that replacing the RRE for the CTE has downstream consequences on viral assembly^{34, 35}. Other labs have shown that these pathways depend on different helicases in the cytoplasm to release messages from their nuclear export receptors^{36, 37}.

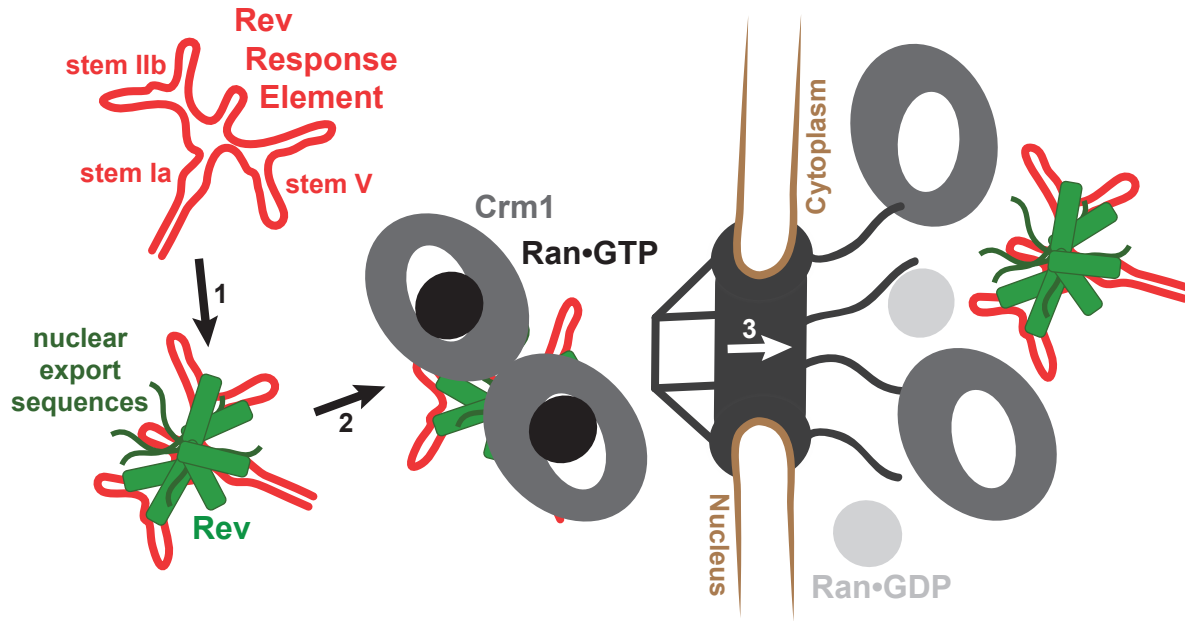


Figure 2: Rev bridges the interaction between HIV RNA and the host nuclear export machinery.

(Step 1) Rev assembles on the Rev Response Element (RRE), and (Step 2) presents nuclear export sequences for Crm1 to recognize in conjunction with Ran•GTP. (Step 3) The assembled particle peragrates the nuclear pore complex and arrives in the cytoplasm where Ran GTP-hydrolysis stimulates the dissociation of Rev-RRE from Crm1.

The differences between mRNA export mechanisms based on cis-acting sequences that direct the assembly of protein cofactors highlights that the composition, assembly, and dismantling of ribonucleoprotein complexes (RNPs) can determine mRNA function, so dissecting how RNPs form and disassemble can help define gene regulatory mechanisms.

Rev assembles into discrete, asymmetric complexes with the Rev Response Element

Nuclear export of unspliced and partially spliced HIV RNA begins with the assembly of the Rev-RRE complex. In a long-standing model for Rev-RRE assembly, Rev initially recognizes a high affinity site, known as stem IIb (Figure 2), and then forms a filament along the length of the RRE, and possibly the entire viral RNA, guided by irregularities in the RRE structure and protein-protein interactions between Rev monomers³⁸⁻⁴². Rev is able to recognize so many different portions of the RRE through an arginine rich motif (ARM), which is an alpha helix made almost entirely of arginine residues, and hydrophobic domains on both sides of the ARM allow Rev to oligomerize with or without RNA⁴²⁻⁴⁸ (Figure 3). Fascinated by the plasticity in RNA recognition that an ARM motif would allow, Matt Daugherty, a previous graduate student in the Frankel Lab, recognized that some RNA and DNA sequences from in vitro selection experiments looked like portions of the RRE that were previously uncharacterized^{49, 50}. With the knowledge that a single ARM could specifically recognize different sequences by using different faces of the helix, Matt discovered another Rev binding site, stem Ia (Figure 2), on the RRE that helped facilitate the assembly of the Rev-RRE complex⁵¹. This was one of the first results that indicated the long-standing model of Rev-RRE assembly needed revision.

In addition to finding the stem Ia binding site, Matt also uncovered biochemically tractable conditions for Rev. Because of the propensity for Rev to oligomerize, previous work described

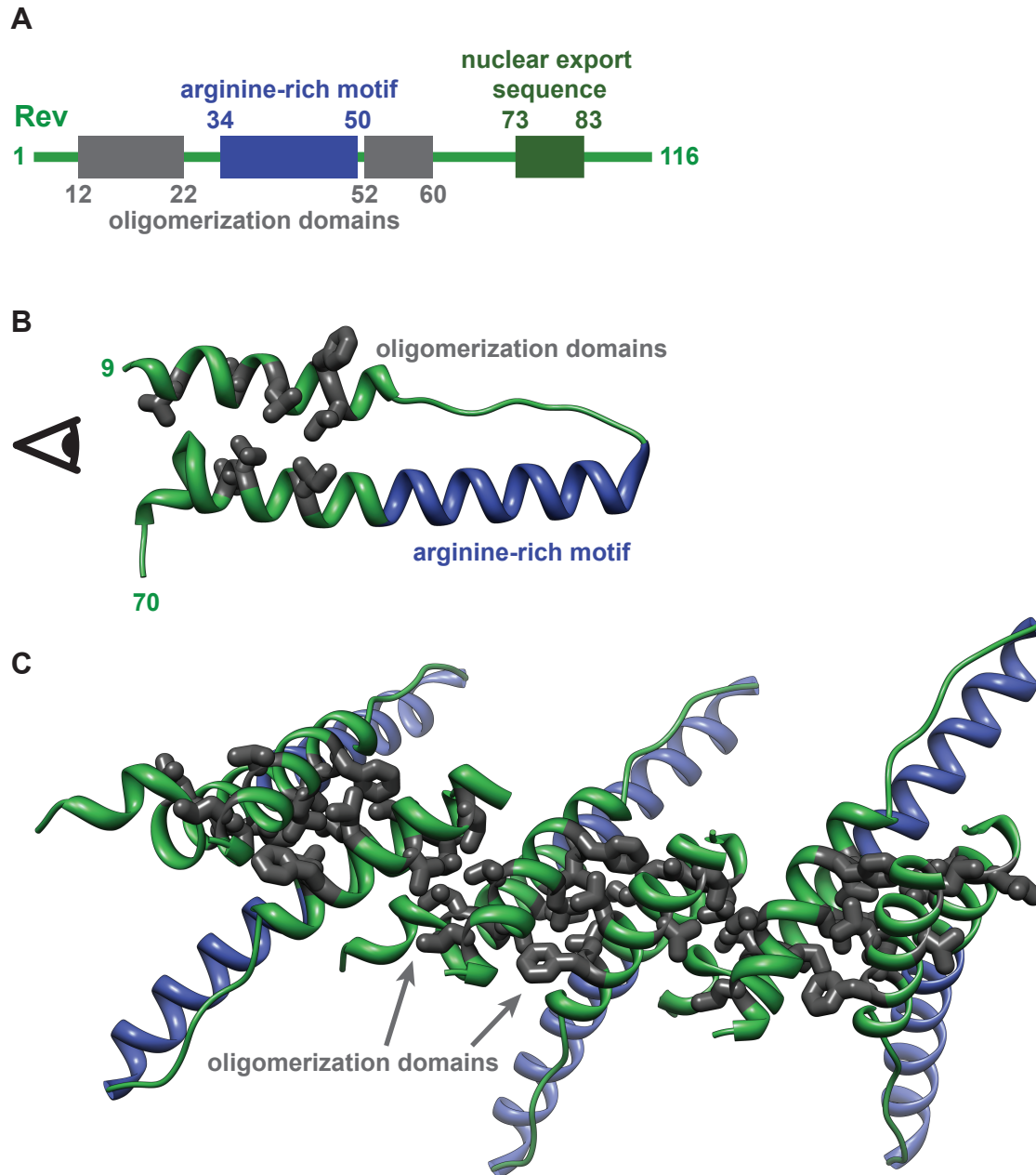


Figure 3: Rev domain organization and higher order assembly.

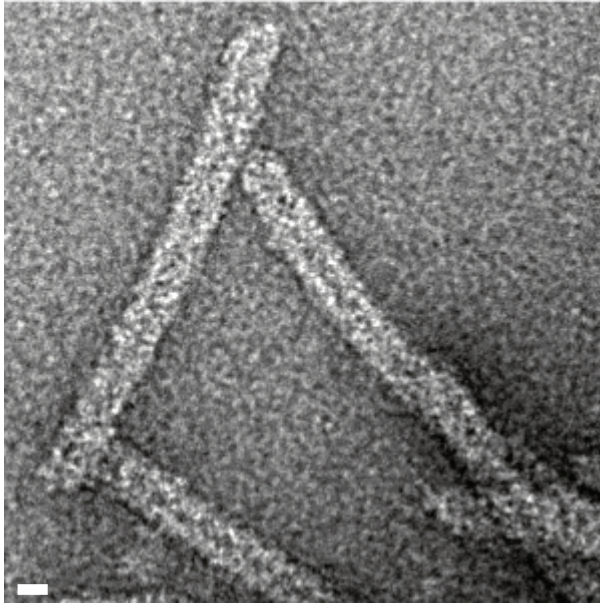
(A) Domain organization of Rev. (B) A monomer of Rev from the dimer crystal structure (PDB 3LPH) shows the organization of the arginine-rich motif and oligomerization domains in the helix-turn-helix fold. (C) Oligomerization domains facilitate the assembly of higher-order Rev assemblies. A Rev hexamer is visualized from one side as indicated in panel B.

Rev as insoluble at concentrations above $\sim 10 \mu\text{M}$ ^{39, 40, 52-54}. After testing different amino terminal tags, Matt found that the small anionic protein GB1 helped keep Rev soluble to concentrations greater than $200 \mu\text{M}$ ⁵⁵. When Rev was purified with this tag while retaining co-purified RNAs, Rev was able to bind the RRE with extremely high affinity ($K_D \approx 100 \text{ pM}$)—500 times higher than binding stem IIb alone—and unprecedented cooperativity⁵¹. Cleaving the tag in the presence of RNA or oxyanions such as sulfate or phosphate maintained this solubility when RNA was purified away from Rev⁵⁵. Together, these observations indicated that Rev does not gradually grow as a filament on the RRE but more likely assembles into defined complex.

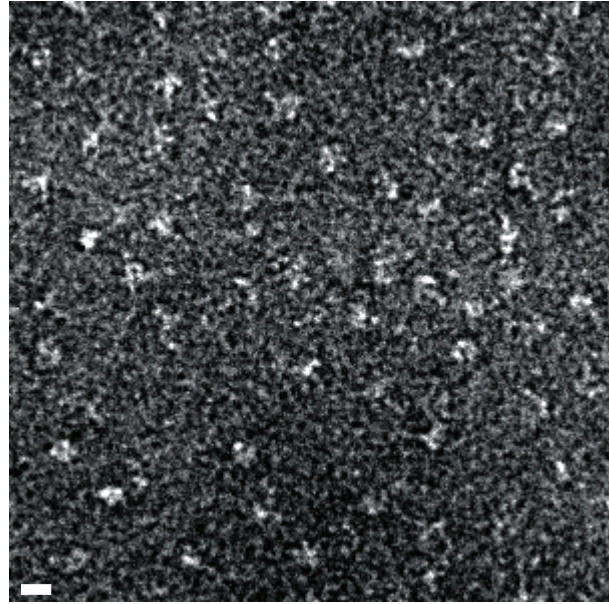
To directly show that the Rev-RRE complex formed a discrete particle, Matt and I worked together to visualize the complex. Even under better biochemical conditions, Rev by itself appeared as long filaments that had previously been observed by negative stain electron microscopy^{55, 56} (Figure 4). By mutating oligomerization interfaces and/or adding shortened RNA constructs, Matt realized that the interplay of protein-protein interactions and the availability of RNA binding sites helped determine the stoichiometry of a Rev oligomer⁵⁵, consistent with previous hypotheses for the Rev filament. In striking contrast to previous reports that visualized amorphous filaments when Rev bound the RRE⁵⁶, we observed that the RRE, without any mutations to Rev oligomerization interfaces, was able to direct the assembly of a Rev hexamer into discrete, asymmetric complexes⁵⁵ (Figure 4). This result indicated that the RRE was not just a passive scaffold on which the Rev oligomer binds; instead, the RRE plays a more active role by stabilizing a particular stoichiometry of the oligomer or by remodeling the oligomer or by doing both.

Overall, these biochemical data enhanced understanding how Rev coordinates RNA export with the viral lifecycle. Previous models appreciated that multiple copies of Rev binding the

Rev



Rev + RRE



Class Averages

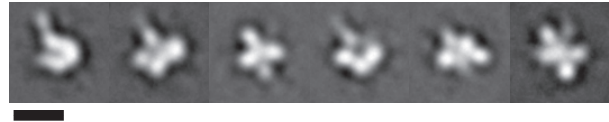


Figure 4: The RRE assembles a Rev hexamer into discrete, asymmetric complexes.

(Left) Rev forms into long filaments without RNA as seen in electron micrographs of negatively stained protein. (Right) Upon adding the RRE, Rev assembles into discrete particles containing six-copies of Rev as seen in negative-stain electron micrographs and class averages of individual particles. Scale bars denote 10 nm.

RRE is required for nuclear export⁴⁵. Those models detailed that a sufficient amount of Rev is required to drive the equilibrium for Rev to bind stem IIb and then add multiple copies on the RRE, which means passing a certain threshold of Rev concentration will switch the viral lifecycle from a latent state to an actively replicating state^{41, 42, 45}. The more recent biochemical experiments from our lab indicate that assembly occurs with lower concentrations of Rev than previously appreciated, and the cooperative assembly of Rev on the RRE indicates that the transition to active replication is sharper⁵¹. Therefore, Rev is a much more potent activator of RNA export than previously thought, and the ultra-sensitivity conferred by tight and cooperative assembly diminishes the delay between latency and active replication.

Crm1 recognizes nuclear export sequences dangling from the Rev-RRE complex

While we were gaining a clearer understanding about Rev-RRE assembly, including a crystal structure of Rev dimers^{57, 58}, we had less knowledge about the interaction between Rev and host co-factor Crm1. Molecular genetics had identified that a portion of Rev with regularly spaced leucine residues was required for nuclear export^{44, 59}. This sequence was later used to identify Crm1 as a nuclear export receptor that requires Ran bound to GTP for NES recognition²⁶. As more NESs were identified, it became clear that the Rev NES was peculiar. Proline residues that usually disrupted other NESs from binding Crm1 were required for Rev⁵⁹⁻⁶³. At the beginning of my project, a major question was how Crm1 was able to recognize such degenerate NESs. The answer came from a structure of human Crm1 bound to the Snurportin1 cargo. This structure identified hydrophobic pockets in a cleft of Crm1 that accommodated a regular spacing of leucine residues in the Snurportin1 NES^{64, 65}. Later crystal structures took advantage of this complex to replace the Snurportin1 NES with other NESs in order to determine

how Crm1 could recognize other sequences⁶³. These structures revealed that the Rev NES has a shorter sequence where proline residues eliminate a full turn of an alpha-helix and serve as a substitute for a leucine residue⁶³ (Figure 5). This result provides an interesting example for molecular mimicry where viruses find a different way to recapitulate essential features from the host in order to coopt cellular processes.

A synthesis of biochemical data and structures of the Rev oligomer, the Rev ARM bound to a portion of the RRE, and the Rev NES bound to Crm1 yielded a model for the overall architecture of the complex. This structural model resembles a jellyfish where the NESs dangled like tentacles from the Rev-RRE complex, which is like the bell of the jellyfish, in order to bind Crm1⁵⁷ (Figure 6). Importantly, the dimensions of the Rev hexamer sterically excludes more than two copies of Crm1 from binding the Rev-RRE complex as opposed to each Rev NES binding a Crm1 molecule proposed in other models⁶⁶. Because a protein fused to a single Rev NES is competent for nuclear export^{51, 57, 67}, it was unclear if one or two copies of Crm1 are required for nuclear export of HIV RNA.

How does the Rev-RRE complex engage Crm1 with RanGTP for the nuclear export of HIV RNA?

Before the structure of the Rev oligomer indicated that potentially two copies of Crm1 bound the Rev-RRE complex, I wondered whether many copies of Crm1 were required to facilitate the nuclear export of HIV RNA from a biophysical standpoint. Most nuclear to cytoplasmic transport occurs through the nuclear pore complex, which has a hydrophobic meshwork within the pore that forms a selective phase able to exclude molecules larger than ~40 kD from being transported between the nucleus and cytoplasm^{68, 69}. Nuclear transport receptors

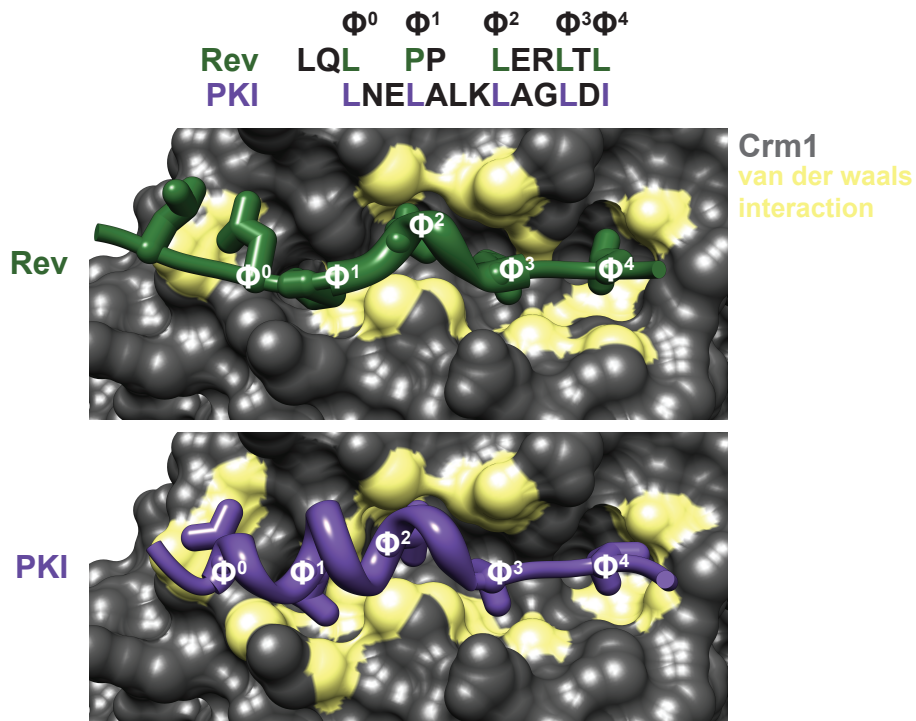


Figure 5: Rev mimics host nuclear export sequences to associate with Crm1.

(Top) A regular spacing of hydrophobic residues (Φ) characterizes nuclear export sequences. The Rev nuclear export sequence (green) has a different spacing than the protein kinase A inhibitor (PKI) nuclear export sequence (purple). (Bottom) The Rev NES establishes similar hydrophobic contacts with Crm1 using a shorter and less-ordered sequence (PDB 3NBZ) than the PKI nuclear export sequence (PDB 3NBY).

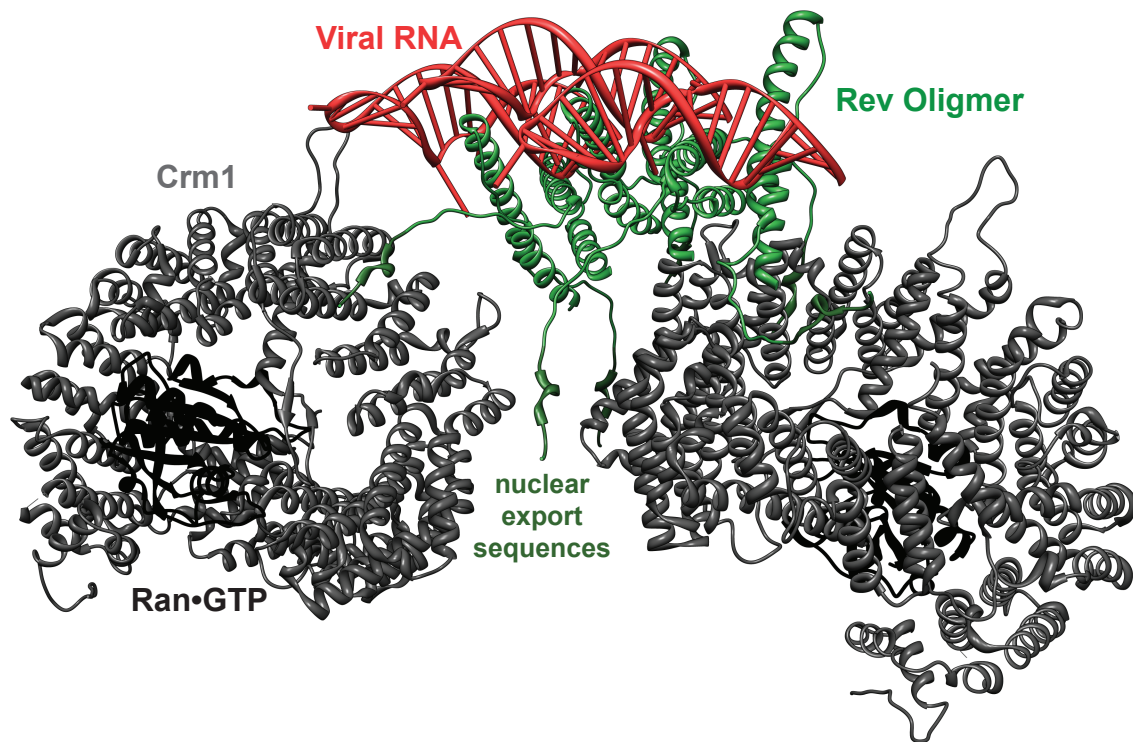


Figure 6: The Rev-RRE complex dangles nuclear export sequences for Crm1 recognition.

A hypothetical model of the entire nuclear export particle from the structures of the Rev oligomer (PDB 3LPH), the Rev arginine-rich motif bound to stem IIb (PDB 1ETF), and the Rev NES bound to Crm1-Ran•GTP (PDB 3NC0) resembles a jellyfish with nuclear export sequences dangling from the body of the Rev-RRE complex for Crm1 recognition.

can partition into the interior of the pore through hydrophobic interactions with the proteinaceous meshwork that allows receptors to ferry large cargoes through the pore^{70,71}. Large cargoes, however, require multiple receptors to facilitate transport⁷¹. A long poly-anionic molecule like an mRNA would have particular difficulty getting through the pore lined with hydrophobic proteins, which explains the deposition of proteins along the length of an mRNA during processing events—like beads on a string—in order to facilitate transport⁷². The RRE presents a particularly large barrier with a size almost twice that of structured RNAs transported by Crm1⁷³, so it would make sense that transporting the RRE would require more copies of Crm1 for nuclear transport. There was, however, no indication that the Crm1 stoichiometry would impact the viral lifecycle or nuclear transport in general.

Some previous evidence did indicate that there was additional complexity to the interaction between Rev-RRE and Crm1. Work that tried to recapitulate nuclear export using tandem arrays of the stem IIB site to scaffold the Rev oligomer was unable to elicit nuclear export to the same degree as the RRE⁷⁴, suggesting that the unique fold of the RRE did not simply scaffold Rev but played a more active role in transport. Consistent with the importance of the RRE fold for export activity, the Rekosh and Hammarskjöld labs identified a mutation in the RRE that was able to suppress deleterious mutations in the Rev NES⁷⁵. Studying a Rev-like protein in a different retrovirus, the Shida lab identified cluster of residues found in human Crm1 but not rat Crm1 that was important for facilitating Rev oligomerization and promoting nuclear export⁷⁶⁻⁷⁸. The crystal structure of human Crm1 bound to Snurportin1 also identified an additional interaction surface between these two proteins that the authors proposed may bind Rev⁶⁴, and a different Crm1 surface was also proposed from protease protection experiments⁷⁹. The jellyfish model, however, could not explain these observations because the model does not account for the unique structure

of the RRE and does not provide for additional contacts within the complex. Because the jellyfish model could not fully explain the underlying biology, we were still left asking, “How does the Rev-RRE complex engage Crm1 with RanGTP for the nuclear export of HIV RNA?”

A Crm1 dimer cooperatively recognizes the Rev-RRE complex to facilitate HIV RNA nuclear export

Given the inconsistencies between biological data and structures that captured parts of the complex, we reasoned understanding this important HIV-host interaction would require reconstituting and characterizing the fully assembled complex. Past studies investigating the interaction of the entire particle were faced with biochemical challenges from Rev’s solubility and poor expression of Crm1^{79, 80}. We were uniquely poised to reexamine this interaction because of our work reconstituting the Rev-RRE particle, and we independently determined conditions for properly expressing Crm1 in sufficient quantities. Additionally, I relied on an extensive history of mutations isolated and characterized from molecular genetic screens of Rev-RRE activity and from cell biological studies of Crm1 and Ran to ensure that biochemical observations corresponded to validated *in vivo* activity^{26, 44}. This work, as described in chapter two, forms the foundation for describing the interaction between the Rev-RRE complex and Crm1 with RanGTP. The most striking feature of these assembled complexes was the presence of two copies of Crm1 bound to Rev-RRE as in the jellyfish model. An important difference was the Crm1 molecules were not randomly oriented to each other, as we would have expected from each copy independently recognizing NESs in the flexible carboxy terminus of Rev. Instead, the two copies of Crm1 were symmetrically arranged. We still, however, did not know if two copies of Crm1 were important for nuclear export and it was difficult to suggest mutations that would

have altered the arrangement of the Crm1 dimer to test in cell-based assays since the structure was at a low resolution.

Luckily, three independent studies emerged that helped us interpret our structure in order to conclude that the Crm1 dimer was important for Rev-RRE activity. In the first study, the Shida lab extended their findings from studying HTLV-1 to show that murine orthologs of Crm1 were unable support nuclear export for HIV⁸¹. Next, the Malim lab showed the same result but more carefully interrogated the molecular basis for the interaction and concluded that seven surface-exposed residues were responsible for the gain nuclear export activity with human Crm1⁸². These residues did not seem to be important for Ran or NES binding based on the crystal structure, and most intriguingly, the residues seemed to be positively selected in simian primates and negatively selected in the murine lineage⁸². These results helped explain an old observation that a main block for HIV replication in murine cells is an inability to export HIV RNA⁸³. In the third paper, the Sutton lab showed that expressing human Crm1 with human SRp40 had a synergistic effect on HIV gene expression⁸⁴, which incorporated a previous observation from the Malim lab that showed human SRp40 helped stimulate translation of HIV RNA⁸⁵. This result highlighted that cytosolic cofactors can amplify changes in RNA export to modulate HIV gene expression.

Integrating these insights into the structural model led us to discover that the human Crm1 residues responsible for enhancing Rev-RRE activity formed an interface between the two copies of Crm1. This interface allows human Crm1 to cooperatively recognize the Rev-RRE complex as a dimer, showing that the host supports the assembly of the entire nuclear export complex. It is unlikely that this interface evolved to help a pathogenic virus, so we speculate that a Crm1 dimer may also be important for Crm1 function during nuclear export or in its alternate functions

during mitosis. These results may provide another example for using a relatively simple virus to provide insight into the complex biology of its host.

How does HIV adapt Rev-RRE to modulate nuclear export activity?

Work identifying mutations in Rev, the RRE, and Crm1 have shown that there is a dynamic range in nuclear export activity that ultimately affects the potential for replicating new virions. Most interestingly, one study following Rev and the RRE over the course of an infection showed that the virus can acquire mutations that change nuclear export activity with mutations in the RRE being the major contributor for increased nuclear export⁸⁶. Consistent with this observation, a viral evolution experiment in tissue culture had previously isolated a mutation in the RRE, named RRE61, that can suppress a defective Rev NES⁷⁵. Together these studies suggest that the RRE can evolve to enhance the interaction with Crm1 in order to increase nuclear export.

Because our work defining the architecture of the complex indicates that the RRE is only connected to Crm1 through its interaction with Rev, how could the RRE establish a better interaction between Crm1? A simple hypothesis for enhancing the interaction with Crm1 is that the RRE could directly contact Crm1. To pursue this idea, I tried crosslinking the RRE to proteins using ultraviolet light and then collaborated with Uzma Zaman and Henning Urlaub to identify cross-linked peptides using mass-spectrometry⁸⁷. These experiments only yielded interactions between the RRE and the arginine rich motif from Rev (data not shown). Because I never pursued these experiments with RRE61, we cannot excluded that this mutation does allow an interaction with Crm1. Arguing against this, however, chemical footprinting showed that the RRE61 mutation is in a portion of the RRE protected by Rev^{88, 89}, so it is more likely that this mutation alters Rev oligomerization instead of establishing a new contact with

Crm1. Puzzlingly, studies testing how mutations in the RRE that enhance nuclear export would affect Rev-RRE assembly showed very subtle if not negligible effects^{86, 88}.

The most telling observation to describe how the RRE could act through the Rev oligomer to alter Crm1 interactions came from a crystal structure of a Rev dimer bound to a portion of the RRE that was solved in our lab by Bhargavi Jayarman. She immediately recognized a dramatic conformational change in the structure of the Rev dimer where the crossing angles between Rev monomers became more acute when bound to the RRE⁹⁰ (Figure 7A). This structure now suggested that the RRE could change nuclear export activity by allosterically controlling the Rev oligomer by revealing a range of conformations that the oligomer could adopt (Figure 7B). Satisfyingly, this concept would explain how mutations that had minimal impact on binding Rev could potentially alter the interaction with Crm1. In fact, one of several models of the Rev oligomer based on different crystal structures had a distance between the carboxy termini of the first and last copy of Rev within the oligomer that was compatible with the distance between NES binding sites in the Crm1 dimer.

If mutations in the RRE could alter the conformation of the Rev oligomer to change Crm1 interactions, then testing how RRE mutations affect the assembly of the Rev-RRE complex with the Crm1 dimer would provide insight into adaptations HIV could make to enhance nuclear export. I investigate this hypothesis in chapter three by interrogating how Crm1 assembles with Rev-RRE complexes containing an RRE61 mutation, which serves as an example of a mutation of the RRE that enhances nuclear export. In this initial work, we find that Crm1 can bind to Rev-RRE complexes containing this mutation with similar affinity as wild-type RRE. Surprisingly, this mutation enhances the Rev-RRE density in a negative stain electron microscopy reconstruction. Rev-RRE was nearly invisible in the reconstruction shown in chapter two partly

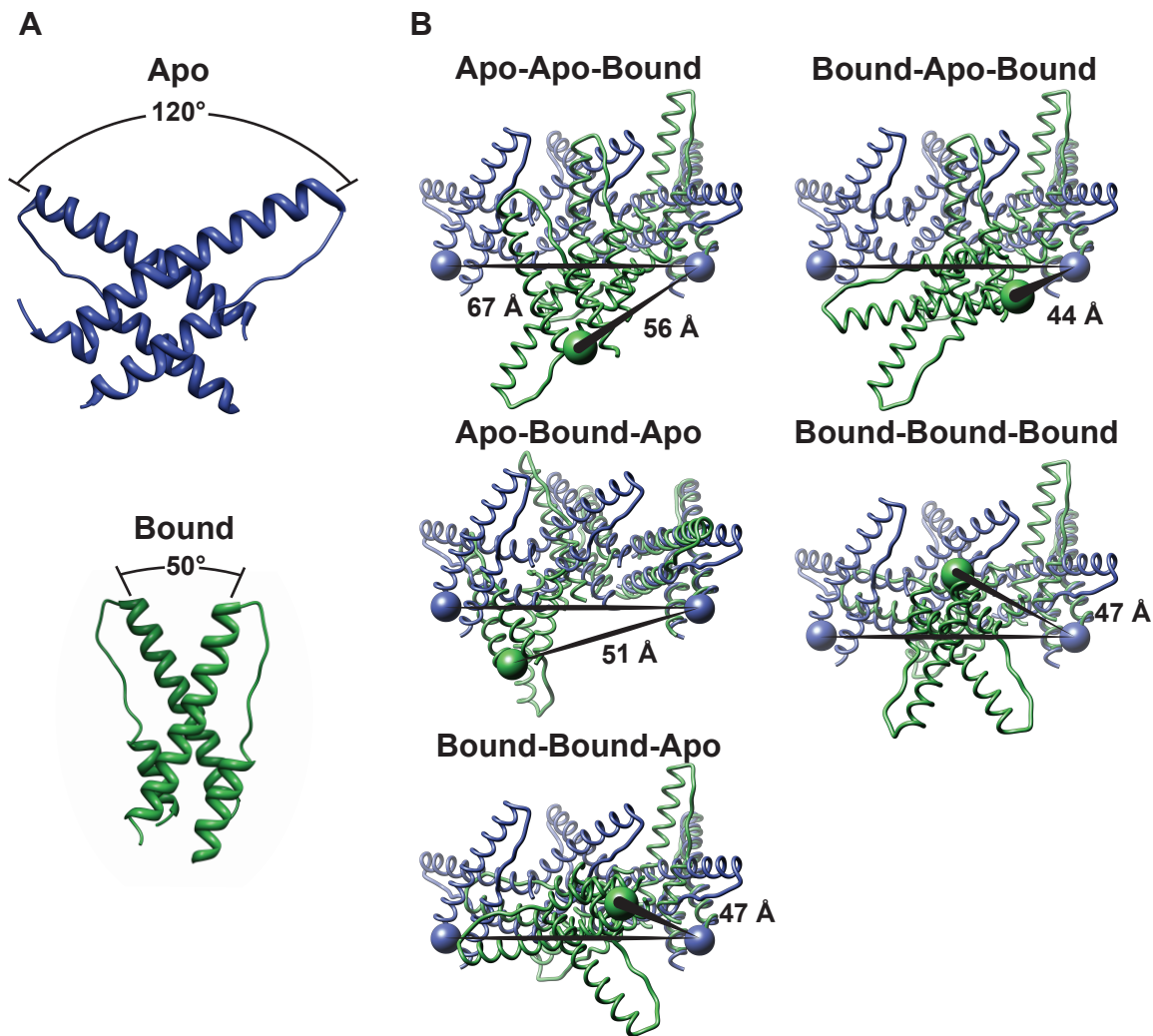


Figure 7: Conformational changes in the RNA-bound Rev dimer lead to a range of hexamer conformations.

(A) When bound to a portion of the RRE⁹⁰ (green), the Rev dimer adopts a more compact structure with a more acute crossing angle between monomers than in the Apo dimer (PDB 3LPH). (B) The RNA-bound Rev dimer yields a range of more compact hexamer conformations (green) as compared to a hexamer built from only Apo dimers (blue). Models of Rev hexamers were built by connecting different arrangements of Apo or RNA-bound dimers through the structure of the Rev oligomeric interface (PDB 2X7L).

because of conformational heterogeneity that potentially arises from different modes of Rev-RRE binding the Crm1 dimer. These data suggest that the RRE61 mutation may help stabilize a particular conformation of Rev oligomer to interact with the Crm1 dimer. This insight and advances in electron cryomicroscopy⁹¹ could potentially lead to an atomic resolution structure of the Rev-RRE complex while helping us understand possible adaptations the virus makes to enhance viral replication.

Altogether, the work within this dissertation helps define determinants from the host and virus for HIV to export unspliced and partially spliced RNA through the Crm1 pathway. The reconstitution of the complex formed between Rev, the RRE, Crm1, and RanGTP and the visualization of the Crm1 interface has revealed an important aspect about the evolution of Crm1 that allows simian primates to serve as hosts for HIV replication. Perhaps, this property of Crm1 has evolved to modulate nuclear export in order to regulate gene expression, or it may serve as a potential target for developing therapeutics to impede a critical step in the HIV lifecycle. Potential directions for researching these implications are further discussed in chapter four as well as ways to finally determine a high-resolution structure for the entire Rev-RRE complex. These structural insights would provide a framework for interrogating how adaptations in Rev or the RRE augment nuclear export and stimulate virion production. Investigating such adaptations, as proposed in chapter four, may reveal the range of activity available to a given virus that would influence the course of an infection. This perspective could apply our appreciation of the central role Rev plays in the viral lifecycle to treat people afflicted with HIV.

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CHAPTER 2: HIV utilizes a Crm1 dimer to promote RNA nuclear export

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ABSTRACT

The HIV Rev protein routes viral RNAs containing the Rev Response Element (RRE) through the Crm1 nuclear export pathway to the cytoplasm where viral proteins are expressed and genomic RNA is delivered to assembling virions. Rev acts as oligomer assembled on the RRE that displays nuclear export sequences (NES) for recognition by the Crm1-Ran•GTP nuclear receptor complex. Here we present the first view of the entire virus-host complex by single-particle electron microscopy. Unexpectedly, Crm1 forms a dimer with an extensive interface that enhances association with Rev-RRE and poises NES binding sites to interact with a Rev oligomer. The interface between Crm1 monomers explains differences between Crm1 orthologs that alter nuclear export and determine cellular tropism for viral replication. The arrangement of the export complex identifies a possibly novel binding surface to target an HIV inhibitor and may point to a broader role for Crm1 dimerization in regulating host gene expression.

INTRODUCTION

Human immunodeficiency virus (HIV) replication depends on the coordinated expression of viral proteins from fully spliced, partially spliced, and unspliced forms of its RNA genome. Only fully spliced RNAs encoding viral regulatory proteins initially reach the cytoplasm for translation¹. Later in the life cycle, the regulatory protein Rev directs the nuclear export of fully spliced and partially spliced viral RNAs by engaging the Crm1 nuclear export pathway typically used to transport host protein cargoes and small RNAs²⁻⁶.

Rev coopts Crm1 to export viral RNAs by bridging the interaction between Crm1 and the Rev Response Element (RRE), a structured RNA located within the introns of unspliced and singly-spliced viral RNAs. The amino terminal portion of Rev recognizes the RRE through an arginine rich motif flanked by hydrophobic domains that facilitate Rev oligomerization on the RRE⁷. The Rev nuclear export sequence (NES), located near the carboxy terminus, recruits Crm1 in cooperation with Ran bound to GTP (Ran•GTP) to elicit nuclear export of viral RNAs⁸.

Recent structures detail the architecture of the RRE⁹, Rev binding to a single sites in the RRE¹⁰, Rev oligomerization^{11, 12}, and the Crm1-NES interaction¹³, but the organization of the entire export complex has remained unknown. Without a complete structure, it is unclear, for example, how HIV modulates Rev-RRE activity through mutations in the RRE that change the RNA fold yet minimally affect Rev binding^{14, 15} or how subtle amino acid substitutions between nearly identical orthologs of Crm1 restrict HIV replication in different species¹⁶⁻¹⁸. Here we report the single-particle electron microscopy (EM) structure of the Rev-RRE/Crm1-Ran•GTP export complex. Unexpectedly, Rev-RRE recruits an ordered Crm1 dimer, unlike characterized cargoes that bind a Crm1 monomer¹⁹. Residues unique to simian primates form a dimerization interface between Crm1 monomers that is seen as a crystal contact in the structure of human

Crm1¹⁹. We suggest the RRE molds the Rev oligomer to space Rev NESs in a configuration that matches the defined architecture of the Crm1 dimer, and we further suggest that some cellular protein-RNA complexes may also utilize Crm1 dimerization to tune nuclear export.

RESULTS

Two copies of Crm1 and Ran•GTP bind the Rev-RRE complex

In order to define the arrangement of HIV RNA export complexes for structural studies, we first determined the biochemical requirements for complex formation using recombinant Crm1, Ran, Rev tagged at the amino-terminus with a GB1 domain (GB1-Rev), and a 251 nt portion of RRE RNA transcribed *in vitro*. Crm1 co-purified with GB1-Rev in the presence of both Ran•GTP and the RRE (Figure 1A, lane 11), but showed only a weak interaction with Ran•GDP or without the RRE (compare lane 11 to lanes 12 and 9). The interaction between Crm1 and Rev requires a functional NES, as GB1-Rev bearing a dominant-negative M10 mutation in the NES, Leu78Asp and Glu79Leu²⁰, did not bind Crm1 under any condition (lanes 17-18 and 21-24). These results mirror *in vivo* and *in vitro* experiments showing that Rev uses an intact NES to bridge the interaction between the RRE and Crm1 utilizing Ran•GTP^{8, 21, 22}. Furthermore, our data directly show for the first time that the RRE enhances the Rev-Crm1 interaction, supporting the hypothesis that the Rev NES must be recognized in the proper Rev-RRE context to function²³.

Previously, we demonstrated that the RRE assembles six subunits of Rev into discrete, asymmetric complexes with a total mass of 160 kD²⁴. We used size-exclusion chromatography to further determine if these Rev-RRE complexes can associate with Crm1 and RanQ69L, a mutant that stabilizes the GTP-bound form of Ran²⁵. When increasing amounts of Crm1-RanQ69L were added to a fixed concentration of Rev-RRE, all four components eluted in a 462 kD fraction (Figure 1B and C), corresponding to two copies of Crm1 and RanQ69L per Rev-RRE complex. In fact, negatively stained electron micrographs of complexes from this fraction clearly show two Crm1 molecules per single particle, compared to images of Crm1 monomers

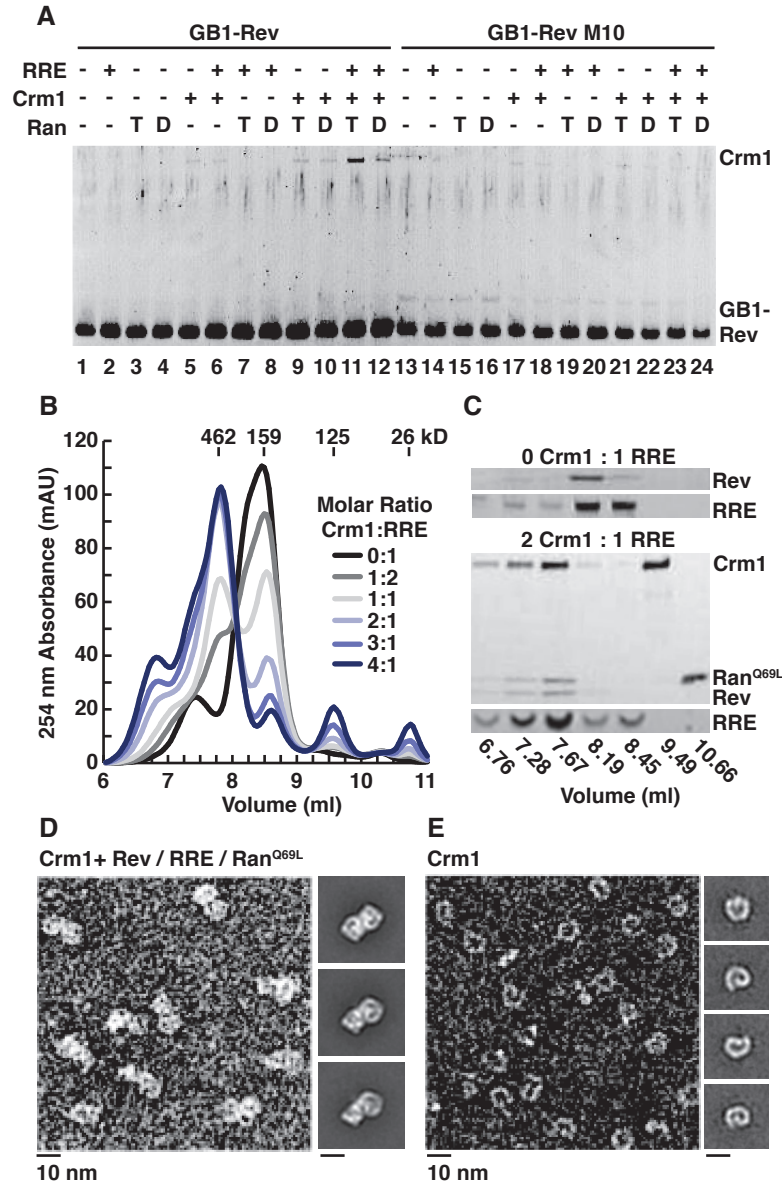


Figure 1: A dimer of Crm1- Ran•GTP binds the Rev-RRE complex.

(A) Crm1 copurifies with GB1-Rev in the presence of Ran•GTP and RRE. The gel shows the protein composition from affinity purifications of GB1-Rev incubated in the presence (+) or absence (–) of the RRE, Crm1, and Ran. T or D denotes Ran exchanged with GTPγS or GDP, respectively. RevM10 is a mutation within the NES of Leu78Asp and Glu79Leu²⁰. This gel is representative of three independent experiments.

Figure 1-continued: A dimer of Crm1- Ran•GTP binds the Rev-RRE complex.

(B-C) Reconstituted Rev-RRE particles preferentially bind two copies of Crm1 and Ran•GTP.

(B) Size-exclusion chromatograms of Rev-RRE with increasing molar ratios of Crm1 and RanQ69L show saturation at a molar ratio of two Crm1 per RRE. Excess Crm1 eluted as free monomers (9.49 ml fraction) or in a small population of larger complexes (6.76 and 7.02 ml fractions). Molecular masses were determined using multi-angle laser light scattering (MALLS) after size-exclusion chromatography and have a technical error of three percent. (C) The composition of peak fractions was analyzed by SDS-PAGE for proteins and by Urea-PAGE for RNA. These results are representative of two independent experiments. (D-E) Negative-stain electron micrographs show two copies of Crm1 in complexes reconstituted with Rev-RRE and Ran•GTP. Representative fields and class averages of 7196 nuclear export particles (d) and 6445 Crm1 molecules alone (E) are shown.

alone (Figure 1D and E). A Crm1 dimer explains the need for at least two NESs in the Rev oligomer to function²⁶, and it may also be important for efficiently transporting the RRE through the nuclear pore complex since the number of receptors required for nuclear transport scales with the size of the cargo²⁷. A Crm1 monomer is thought to use a protein adaptor to export relatively small, structured RNAs⁶. In contrast, the RRE is almost twice size of host RNA cargoes, is embedded in a much larger mRNA, and is bound to the Rev oligomer.

Species-specific residues define an interface between Crm1 monomers that enhances recognition of Rev-RRE

Previous structures of Crm1 bound to host cargo proteins have shown monomeric binding, so we sought to understand how Rev-RRE organizes a Crm1 dimer. We determined the structure of the entire export complex by single-particle negative-stain EM using random conical tilt and projection matching to yield a 25-30 Å reconstruction. We observed two connected bowl-shaped densities arranged with two-fold symmetry (Figure 2-figure supplement 1). Fitting the structure of Crm1-Ran•GTP monomers into the EM density (Figure 2-figure supplement 2) prompted us to examine the unit cell from a crystal structure of human Crm1 bound to Snurportin1¹⁹, where we discovered the presence of a Crm1 dimer that unambiguously fit as a rigid body into the EM map (Figure 2A). Remarkably, seven residues specific to simian primates that lie outside the NES-binding site and are known to be important for Rev function and HIV biogenesis¹⁶⁻¹⁸ were symmetrically apposed in the Crm1 dimer. This Crm1 dimer interface was not previously noted because Crm1 was known to bind the host Snurportin1 cargo as a monomer and was seen as a monomer in the asymmetric unit of the crystal. Subsequent studies interpreted species-specific residues in the context of the Crm1 monomer structure¹⁶⁻¹⁸. Based on steric considerations from the structure of a Rev oligomer, we previously proposed a ‘jellyfish’ model in which a Rev

hexamer with dangling unstructured NES peptides could accommodate no more than two Crm1 molecules¹¹. As described below, our data now suggest that Crm1 forms an intimate dimer interface in the context of the Rev-RRE particle, permitting us to propose a more comprehensive model of the export complex.

A detailed examination of the Crm1 dimer reveals an extensive interface that buries a total surface area of 2170 Å² in the crystal structure (Figure 2B). Central to the interface are symmetric hydrogen bonds between Arg474 and Glu478 across the dimer (2.9 Å donor to acceptor; Figure 2B) and, indeed, substituting these residues decreases Rev function¹⁸. The surrounding interactions are largely hydrophobic: Phe414 fills a cavity between Glu478 and His481 from the opposite subunit while Pro411, Met412, and Met 348 interact with the apposing Val484. Consistently, swapping murine residues for Pro411, Met412, and Phe414 results in the largest reduction in activity^{16, 18}. The ends of the interface near Thr346 utilize Glu345 to form a salt bridge with Lys531 (2.8 Å) and hydrogen bond with Gln530 (2.8 Å) on the other monomer. The sum of these interactions enables human Crm1 to form an ordered dimer interface upon Rev-RRE binding.

To assess the importance of this interface for Rev-RRE recognition, we compared the binding of murine and human Crm1 to Rev-RRE complexes using gel mobility shift assays (Figure 2C and D). We consistently observed two-fold higher affinity and increased cooperativity for the human protein, in agreement with the four-fold enhancement in Rev activity when human Crm1 is expressed in murine cells^{16, 18}. Furthermore, the weaker dimers formed with murine Crm1 eluted from size-exclusion chromatography with a larger apparent mass than human Crm1 particles (Figure 2E) and had a larger separation between Crm1 subunits in class averages of negatively-stained particles (Figure 2F). Together, these data indicate that Rev-RRE

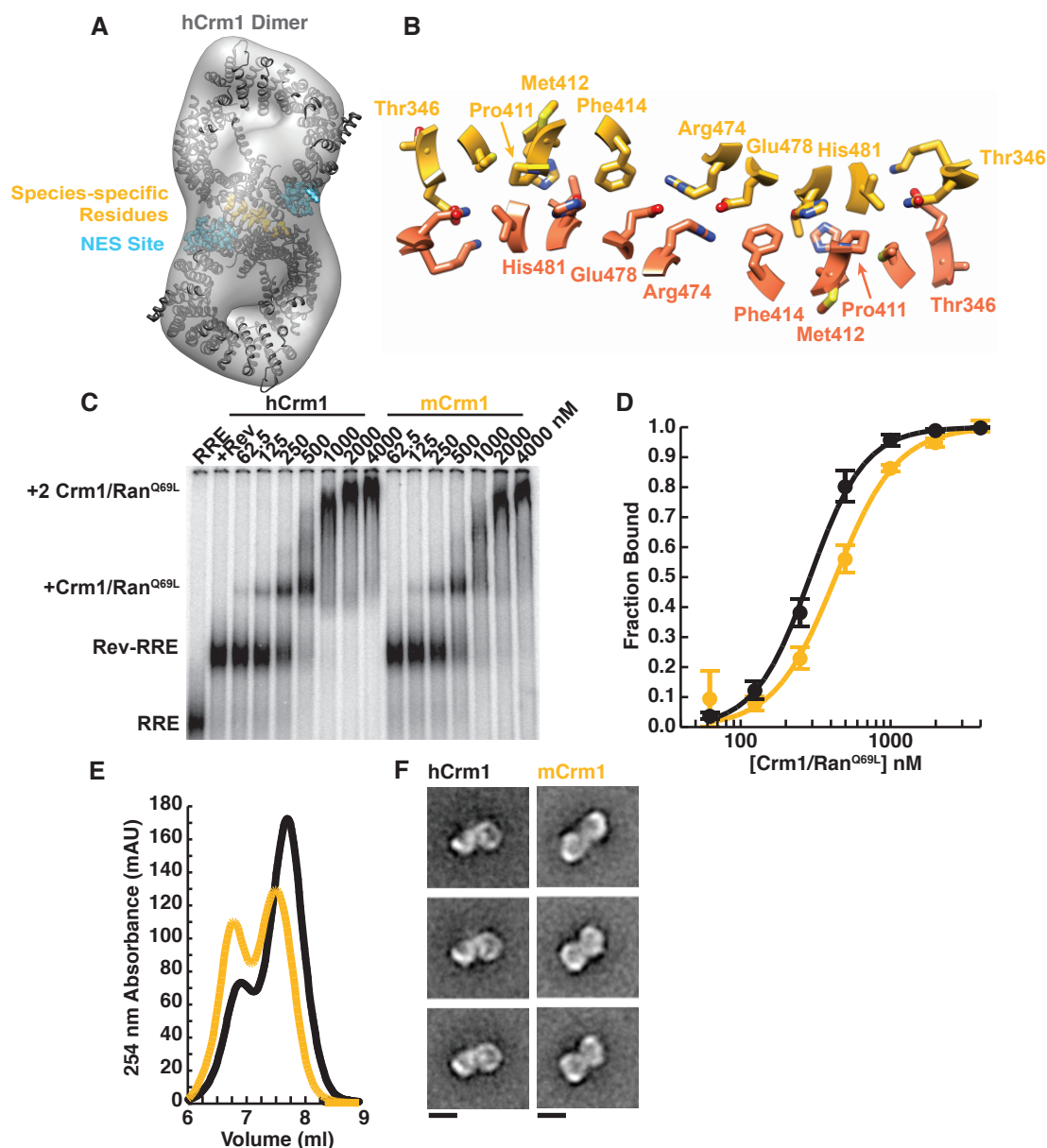


Figure 2: Species-specific residues define a Crm1 dimerization surface that enhances Rev-RRE binding.

(A) A dimer of human Crm1 extracted from a unit cell of a Crm1-Snurportin1 complex that lacks Ran•GTP (PDB 3GB8) was fit into the EM reconstruction (Correlation = 0.97). Residues that differ between murine and human Crm1 and enhance Rev-RRE activity^{16,18} are shown in gold and form an interface between two Crm1 monomers (detailed in panel B). Two NES binding sites poised to engage two Rev NES peptides are shown in cyan.

Figure 2-continued: Species-specific residues define a Crm1 dimerization surface that enhances Rev-RRE binding.

(C-D) Human Crm1 recognizes Rev-RRE with higher affinity and increased cooperativity than its murine ortholog, shown by gel mobility shift assays with radiolabeled RRE, a molar excess of Rev, and increasing concentrations of Crm1-Ran•GTP. The data were quantified and fit ($\chi^2_{\text{hCrm1}} = 0.961$ and $\chi^2_{\text{mCrm1}} = 1.89$) to the Hill equation, Fraction Bound = $[\text{Crm1-Ran}\bullet\text{GTP}]^h / ([\text{Crm1-Ran}\bullet\text{GTP}]^h + K_{\text{app}}^h)$ where K_{app} is the apparent dissociation constant and h is the Hill coefficient, as shown in panel D and described in Methods. Human Crm1 bound Rev-RRE with higher affinity ($P < 1 \times 10^{-10}$) than murine Crm1, $K_{\text{app}} = 291 \pm 15$ nM for human Crm1 compared to $K_{\text{app}} = 432 \pm 36$ nM for mouse Crm1, and increased cooperativity ($P < 1 \times 10^{-4}$), $h = 2.37 \pm 0.13$ for human Crm1 compared to $h = 2.12 \pm 0.25$ for murine Crm1. Error bars show standard deviations of three independent experiments and P -values are from one-tailed t-tests. (E) Size-exclusion chromatograms of Rev-RRE complexes assembled with murine Crm1 or human Crm1 show that murine Crm1 elutes with a larger apparent mass, presumably due to a larger hydrodynamic radius in the absence of Crm1-Crm1 interactions. (F) Class averages of Rev-RRE complexes bound to a dimer of human (426 particles) or murine (617 particles) Crm1 from negatively-stained micrographs of particles from (E). Murine Crm1 particles showed a wider range of sizes, with most being larger, and different features, such as an hourglass shape, than human Crm1 particles. Scale bars show 10 nm.

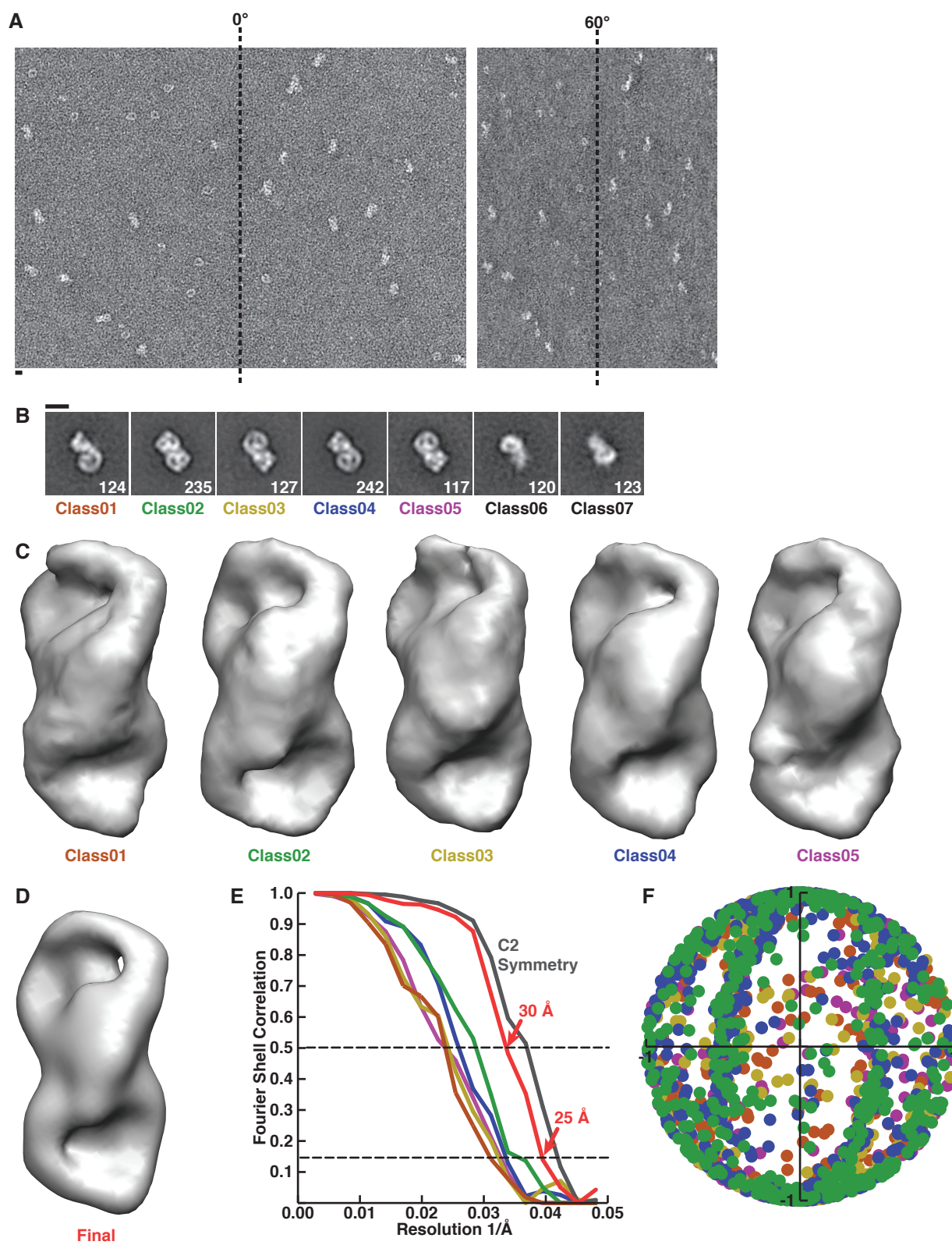


Figure 2-figure supplement 1: Random conical tilt reconstruction and refinement.

Figure 2-figure supplement 1: Random conical tilt reconstruction and refinement.

(A) Representative fields from a random conical tilt pair of particles assembled with Rev-RRE, Crm1, and Ran•GTP. (B) Class averages from untilted particles used for random conical tilt. Particle numbers are inset in each image. Classes six and seven were observed with lower concentrations of sample and resemble partially disassembled complexes. Scale bars show 10 nm. (C) Volumes from each class after random conical tilt refinement show similar features among all classes suggesting the classes are different views of the same particle. The volume from the final reconstruction is shown in (D). (E) Fourier shell correlation curves for each random conical tilt class and the final reconstruction. The boost in Fourier shell correlation when C2 symmetry is applied during refinement confirms the symmetric arrangement of bowl-like densities. (F) Angular distribution from projection matching shows no major gap in Euler space.

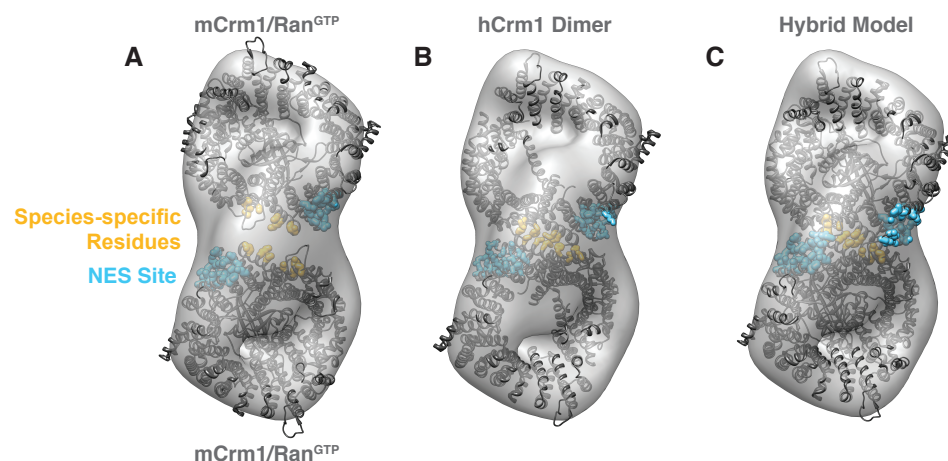


Figure 2-figure supplement 2: Comparison of fitting murine Crm1 or human Crm1 crystal structures in the EM reconstruction.

(A) Two murine Crm1-Ran•GTP (PDB 3NC0) monomers individually fit well (Correlation > 0.98) into two bowl-like densities from the reconstruction of Crm1-Ran•GTP bound to Rev-RRE. The final fit places residues different between murine and human Crm1 (gold) to potentially form an interface between two Crm1 monomers, but fitting these structures into the low-resolution map introduces a gap between the two monomers. (B) From the fit of murine Crm1, we inferred that the same residues might form a crystal contact between Crm1 monomers in the crystal structure of human Crm1 bound to Snurportin-1 (PDB 3GB8) and this, indeed, was observed. The fit of the human Crm1 dimer structure is shown. Although the crystal structures of murine Crm1 either have two copies of Crm1 in the asymmetric unit or other dimers in the unit cell, these alternate dimers use different crystal contacts to build the unit cell and do not have the same conformation as the human Crm1 dimer (data not shown). (C) A hybrid model of the Crm1 dimer bound to Ran•GTP. The human Crm1 interface was used to orient two copies of murine Crm1 bound to Ran•GTP. The final model fits well (Correlation = 0.97) into the EM map and was used to search for Rev-RRE density shown in figure 3.

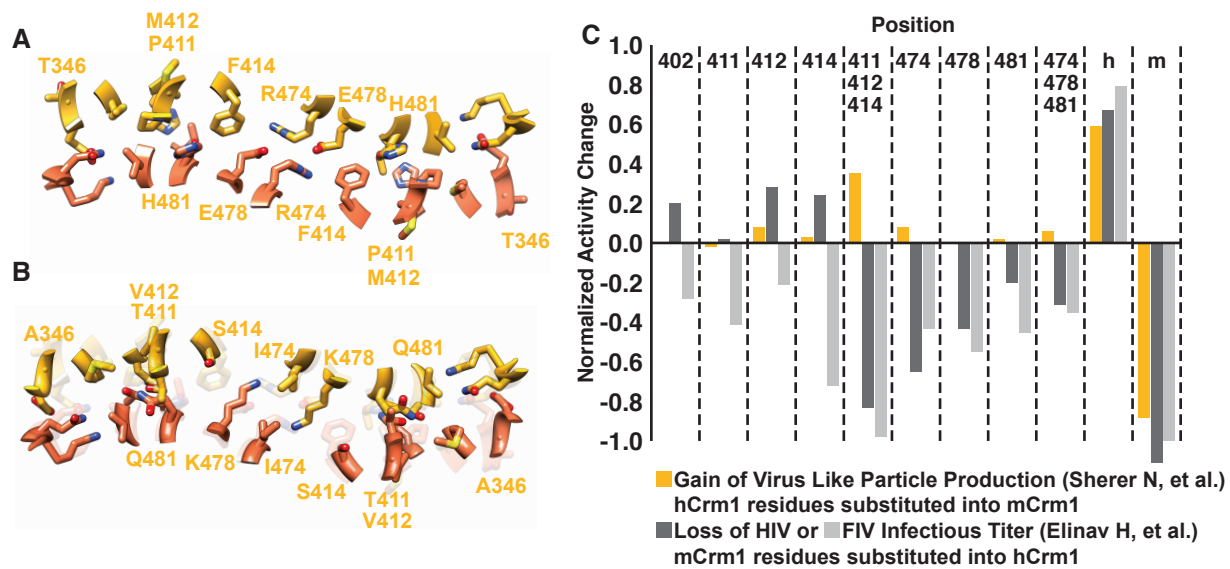


Figure 2-figure supplement 3: The inability of murine Crm1 to form a dimerization interface results in lower nuclear export activity.

A comparison of divergent residues at the interface of the Crm1 dimer shows many interactions at the human interface (A) but few at the analogous murine positions. Aligning the C α from murine Crm1 with human Crm1 residues at the dimer interface (B) shows steric clashes between side chains near Thr411 in murine Crm1 interface and between the side chains of His352 and Thr487 that would prevent murine Crm1 dimerization at this site. (C) Swapping residues between human and murine Crm1 in the dimer interface alters virus production. A summary of data from references 16 and 18. Scherer et al. substituted human residues into murine Crm1 to determine which residues enhanced VLP production¹⁶. Elinav et al. substituted mouse residues into human Crm1 and scored loss of function¹⁸. In both studies, the gain in activity denoted by ‘h’ is a substitution of all human interface residues into murine Crm1 and vice versa for the loss of activity denoted by ‘m’. Data were normalized according to the following equations: Gain of Function = (mutant activity – murine Crm1 activity)/(human Crm1 activity – murine Crm1 activity) and Loss of Function = Gain of function-1.

establishes the Crm1 stoichiometry¹¹ and the Crm1 dimer interface helps promote cooperative assembly of the complex. The added stabilization of the Rev-RRE export complex imparted by the human Crm1 dimer interface may explain how human Crm1 enhances oligomerization of a Rev-like protein from Human T-cell Leukemia Virus-1²⁸ or why it enhances the nuclear export activity of Rev for other lentiviruses, such as feline immunodeficiency virus¹⁸. The dimer interaction may also influence disassembly of the particle in the cytoplasm to regulate translation and virion assembly^{17, 18, 29}.

Rev-RRE binds between NES binding sites on one side of the Crm1 dimer

To reveal how the Rev-RRE particle docks onto the export complex, we fit a dimer of Crm1 with bound Ran•GTP (Figure 2-figure supplement 2) into the EM map and then searched for extra density. Rev-RRE appears to sit on one side of the Crm1 dimer between the two NES binding sites (Figure 3A). This arrangement contrasts with how a host cargo, Snurportin1, binds to monomeric Crm1, where the main docking surface is on the opposite side of Crm1 (Figure 3B). The extra density observed is too small to fully accommodate a Rev hexamer bound to RNA, likely reflecting the poor visualization of RNA in uranyl-stained images as well as conformational heterogeneity (Figure 3-figure supplement 1) arising from the disordered carboxy-terminus of Rev where the NES is located²⁴.

To be certain of the locations of Rev and the RRE in the particle, we compared class averages of the complex to those formed either with GB1-tagged Rev or with streptavidin-tagged RRE (Figure 3C). Despite heterogeneity arising from flexible linkers to the tags and from different conformations within each class, additional density was plainly visible in both tagged complexes (Figure 3-figure supplement 2). Rev and the RRE are unambiguously positioned on one side of the Crm1 dimer, with Rev close to Crm1 and the RRE slightly further away,

emphasizing the role of Rev as a bridge between the RNA and export machinery. Class averages from cryo-electron micrographs, where RNA staining is not an issue, also show extra density in the same location (Figure 3C). Together these data indicate that the positioning of NES sites constrains Rev-RRE to flexibly dock on one side of the Crm1 dimer.

To understand how the Crm1 dimer arrangement relates to binding of the Rev oligomer, we compared the size of Rev hexamer models³⁰ to the distance between NESs in order to evaluate which would plausibly match the fixed dimension of the Crm1 dimer. Strikingly, the conformation of a Rev hexamer model positions the NESs from the two outer Rev subunits to closely match the distance between the two NES-binding sites of the Crm1 dimer (Figure 3D). Given the flexible nature of the Rev carboxy-terminus, there may be some variation in this distance that allows an NES from a neighboring subunit to be utilized, potentially increasing avidity from a high local concentration of NESs. Changes to the architecture of the Rev oligomer would affect this local concentration and shape complementarity with the Crm1 dimer. Within a single host, HIV can evolve to fine-tune the levels of nuclear export through changes in the RRE^{14, 15}. Such changes may reflect an RNA-driven remodeling of Rev dimers and oligomers^{24, 30}, allowing access to conformations compatible with the defined architecture of the Crm1 dimer.

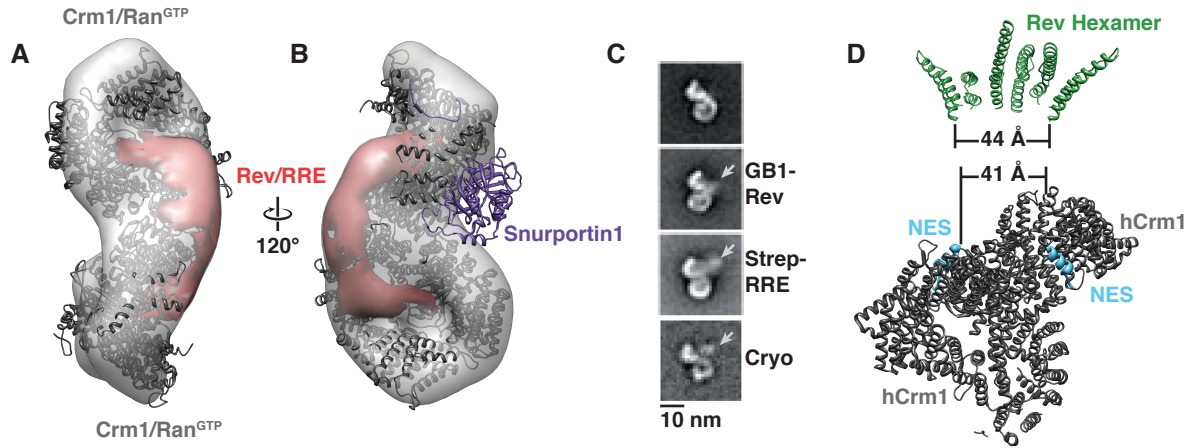


Figure 3: Rev-RRE binds between NES binding sites on the Crm1 dimer.

(A) A model of the Crm1-Ran•GTP dimer displays extra density (red) on one side of the structure that locates the Rev-RRE complex. The Crm1-Ran•GTP dimer model was built by aligning two murine Crm1-Ran•GTP complexes onto the human Crm1 dimer. (B) Rotated view of the structure showing the location of Snurportin1 bound to a Crm1 monomer on the opposite side of the protein. (C) Class averages of particles containing Rev tagged with GB1 or RRE tagged with streptavidin, and cryo-EM of the complex, confirm the location of Rev-RRE. Raw micrographs, class averages, and particle numbers are shown in Figure 3-supplement 1. (D) The distance between two NESs (cyan) bound to the Crm1 dimer is similar to the distance between the terminal subunits of the Rev hexamer (green).

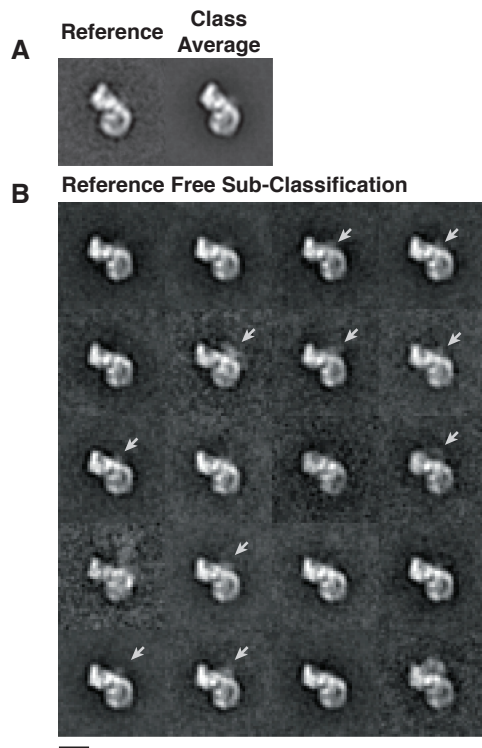


Figure 3-figure supplement 1: Heterogeneity within class averages reveals Rev-RRE density.

Particles collected from an independent data set were aligned to references from random conical tilt class averages (A), and 1150 particles from one class were sub-classified using a reference-free alignment (B). Some of the sub-classes further reveal extra densities (arrows) at the Rev-RRE location. Scale bar shows 10 nm.

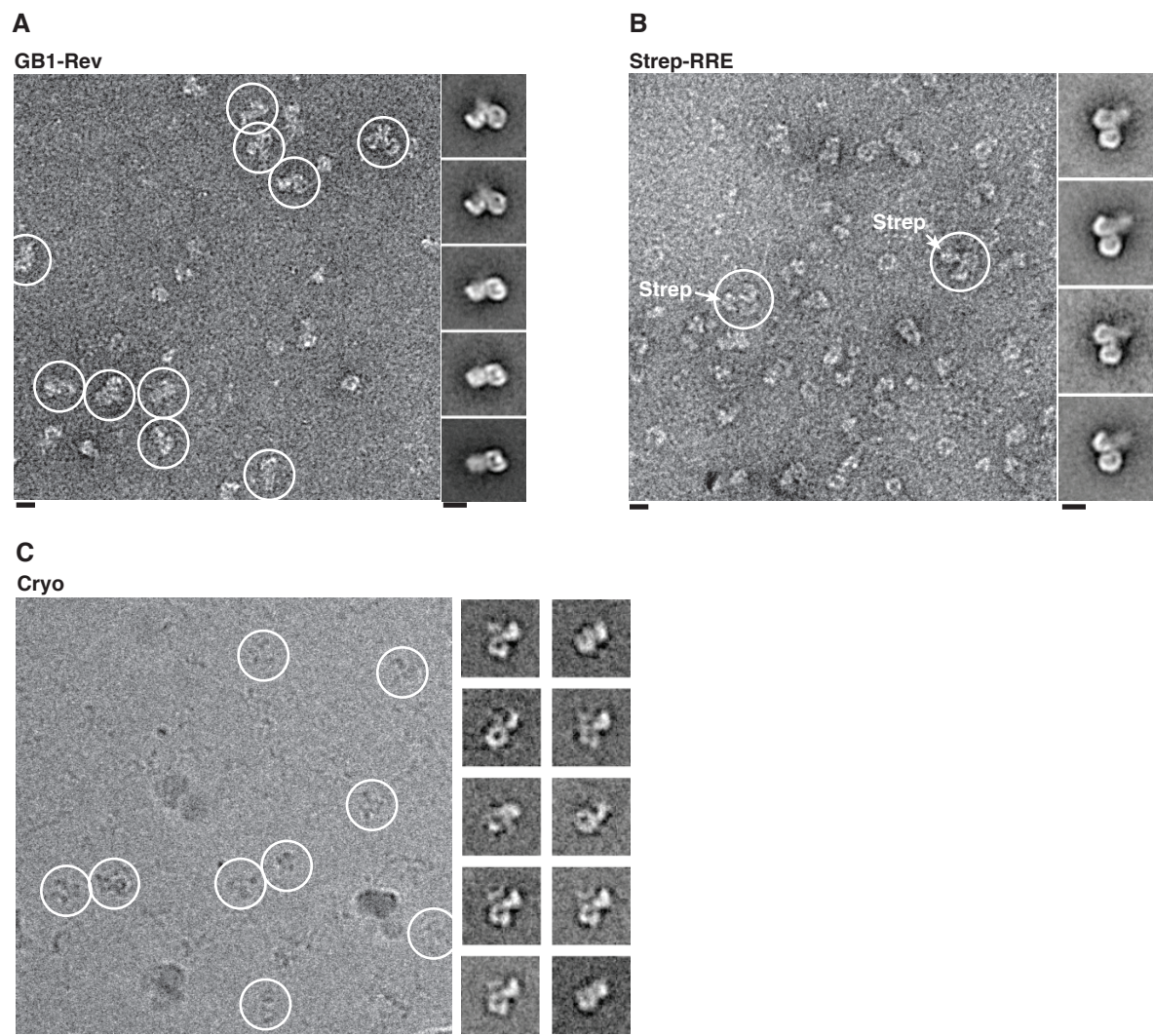


Figure 3-figure supplement 2: Localization of Rev-RRE density.

Representative fields and class averages from negative stain (A-B) or cryo-electron micrographs (C) show extra density from Rev-RRE when Rev is tagged with GB1 (A, 794 particles) or the RRE tagged with Streptavidin (B, 383 particles). Extra density from Rev-RRE also is apparent in cryo-EM images (C, 3262 particles), where the contrast is generated from the particles themselves rather than from uranyl ions that typically obscure nucleic acids. Scale bars show 10 nm.

DISCUSSION

This work provides the first glimpse of assembled HIV nuclear export complexes, involving an essential HIV-host interaction between the Rev-RRE complex and Crm1. Most surprisingly, we revealed the existence of a Crm1 dimer, whose details could be inferred from crystal contacts observed in an existing Crm1 structure¹⁹. The details of its interface with Rev-RRE and possible significance as a therapeutic target will require a higher resolution structure of the entire complex and a better understanding of whether the Crm1 dimer is utilized in host cell biology. Nonetheless, the ability to assemble these complexes *in vitro* provides a good starting point to investigate how additional host proteins may work with Crm1 during viral RNA trafficking.

It is clear that the Crm1 dimer architecture is functionally important for HIV replication since mutations in Crm1 that decrease Rev-dependent export and restrict viral replication correspond to residues that form the interface between Crm1 monomers¹⁶⁻¹⁸. Changes in Crm1 dimerization may also modulate levels of nuclear export for host genes. For example, Crm1 mediates nuclear export of a subset of mRNAs via proteins that bind elements within their 3' ends³¹⁻³⁴. Unspliced HIV RNA resembles this organization, with the RRE acting like an element at the 3'-end. Perhaps analogous to how the RRE directs assembly of a Rev oligomer, the positioning of cis-acting elements within a cellular mRNA may define a topology for RNA-binding proteins to recruit a Crm1 dimer. Such a mechanism would allow Crm1-mediated export to integrate different protein signals to tune the levels of nuclear export for a given mRNA.

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MATERIALS AND METHODS

Molecular Cloning

All plasmids used in this study are listed in Table 1. Crm1 and Ran were cloned between the NdeI and XhoI sites in a pET19b vector (Novagen) that encodes an amino-terminal deca-histidine tag followed by a tobacco etch virus (TEV) protease site. Genes were amplified from their source vectors by polymerase chain reaction with phusion polymerase (New England Biolabs - NEB) using forward and reverse primers (IDT DNA, Table 2) containing NdeI or XhoI restriction sites, respectively. PCR products and the target vector were digested with restriction enzymes (NEB), purified (Qiagen), ligated (Roche), and then transformed into *E. coli DH5α*. Plasmids from positive transformants were isolated (Qiagen), screened by restriction digests, and sequenced (Berkeley DNA Sequencing Facility).

Site-directed Mutagenesis

Mutations were introduced using a single primer method according to ref. 35. Briefly, DNA was synthesized from the primer (IDT DNA) using Pfu Turbo (Agilent), purified, DpnI digested, and transformed into *E. coli DH5α*. Plasmids from transformants were isolated and sequenced.

RNA Transcription and Purification

RNA was transcribed by T7 polymerase from a linearized plasmid and then purified by Urea-PAGE³⁶. A single-stranded segment that was a cloning byproduct at the 5'-end of the RRE was removed from the original template by site-directed mutagenesis. Pure RNA pellets were resuspended in buffer A and stored at -20°C. RNA was annealed by heating to 95°C and cooling to 30°C in an aluminum heat block placed at room temperature. RNA was quantified by UV spectroscopy using a 260 nm extinction coefficient of $2.5 \times 10^6 \text{ M}^{-1}\text{cm}^{-1}$ for the RRE.

5'-end labeling RNA

Purified RNA was treated with calf intestinal phosphatase according to the manufacturer (NEB). RNA was purified by phenol-chloroform extraction, ethanol precipitated in 300 mM NaCH₃CO₂, washed with 70% ice-cold ethanol, and resuspended in buffer A [1 mM HEPES-KOH, pH 7.5 @ 20°C]. RNA was labeled for ≥1 h at 37°C in buffer B [50 mM imidazole-HCl, pH 6.4 @ 20°C, 10 mM MgCl₂, 5 mM DTT] with 10 Units of Polynucleotide Kinase (NEB) by adding either γ -³²P-ATP (Perkin Elmer, 3000 Ci/mmol) or ATP γ S (Sigma Aldrich) to final concentrations of 450 nM and 1 mM, respectively. For radiolabeling, RNA was purified by phenol chloroform extraction, diluted in buffer A, and desalted with PD Spintrap G-25 column (GE Lifesciences) according to manufacturers instructions. For biotin labeling, RNA was purified by adding NH₄CH₃CO₂ to a final concentration of 2.5 M and centrifuging at >20,000 x g and 4°C for 1 h to pellet the protein. RNA in the supernatant was ethanol precipitated and resuspended in buffer A. Maleimido-biotin was added at a final concentration of 100 μ M, incubated at room temperature for 4 h, and quenched with an excess of 2-mercaptoethanol. The reaction was diluted in buffer A, desalted as above, and ethanol precipitated twice in NaCH₃CO₂ in order to get rid of excess label. The washed RNA pellet was resuspended in buffer A. After labeling, RNAs were annealed as above and evaluated on Urea-PAGE to ensure no degradation. Radiolabeled RNA was quantified by scintillation counting, aliquoted, and then stored at -20°C.

Protein Expression

Proteins were expressed in *E. coli* BL21(DE3). Bacteria were grown in Luria-Bertani Miller Formula (LB) media with noted supplements by diluting overnight cultures 50-fold into 1 L of media and by shaking at 200 rpm in baffled Fernbach flasks to the indicated OD₆₀₀. Expression was induced by adding isopropyl- β -D-thiogalactopyranoside (IPTG) to 200 μ M.

Bacteria were harvested by centrifugation at 4,500 x g and 4°C for 20 min. Pellets were frozen in N₂ (l) and stored at -80°C.

Rev Expression

Rev was expressed as previously described³⁶.

Crm1 Expression

LB media and agar plates were supplemented after autoclaving with 1% (w/v) glucose, 100 µg/ml Ampicillin, and 34 µg/ml chloramphenicol. Crm1 expression vectors were co-transformed with pLysS-Rosetta2 (Novagen), and colonies were selected on LB agar plates. Bacteria were grown for protein expression with chloramphenicol reduced to 17 µg/ml at 37°C until OD₆₀₀=1.0-1.2. Cultures were shifted to 20°C for 20 min, induced by adding IPTG to 200 µM, and grown overnight.

Ran Expression

Bacteria were grown in LB with 100 µg/ml Ampicillin at 37°C to OD₆₀₀=0.6-0.8, shifted to 18°C for 20 min, induced with 500 µM IPTG, and grown overnight.

Rev Purification

Each gram of bacterial pellet was resuspended in 10 ml of buffer C [50 mM Tris-HCl, pH 8.0 @ 20°C; 2 M NaCl; 2 mM 2-mercaptoethanol; 10 mM imidazole; 0.1% (v/v) Tween-20]. Proteases were inhibited by adding phenylmethanesulfonylfluoride (PMSF) to a final concentration of 1 mM and one complete, Mini, EDTA-free protease inhibitor cocktail tablet (Roche) was added for every 5 g of bacteria. Bacteria were lysed with an Emulsiflex-C3 homogenizer (Avestin), and the lysate was cleared by centrifugation at 30,000 x g and 4°C for 30 min. The supernatant was loaded on Ni-NTA Agarose resin (Qiagen) with 1 ml of resin for every

2.5 g of bacterial pellet. The resin was washed extensively with buffer C and then buffer D [125 mM K_2HPO_4 - KH_2PO_4 , pH 7.5 @ 20°C; 250 mM NaCl; 2 mM 2-mercaptoethanol; 10% (v/v) glycerol; 0-500 mM imidazole]. A step-wise increase in imidazole concentration eluted proteins from the resin. Pure fractions were combined, supplemented with TEV protease at a 1:20 molar ratio, and dialyzed overnight in a 3,500 Da MWCO Slide-A-Lyzer Cassette (Thermo Scientific) against buffer D without imidazole. Dialyzed protein was passed over Ni-NTA resin. The flow-through was collected and filtered through a 0.22 μm syringe filter. The protein was then loaded onto a Mono-S 5/50 GL column (GE Lifesciences), washed, and eluted with a linear gradient of buffer D into buffer E [500 mM K_2HPO_4 - KH_2PO_4 , pH 7.5 @ 20°C; 1 M NaCl, 2 mM 2-mercaptoethanol; 10% (v/v) glycerol]. After analysis by SDS-PAGE, pure fractions were pooled, frozen in N_2 (*l*) and stored at -80°C. Rev concentration was determined by UV spectroscopy using 280 nm extinction coefficients of 20,000 $\text{M}^{-1}\text{cm}^{-1}$ for GB1-Rev and 8,480 $\text{M}^{-1}\text{cm}^{-1}$ for Rev.

Crm1 Purification

Crm1 was resuspended in 4 ml of buffer F [50 mM Tris-HCl, pH 7.5 @ 4°C; 600 mM NaCl; 2 mM $\text{Mg}(\text{CH}_3\text{CO}_2)_2$; 2 mM 2-mercaptoethanol; 10-200 mM imidazole; 20% (v/v) glycerol] containing 0.1% (v/v) Tween-20 and 5% glycerol for each gram of bacterial pellet. A protease inhibitor tablet was added for every 40 g of bacteria with PMSF. Lysis was as for Rev. Contaminating nucleic acids were removed from the supernatant of the clarified lysate by adding polyethylenimine to 0.2% (w/v) and cleared by centrifugation at 20-25,000 x g and 4°C for 30 min. Ni-NTA chromatography was as for Rev with with 1 ml of resin for every 20 g of bacterial pellet and washes and elutions were in buffer F. TEV protease was added to pooled fractions in a 1:20 molar ratio and incubated at 4°C overnight. Protein was concentrated with a 10 kD MWCO Amicon Ultra Free Centrifugal Concentrator (Millipore) and filtered through a 0.22 μm syringe

filter. Crm1 was further purified by size-exclusion chromatography with buffer F containing 10% glycerol and 20 mM imidazole on a Superdex 200, HiLoad 16/60 column (GE Lifesciences) followed in tandem by a HisTrap Fast Flow, 5 ml column (GE Lifesciences). Fractions were analyzed by SDS-PAGE. Pure fractions were pooled, concentrated, frozen in N₂ (l), and stored at -80°C. Prior to use Crm1 was exchanged into buffer G [50 mM HEPES-KOH, pH 7.5 @ 20°C, 50 mM KCl, 2 mM Mg(CH₃CO₂)₂, 2 mM 2-mercaptoethanol, 2% (v/v) glycerol] using a 7 kD MWCO, Zeba Spin column (Thermo), and the concentration was determined using Bradford assays with a bovine serum albumin (BSA) standard (Thermo Scientific).

Ran Purification and Nucleotide Exchange

Ran was purified in a similar manner as Rev with the following modifications: Lysis, Ni-NTA purification, and dialysis were performed with buffer H [64 mM Tris-HCl, pH 7.5 @ 4°C, 400 mM NaCl, 5 mM MgCl₂, 2 mM 2-mercaptoethanol, 0-250 mM Imidazole]. Prior to cation exchange, protein was concentrated with a 3 kD MWCO Amicon Ultra Free Centrifugal Concentrator (Millipore), nucleotides were removed by adding EDTA to a final concentration of 10 mM, and exchanged into buffer I [25 mM MES-HCl, pH 6.5 @ 4°C, 0.020-1 M KCl, 1 mM EDTA, 4 mM 2-mercaptoethanol, 10% (v/v) glycerol] using a PD-10 column (GE Lifesciences). Any precipitate was removed by centrifugation at 20,000 x g followed by filtration through a 0.22 µm syringe filter. Cation exchange was performed with buffer I and eluted with a linear gradient of KCl in buffer I. Prior to use Ran nucleotides were exchanged into Ran by adding nucleotide and MgCl₂ to final concentrations of 1 mM and 5 mM, respectively, and then incubating at room temperature for 30 min. Excess nucleotide was removed by exchanging the protein into buffer G using a 7 kD MWCO, Zeba Spin Column (Thermo). Protein concentration was determined with a BSA standard as for Crm1.

Affinity co-purification with GB1-Rev

Ran was exchanged with GTP γ S or GDP as described above. Rev was diluted with buffer G containing 30 mM imidazole, and Crm1 and Ran were exchanged into the same buffer in order to reduce non-specific interactions with the resin. 1.6 μ M GB1-Rev was mixed with 200 nM RRE, 400 nM Crm1, and/or 800 nM Ran in 50 μ L. Reactions were incubated with 50 μ L of Ni-NTA agarose resin for 2 hrs at 25°C. The supernatant was removed and the beads were washed three times with 500 μ L of buffer. Proteins were eluted by rotating the resin for 5 h with 100 μ L of assembly buffer containing 750 mM imidazole. Eluates were separated on a 4-12% Novex Bis-Tris Gel in SDS-MES buffer (Life Technologies) and stained using SYPRO Ruby (Lonza). In these gels, only Rev and Crm1 are easily visible because Rev and Ran migrate with the same apparent mass.

Size-Exclusion Chromatography and Multi-angle Laser Light Scattering (MALLS)

Rev-RRE complexes were assembled with a ten-fold excess of Rev added to the RRE. Crm1 and RanQ69L were then added to Rev-RRE and diluted to 70 μ L. The final concentration of the RRE was 4 μ M for stoichiometric titrations. Based on the affinity of a nuclear export sequences near 100 nM¹³, Rev-RRE completely assembles with Crm1 and RanQ69L at these concentrations but disassembles through dilution on the column, as supported by native gel electrophoresis (data not shown). The assembly reactions were incubated at 37°C, filtered through a 0.22 μ m centrifugal filter (Amicon), and then kept on ice. A KW-804 (Shodex) column equilibrated in buffer G was loaded with 50 μ L of assembly reaction. Complexes were separated at a flow rate of 0.35 ml/min, and the 12.5 ml column volume was collected in 135 μ L fractions. Fractions were analyzed by SDS-PAGE as for in vitro pulldowns. Molecular masses were determined using the program ASTRA from MALLS and refractometry (Wyatt)

measurements following size-exclusion chromatography. For these experiments, the concentration of Rev-RRE was increased to 8 μM for accurate mass determination. The masses of Crm1 and RanQ69L were determined at 20 μM and 50 μM , respectively. Because the technical error of measuring the mass of the nuclear export particle was nearly the same as a Rev monomer, we validated the stoichiometry by densitometry of SDS-PAGE gels stained with Sypro-Ruby, scanned on a Typhoon (GE Lifesciences), and quantified with ImageQuant. Concentrations for standards of each protein were determined by amino-acid analysis (UC-Davis Proteomics Facility).

Native Gel Electrophoresis

Crm1 and Ran were exchanged into buffer J [50 mM HEPES-KOH, pH 7.5 @ 20°C, 100 mM KCl, 2 mM $\text{Mg}(\text{CH}_3\text{CO}_2)_2$, 0.5 mM EDTA, 2 mM 2-mercaptoethanol, 12% (v/v) glycerol] and protein concentrations were determined by quantitative SDS-PAGE. Stocks of Rev and the RRE were diluted in buffer J and then combined at 80 nM and 2000 cpm/ μl (~0.5 nM), respectively. Crm1 and Ran•GTP were serially diluted in buffer J containing 100 $\mu\text{g}/\text{ml}$ BSA, 10 μM yeast tRNA, and 200 nM methionine. 6 μl of Crm1-Ran•GTP was added to 6 μl of Rev-RRE and assembled at 37°C for 15 min. 10 μl of the assembly reaction was loaded onto a 5% acrylamide (Protogel, 37.5 acrylamide to 1 bisacrylamide) native gel in buffer K [25 mM HEPES-25 mM imidazole, pH 7.4 @ 20°C] and continuously running at ~10 V/cm and 20°C. The gel was dried onto filter paper, exposed to a phosphor-screen overnight, and imaged on a Typhoon. Intensities (I) were quantified using ImageQuant and normalized with the equation $I_{\text{Norm}} = 1 - (I_j^{\text{Rev-RRE}} / I_j^{\text{LaneTotal}}) / (I_0^{\text{Rev-RRE}} / I_0^{\text{LaneTotal}})$, where I_j is the intensity at the indicated concentration of Crm1-Ran•GTP and I_0 is the intensity of Rev-RRE alone. A modified Hill equation, $I_{\text{Norm}} = I_{\text{max}} \cdot [\text{Crm1-Ran}\bullet\text{GTP}]^h / ([\text{Crm1-Ran}\bullet\text{GTP}]^h + K_{\text{app}}^h)$ where K_{app} is the apparent

equilibrium constant and h is this Hill coefficient, was fit to data by chi-squared (χ^2) minimization using the Solver tool in Microsoft Excel and converted to fraction bound (θ) with the equation $\theta = I_{\text{Norm}}/I_{\text{max}}$. Parameter errors were calculated using jackknife resampling^{37, 38}. We also used a sum-of-squares minimization to fit the model to data from individual experiments and then calculated the average parameter values and standard errors. We obtained good fits ($\chi^2_{\text{hCrm1}} = 1.69$ and $\chi^2_{\text{mCrm1}} = 3.13$, model compared to averages for θ) with similar values as reported for K_{app} , a larger value for h with hCrm1, a smaller value for h with murine Crm1, and larger errors in all of the values. hCrm1 still has statistically significant higher values for K_{app} ($P < 0.01$) and h ($P < 0.05$) than mCrm1. Overall, testing different fitting procedures indicates that the reported values for the model are conservative estimates that demonstrate hCrm1 binds Rev-RRE with higher affinity and cooperativity than mCrm1.

Negative-Stain Electron Microscopy

2.5 μl of 100-400 nM samples were adsorbed to glow-discharged, carbon-coated, 400 mesh grids and stained with 0.75% uranyl formate as previously described³⁹. For random conical tilt, 200 mesh grids were used. A substoichiometric amount of streptavidin was added to biotinylated RNA immediately before adsorbing onto grids. Images were acquired at 1.5 μm defocus for untilted images and ~ 2.0 μm for tilted images using low dose procedures on a T12 microscope (FEI) operating at 120 kV and recorded on a 4096 x 4096 CCD camera (Ultrascan 4000, Gatan) with a 52k magnification corresponding to a pixel size of 2.21 Å. Tilt pairs were manually collected at 60° and then 0° angles.

Cryo-Electron Microscopy

2 μl of sample was applied to 1.2/1.3 μm Quantifoil Grids (Electron Microscopy Sciences), blotted, and frozen in liquid ethane with a Vitrobot (FEI). Images were recorded with

a 2-4.5 μm defocus range using low dose procedures on a TF20 microscope (FEI) operating at 200 kV and recorded on a 8192 x 8192 CMOS camera (TVIPS, Germany) at 62k magnification corresponding to a pixel size of 1.29 $\text{\AA}$.

Image Processing

Negative-stain images were binned two-fold for a pixel size of 4.42 $\text{\AA}$. Cryo-images were binned four-fold for a final pixel size of 5.17 $\text{\AA}$. The defocus astigmatism, tilt angle, and tilt axis were determined using CTFFind and CTFTilt⁴⁰. Tilted images were rotated to align the tilt axis with untilted images. Particles were manually selected using Samviewer⁴¹. The tilt angle for each pair was further refined based on particle positions. Particles were boxed from images, combined into a single stack, normalized, and contrast transfer function (CTF) corrected. For cryo images, the contrast was inverted to have white particles against a black background as required for SPIDER⁴². Untilted particles were subjected to reference free classification followed by multiple rounds of correspondence analysis, k-means classification, and multi-reference alignment using SPIDER^{41, 42}.

3-D Reconstruction

For each class average of untilted images, we calculated a random conical tilt (RCT) reconstruction from tilted particles with angular parameters from the in plane rotation of class averages and from the tilt angle determined during particle picking. This initial RCT reconstruction was first refined for translational alignment of individual particles. Images of untilted particles were then added to the reconstructions followed by translational alignment. All refinements were performed using FREALIGN⁴³ in a frequency-limited range of 240-38 $\text{\AA}$. Volumes were low-pass filtered after each cycle of refinement using the FCREF option. When we compared RCT reconstructions using UCSF Chimera, we concluded the first five classes

were different views of the same structure and the remaining two classes were substoichiometric particles (Figure 2-figure supplement 1). Untitled and tilted particles from the first five classes were merged into a single dataset for further projection matching refinement. The RCT reconstruction from Class02 was used as an initial model to determine Euler angles and in-plane shifts of all particles. In the subsequent iterative refinement, the frequency was limited between 240 Å and 34 Å. The final resolution was estimated to be 25 Å (FSC=0.143) to 30 Å (FSC=0.5) (Figure 2-figure supplement 1). The Euler angle distribution of all particles suggests there is no major gap in Euler space. A b-factor of -1000 Å^2 with a cosine-edge, low-pass filter to 25 Å and a 2-pixel width was applied to the final volume using bsoft (N. Grigorieff). We also tested applying C2 symmetry during the refinement and reconstruction. Symmetry improves the resolution of the overall reconstruction but weakens the features from Rev-RRE. We, therefore, only interpreted the volume with no symmetry applied.

Molecular Modeling

All molecular modeling was performed using UCSF Chimera⁴⁴. The volume of the EM reconstruction was set to a threshold that corresponded to a 372 kD mass, which is the mass of the particle without the RRE. Volumes were calculated from molecular masses using published measurements of protein⁴⁵ or RNA⁴⁶ density, respectively. To obtain rigid body fits of crystal structures into the EM map, we filtered crystal structures to 25 Å, set the volume threshold to correspond to the molecular mass of the crystal structure, placed the structure into the map, and optimized the correlation between EM and crystal structure volumes using the ‘Fit in Map’ function in Chimera.

Table 1 – Plasmids for protein and RNA expression

Name	Description	Reference
pHG-Rev	HXB3 Rev	36
pHG-Rev M10	Site directed mutagenesis of pHG-Rev.	This study
pET19b-hCrm1	Amplified from <i>Homo sapiens</i> Crm1cDNA clone (Open Biosystems).	This study
pET19b-mCrm1	Amplified from pCDNA-myc-murCrm1 from M. Malim ¹⁶	This study
pET19b-hRan	Amplified from pQE30-hRan from I. Mattaj ²²	This study
pET19b-hRanQ69L	Site directed mutagenesis of pET19b-hRan	This study
pBluescript-5'ΔRRE	Site directed mutagenesis of pBluescript-SF-2 RRE	

Table 2 – Primers used for cloning and site-directed mutagenesis

Name	Sequence (5'-3')
Crm1 Forward	GCACTGACATATGCCAGCAATTATGACAATGTTAGCAGACCATG
Crm1 Reverse	CTTCTCGAGTTAATCACACATTTCTTCTGGAATCTCATGTGGATTAAAG
Ran Forward	GCACTGACATATGACCGCGCAGGGAGAG
Ran Reverse	CTTCTCGAGTTACAGGTCATCATCCTCATCCGGGAG
RevM10	GTGCCTCTTCAGCTACCACCGGATCTGAGACTTACTCTTGATTG
RanQ69L	GTATGGGACACAGCCGGCCTGGAGAAATTCGGTGGACTG
5'ΔRRE	GCGTAATACGACTCACTATAGGGCTAGTAGGAGCTATGTTCC

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**CHAPTER 3: HIV selects a conformation of the Rev-RRE complex
to bind the nuclear export receptor Crm1 through adaptive mutations in the RRE**

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ABSTRACT

Adaptations in HIV that improve viral replication cause concern for the course of an infection and treatment. By post-transcriptionally regulating the expression of viral genes, the viral protein Rev acts at a critical time in the viral lifecycle to enhance the capacity for producing new virions. Rev binds to the Rev Response Element (RRE) within viral RNAs and interacts with the host proteins Crm1 and Ran through a nuclear export sequence (NES) in order to facilitate the nuclear export of unspliced and partially spliced RNAs. In preliminary experiments, we examine how one mutation in the RRE that has been shown to enhance viral fitness affects the association of the Rev-RRE complex with Crm1 and Ran. We find that this mutation does not perturb the affinity of the host-virus interaction but does stabilize a conformation of Rev-RRE bound to Crm1 and Ran as seen by negative stain electron microscopy. Based on the augmentation of Rev-RRE features in low-resolution models, RRE mutations may provide a path towards a high-resolution structure of the entire host-virus complex. Overall, these results posit that RRE mutations enhancing nuclear export may alter the dynamics rather than the equilibrium of complex formation.

INTRODUCTION

Viruses have an astounding capacity to evolve beneficial adaptations during the course of infecting their hosts¹. For human immunodeficiency virus (HIV), the ability to evolve drug resistance mutations shows a clinically concerning example of viral evolution². In addition to these mutations in the structural and enzymatic proteins targeted by most therapies, accessory and regulatory proteins can acquire mutations that alter the ability for HIV to replicate. Studies of patients with long-term HIV infections that do not progress to acquired immunodeficiency syndrome have demonstrated that mutations diminishing the function of accessory and regulatory proteins can prevent replication³. One of these proteins, Rev, serves a central role in the HIV lifecycle by recruiting the nuclear export receptors Crm1 and Ran to the Rev Response Element (RRE), a cis-acting element in unspliced and partially spliced viral RNAs, to facilitate transport to the cytoplasm for gene expression⁴. Interestingly, studies have not only identified mutations that can inhibit the function of Rev⁵ but also have found changes in the RRE that augment nuclear export in order to increase viral replication⁶. These studies suggest that HIV can evolve beneficial mutations in the Rev-RRE module to enhance the replicative capacity of the virus, but how such mutations function is unknown since they minimally impact RRE directing the assembly of a Rev homo-oligomer, a step required for eliciting nuclear export^{6,7}.

Because previous work has shown that nuclear export enhancing mutations in the RRE negligibly impact the assembly of Rev with the RRE, we thought that these mutations might alter the association of Rev-RRE with Crm1 and Ran. A plausible mechanism for RRE mutations to enhance the interaction with Crm1 comes from a crystal structure of the Rev dimer bound to a portion of the RRE⁸. This structure revealed a conformational change in the Rev dimer that could produce a variety of shapes for the functional Rev oligomer, emphasizing that the RRE could act

as an allosteric modulator of the Rev oligomer. In chapter two, we suggested that the conformation of the Rev oligomer might affect Crm1 binding to the Rev-RRE complex since the Crm1 dimer sets a fixed distance between nuclear export sequence (NES) binding sites. Mutations in the RRE might stabilize different conformations of the Rev oligomer to alter the spacing of available NESs for Crm1 to bind. Arguing against this, however, is the observation that the interaction between Rev-RRE and Crm1 is flexible, arising from the NES lying in an unstructured portion of Rev⁹ and the choice of six different NESs for the Crm1 dimer to bind¹⁰. The flexibility may provide such a wide range of options for assembling the complex that variations in the Rev oligomer would do little to perturb binding. Yet, without a high-resolution view of the Rev-RRE complex, we do not know how available NESs may be for Crm1 binding.

Altered conformations of the Rev oligomer may augment Crm1 binding in two possible ways. On one hand, the Rev-RRE complex may present only a few options for engaging the Crm1 dimer, so mutations in the RRE could change the oligomer to provide more possible interactions with Crm1. In this case, we would expect that changes in the RRE would provide many degenerate binding modes that result in a higher affinity binding with the Crm1. Because there would be an array of Rev-RRE conformations bound to Crm1, we would also expect a high degree of structural heterogeneity introduced from these RRE mutations. On the other hand, the Rev-RRE complex may have a plethora of conformations, some of which do not associate well with Crm1. Mutations in the RRE could select an active conformation of the Rev oligomer that engages the Crm1 dimer more efficiently. This conformational selection may also result in a higher affinity interaction with Crm1 from a larger fraction of active Rev-RRE complexes but would have a different structural consequence than degenerate binding because the selection of a particular conformation would lead to a more defined complex. Either of these scenarios would

provide an important insight into understanding how HIV can evolve the interaction with Crm1 to enhance viral replication.

A representative mutation for understanding how changes in the RRE might affect assembly with Crm1 is the RRE61 mutation. RRE61 was isolated from a NL4-3 strain of HIV that became resistant to dominant negative inhibition by overexpressing Rev with an M10 mutation¹¹, which is a mutation of Leu78Asp and Glu79Leu that renders the NES inactive¹². RRE61 harbors two mutations (Figure 1A) that result in a synonymous codon for the envelope protein but changes the secondary structure of the RRE⁷. The first mutation, G181A, located near stem III is found in the majority of HIV-1 isolates⁷, including the RRE from the SF-2 strain that we have previously used for structural studies, and does not confer resistance. The second mutation, G262A, located in stem V is responsible for RRE61 resistance and induces a change in the stem V base pairing⁷. In addition to these mutations, a third difference, G269A, between the NL4-3 RRE and the SF-2 strain disrupts base pairing in the stem IV/V junction. Since all of these mutations sit at or near sites protected by Rev during particle assembly^{7, 13}, we anticipate that they may alter the conformation of the Rev oligomer as they change the secondary structure of the RRE.

We engineered the G262A and G269A mutations into our existing version of the SF-2 RRE to interrogate how RRE61 alters the assembly and structure of the complex formed between Rev-RRE, Crm1 and Ran. In a preliminary study, we find that the RRE61 mutations have little impact on the steady-state formation of the particle yet result in a structure with a stronger density corresponding to the location of Rev-RRE. These results are consistent with a model where RRE61 mutations stabilize a particular conformation of the Rev oligomer that associates with Crm1 dimer, but do not explain how the mutations may be beneficial for nuclear export since

they do little to alter Crm1 binding. We suggest that these mutations in the RRE and alternate versions of Rev may help future work in determining the structure of the entire Rev-RRE complex bound to Crm1 and argue that kinetic studies are required for determining how changes in the RRE influence particle assembly and nuclear export.

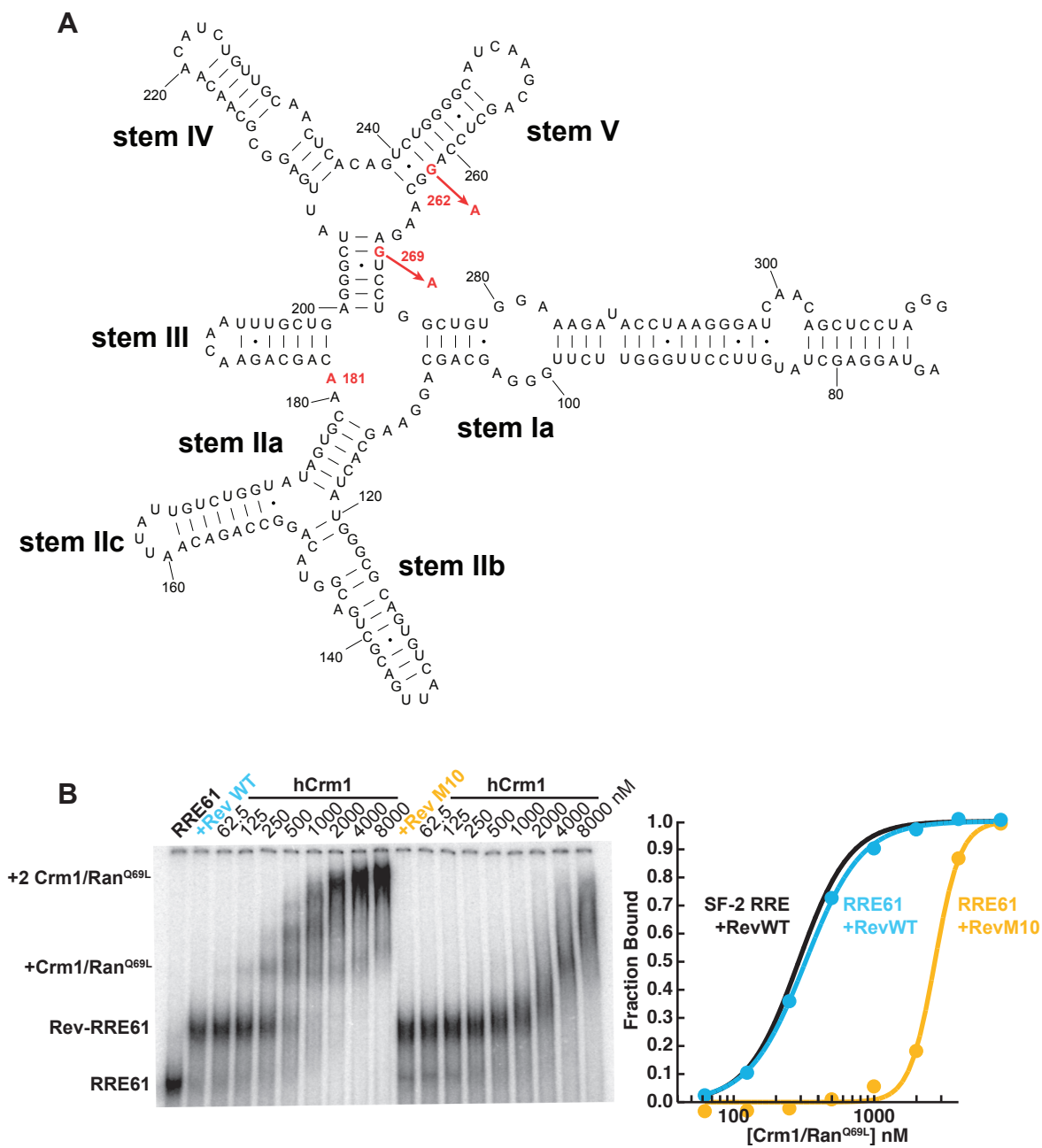


Figure 1: RRE61 does not enhance the association between Rev-RRE and Crm1-Ran•GTP

(A) A secondary-structure of the RRE from the SF-2 strain of HIV¹³ showing the location and identity of RRE61 mutations (red).

Figure 1-continued: RRE61 does not enhance the association between Rev-RRE and Crm1-Ran•GTP

(B) RevM10 abrogates Crm1 binding to Rev-RRE complexes assembled with the RRE61 mutation. RevM10 is a mutation within the NES of Leu78Asp and Glu79Leu (left). Native gel electrophoresis with a fixed amount of Rev-RRE and increasing amount of Crm1 and Ran•GTP shows a significant difference in affinity between Rev and RevM10. (right) Fitting the Hill equation, $\text{Fraction Bound} = [\text{Crm1-Ran}\bullet\text{GTP}]^h / ([\text{Crm1-Ran}\bullet\text{GTP}]^h + K_{\text{app}}^h)$ where K_{app} is the apparent dissociation constant and h is the Hill coefficient, to the quantification of Rev-RRE being incorporated into higher-order complexes shows that complexes formed with the RRE61 mutation (blue) have a similar affinity for binding Crm1-Ran•GTP, $K_{\text{app}} = 326$ nM and $h = 2.17$ for RRE61, as complexes without the RRE61 mutation (black). The isotherm for complexes formed with wild-type RRE was simulated using parameters, $K_{\text{app}} = 291$ nM and $h = 2.37$, determined from previous experiments (Chapter 2).

RESULTS

RRE61 does not enhance the association between Rev-RRE and Crm1-Ran•GTP

We first tested if the RRE61 mutation confers a higher affinity interaction with Crm1. Using native gel mobility shift assays, we monitored the assembly of Rev-RRE into higher-order complexes upon titrating Crm1 and Ran bound to GTP (Ran•GTP). When we compared the affinities of complex formation, we were surprised to find that Crm1 and Ran•GTP bound to RRE61 containing complexes almost the same as it did with the wild-type RRE (Figure 1B, right). Because the RRE61 mutation partially restores nuclear export in the presence of the RevM10 mutation¹¹, we also tested if RRE61 complexes containing the RevM10 mutation were able to bind Crm1. Complex formation with RevM10 was dramatically reduced. At concentrations greater than 2 μ M complexes did form but with more diffuse bands than complexes having a wild-type NES. This result is consistent with previous studies that showed a significant reduction of nuclear export with defective nuclear export sequences even with the RRE61 mutation¹¹. Furthermore, these data exclude a model where RRE61 helps establish a higher affinity interaction between Rev-RRE and Crm1, although the diffuse complexes formed with RevM10 may indicate that degenerate binding occurs in the presence of RevM10.

The RRE61 mutation may enhance nuclear export by stabilizing a particular conformation of the Rev oligomer that is compatible with the dimensions of the Crm1 dimer. Such conformational selection may enhance association by preventing the accumulation of complexes that are non-productive intermediates or decreasing any kinetic barriers along the path to forming an export complex. If RRE61 stabilizes a particular conformation of the Rev oligomer to interact with Crm1, then the density for Rev-RRE in reconstructions from negatively stained electron micrographs would appear stronger than complexes formed with wild-type RRE. A potential

problem, however, for detecting this conformational selection is heterogeneity introduced from flexible portions of Rev.

Before we tested if RRE61 can stabilize a particular conformation of the Rev oligomer, we first wanted to eliminate structural heterogeneity that might arise from the flexible portions of Rev. The portion of Rev with greatest flexibility that would contribute to heterogeneity of the overall complex is a linker between the NES and the arginine-rich motif (ARM) that binds RNA. Although the NES itself is disordered, it adopts structure upon Crm1 binding. In crystal structures of Rev, the last ordered residue is Tyr63, so the stretch between Tyr63 and the NES is the target for reducing flexibility.

HIV-2 Rev is a candidate for reducing heterogeneity from flexible domains of Rev

In order to identify Rev sequences that would reduce heterogeneity, we compared Rev sequences from different isolates of HIV and simian immunodeficiency virus (SIV) available from the Los Alamos National Laboratories HIV Sequence Database¹⁴. Initially, we only examined HIV-1 sequences and did not find a sufficient amount of diversity for Rev (data not shown), so we expanded our search to include SIV and HIV-2 sequences as well. We identified ten sequences that widely represented Rev variation among HIV and SIV isolates (Figure 2A). We examined the ARM, the NES, and the intervening linker to identify the best candidate for assembling complexes with RRE61 (Figure 2B).

We first examined the ARM to ensure that candidate Rev sequences could bind RRE61. We noticed sequences from the SIV lineage that share a common ancestor with SIV_{VER} substitute Asn40, a residue important for recognition of the high affinity binding site stem IIb, for arginine or glutamine residues that may impede RRE binding. It is notable that stem IIb sequences within this lineage differ by substituting a non-canonical G-A base pair for a G-C or G-U pair. These

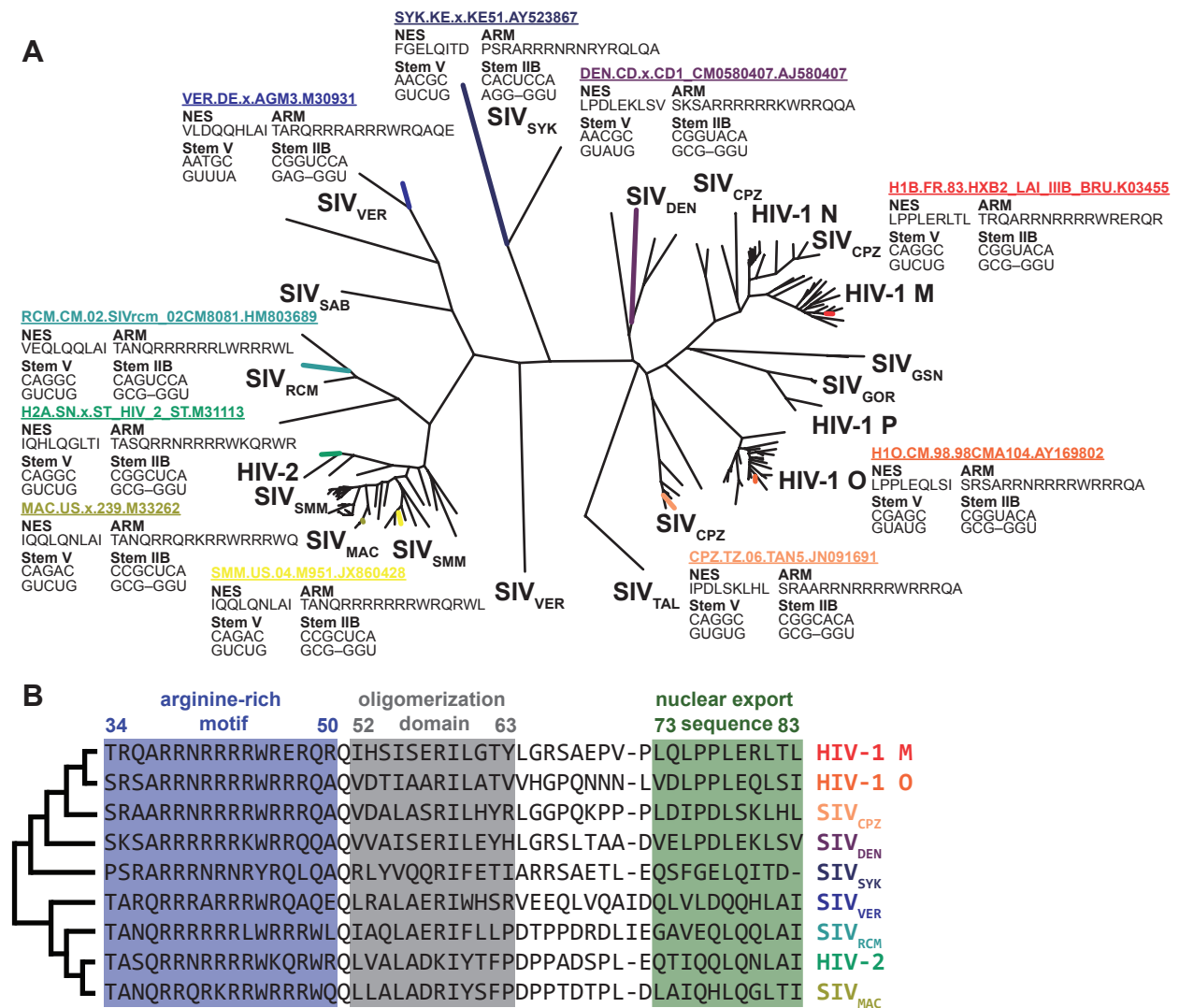


Figure 2: Phylogenetic comparison of HIV and SIV Rev sequences

(A) A cladogram shows the phylogenetic relationships of HIV and SIV sequences obtained from the Los Alamos National Laboratories HIV Sequence Database¹⁴. Ten sequences that comprise a diverse set of Rev variants are indicated in color. Functional features from Rev and the RRE are shown below the identifier for each Rev sequence. (B) A sequence alignment of representative Rev sequences highlight structural features that led to the selection of HIV-2 Rev as a candidate for further structural work.

differences may indicate that RRE has evolved with Rev to accommodate different modes of recognition. We, however, have not investigated if this coevolution occurs, and we have limited our search of these sequences to find candidates of Rev that would be compatible with the existing RRE61 sequence. An exception within this lineage is HIV-2, which has Asn40 and some minor differences from the canonical HIV-1 HXB2 strain. Based on the requirement to maintain Asn40, we narrowed the candidates from ten sequences to four, which includes HIV-2 and sequences that share a common ancestor with SIV_{CPZ}.

Next, we examined NESs from these Rev sequences to ensure they could still bind Crm1. An notable difference between NESs in the lineage sharing a common ancestor with SIV_{CPZ} versus the lineage that shares a common ancestor with SIV_{MAC} is the substitution of Pro, which forms a van der waals interaction with Crm1¹⁵, for a more hydrophilic glutamine residue. While this difference would appear to disrupt an important hydrophobic contact, mutating this residue in silico shows that a rotamer of glutamine could establish a hydrogen bond with a buried carbonyl from the Crm1 backbone (data not shown). From this analysis, it appears that glutamine at this position could still facilitate Crm1 binding. While examining NES differences, we also looked for changes in stem V that would be similar to the RRE61 mutation in order to see if naturally occurring strains of HIV have the capacity to suppress deleterious mutations in the Rev NES. We found only the HIV-1 O subgroup had a mutation similar to that in stem V of RRE61, but the Rev NES within this subgroup was the same as the canonical NES from HIV-1.

Finally, we examined the linker between the ARM and NES to find less flexible sequences that would reduce heterogeneity. When we examined these sequences, we noticed the presence of several glycine residues in this stretch. These residues would probably increase flexibility given the increased angular space sampled by glycine dihedral angles. From this analysis, we

find that of the remaining Rev proteins that have a competent ARM and NES, Rev from HIV-2 appears to be best for reducing the flexibility in the linker sequence because it lacks glycines. Thus, this version of Rev is the best candidate for reducing heterogeneity from the flexible carboxy terminus in order to find a more defined conformation of the Rev-RRE complex when bound to Crm1 and Ran•GTP.

RRE61 enhances the Rev-RRE density in three-dimensional reconstructions of complexes bound to Crm1 and Ran•GTP

In order to assess if RRE61 combined with HIV-2 Rev yields a stable conformer bound to Crm1 and Ran•GTP, we used negative stain electron microscopy to visualize the complex. We assembled complexes with HIV-1 or HIV-2 Rev, the RRE from the SF-2 strain with or without the RRE61 mutation, Crm1, and Ran•GTP in stoichiometric ratios and adsorbed the crude reaction to grids. From these images, we first noticed fewer aggregates when HIV-2 Rev was used in reactions (Figure 3A, left). Although we picked particles outside of these aggregates in all conditions, samples with less aggregation yielded more particles per image. After using semi-automated particle picking to ensure little bias in particle selection, we generated class averages that showed less heterogeneity in complexes formed with HIV-2 Rev as compared to complexes formed with HIV-1 Rev (Figure 3B, right). Overall, forming complexes with HIV-2 Rev has an unexpected consequence for obtaining a more homogeneous sample.

While HIV-2 Rev yielded more homogeneous samples, it was unclear if RRE61 or HIV-2 reduced heterogeneity in associating with Crm1 because it is hard to visualize the Rev-RRE density in negative stain micrographs and class averages. In order to visualize Rev-RRE density in these complexes, we determined the three dimensional structure of complexes containing HIV-2 Rev. We focused on these complexes because more particles persisted after selection

from class averages. First, we examined if substituting HIV-2 Rev for HIV-1 Rev could enhance the Rev-RRE density by comparing its structure to the previous structure of the complex formed with HIV-1 Rev (Figure 3B, left). The structure containing HIV-2 Rev with the SF-2 RRE was similar to the previous structure with the symmetric dimer of Crm1-Ran•GTP being the only visible in the complex (Figure 3B, middle). The invisibility of the Rev-RRE density may indicate that the HIV-2 linker is still quite flexible and/or the Rev-RRE complex binds the Crm1 dimer in multiple conformations that lead to a weaker average density in the reconstruction. In contrast, complexes containing the RRE61 mutation had a much stronger Rev-RRE density (Figure 3B, right). The enhanced density indicates the sample is more homogeneous, most likely resulting from fewer conformations of the Rev oligomer. Together, these results suggest that the RRE61 mutation organizes the complex by stabilizing a particular conformation to bind with the Crm1 dimer.

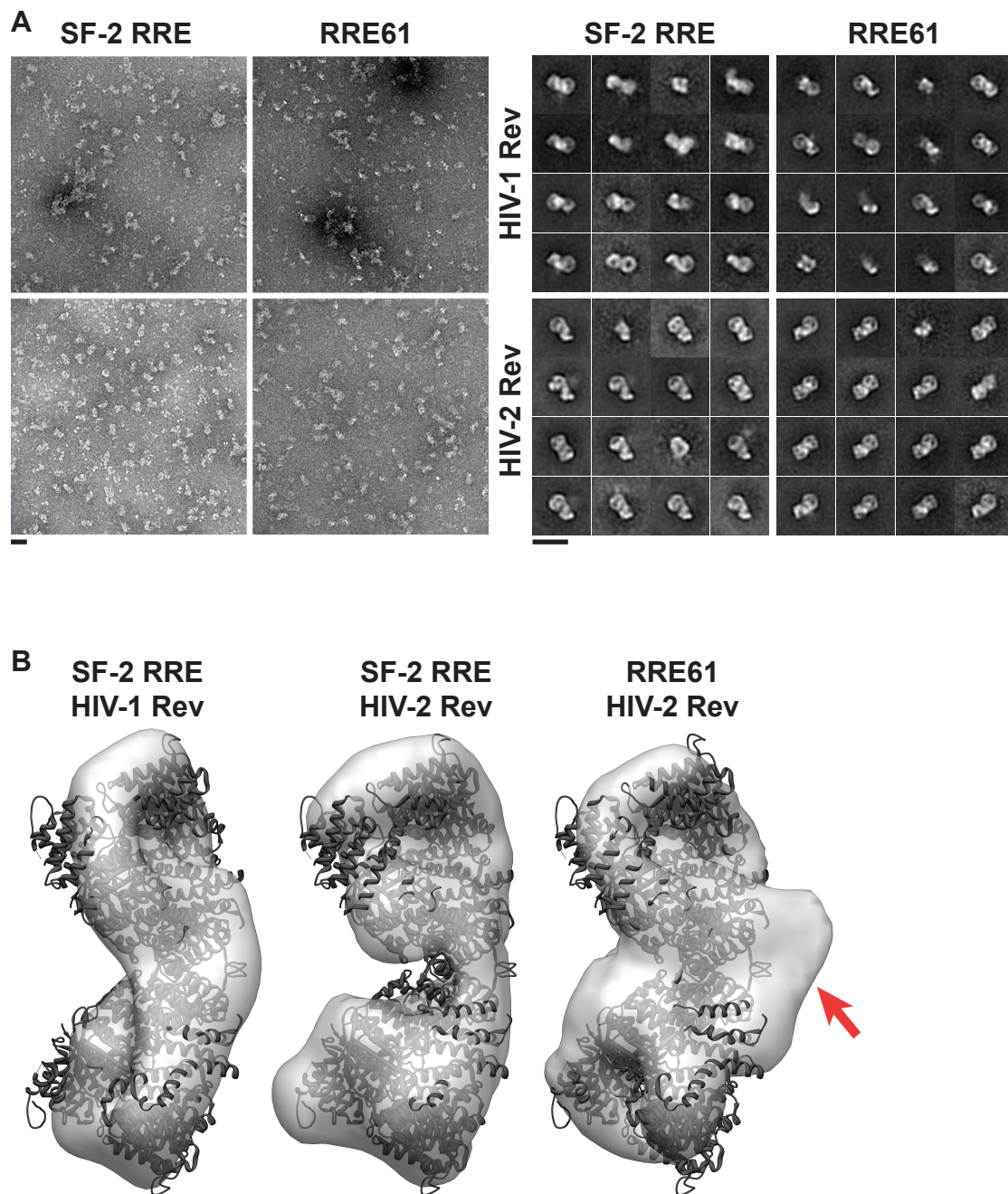


Figure 3: RRE61 enhances the Rev-RRE density in three-dimensional reconstructions of complexes bound to Crm1 and Ran•GTP

Figure 3: RRE61 enhances the Rev-RRE density in three-dimensional reconstructions of complexes bound to Crm1 and Ran•GTP

(A) Negative stain electron micrographs (left) and class averages (right) show that HIV-2 Rev reduces sample heterogeneity when assembled into complexes with the RRE, Crm1, and Ran•GTP. Class averages were generated with 2566 particles for SF-2 RRE and HIV-1, 2398 particles for SF-2 RRE and HIV-2 Rev, 2455 particles for RRE61 and HIV-1 Rev, and 2440 particles for RRE61 and HIV-2 Rev. Scale bars show 20 nm (B) Three-dimensional reconstructions with the indicated combinations of Rev and the RRE show that RRE61 enhances the Rev-RRE density (red arrow) in complexes bound to Crm1 and Ran•GTP. Reconstructions were fit with a model of the Crm1-Ran•GTP dimer from chapter two.

DISCUSSION

RRE61 stabilizing a particular conformation of the Rev oligomer without enhancing the affinity of the Crm1 dimer interaction presents a conundrum for how this mutation could enhance nuclear export. Polyvalent interactions, such as a Crm1 dimer binding two of six possible nuclear export sequences in the Rev oligomer, usually enjoy a large boost in affinity from degenerate binding modes¹⁶. This strategy is commonly found in virus-host interactions: either from the virus trying to bind to a low-abundance cell-surface receptor or from the host trying to thwart a virus bind recognizing antigens, as with IgM¹⁷. If RRE61 acted in a similar manner to stabilize the interaction with the Crm1 dimer, then the affinity of Crm1 binding the Rev-RRE complex should be higher, but that is not the case.

Alternatively, RRE61 could increase the rate at which Rev-RRE forms a complex with the Crm1 dimer. In this way, the RRE61 mutation may lower the transition state in the trajectory of forming a complex with the Crm1 dimer (Figure 4A). To test how RRE61 and other RRE mutations influence the dynamics of complex assembly, an in vitro assay needs to be developed to monitor the assembly of the particle over time. A potential way to monitor complex formation is to label components of the overall complex with dyes that can undergo fluorescence resonance energy transfer (FRET). Ran may be the best of the components to label with FRET donor and acceptor dyes in order to monitor fluorescence over time. Ran has only three cysteine residues, only one of which is surface-exposed, and the putative labeling sites are at an ideal for FRET since they are ~60 Å apart (Figure 4B). Using this system to study the kinetics for assembling the nuclear export particle with variants of the RRE has the potential to reveal how the dynamics of forming a particle competent for nuclear export helps control viral replication.

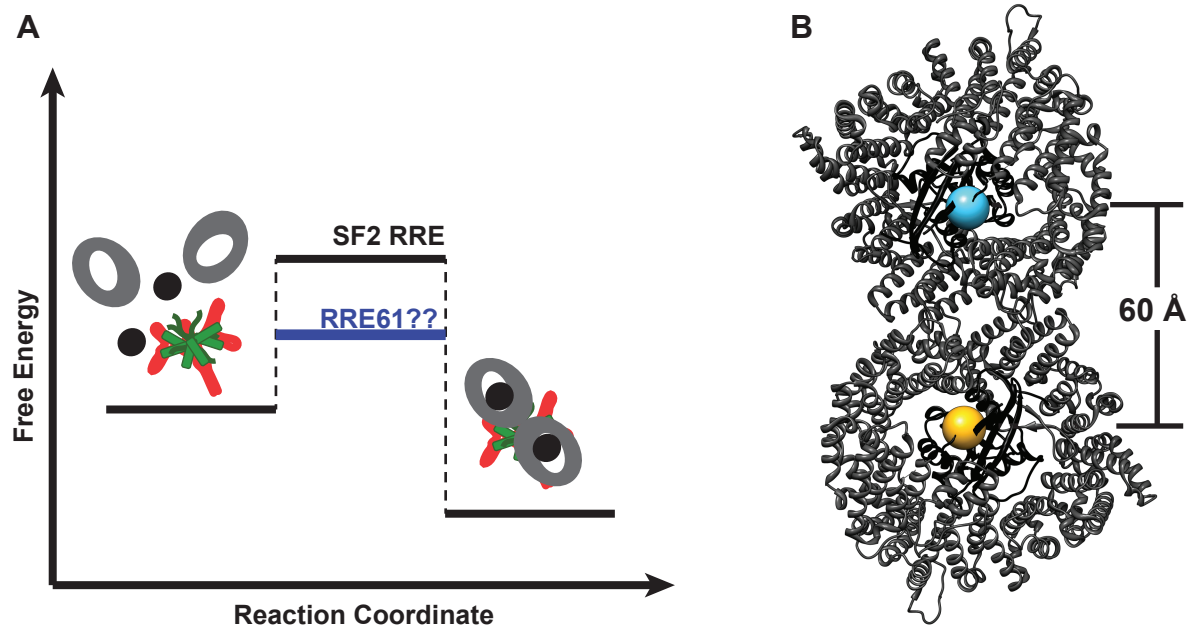


Figure 4: RRE61 potentially alters the dynamics of forming a complex competent for nuclear export

(A) A hypothetical energy landscape for assembling Rev-RRE (green and red) with or without the RRE61 mutation into complexes with Crm1 (grey) and Ran (black) shows that the RRE61 mutation may alter the dynamics of assembly by lowering the activation energy separating the unbound and bound states. (B) A structure of the Crm1-Ran•GTP dimer highlights the putative locations in Ran (blue and gold spheres) for conjugating fluorescent dyes in order to monitor the dynamics of complex assembly using fluorescence resonance energy transfer.

MATERIALS AND METHODS

Rev Phylogeny

Rev sequences from HIV and SIV isolates were downloaded from the Los Alamos National Laboratory HIV Sequence Database as premade alignments from the 2012 update of ‘Other SIV’ sequences which includes 161 sequences from HIV-1, HIV-2, and SIV¹⁴. The protein sequences were then aligned using the MUSCLE program¹⁸ in order to identify sequences that introduced large gaps in the alignment that would introduce errors into phylogenies. After iterative rounds of alignment and sequence removal, we eliminated 27 sequences, yielding 134 sequences. Using the DNA sequences of the proteins, we determined the correct nucleotide substitution model using FindModel with an initial tree constructed with PAUP¹⁴. We then estimated the phylogeny from a maximum-likelihood method implemented in PhyML 3.0¹⁹ using a general time reversible substitution model with gamma shape parameter of 1.00913, default parameters for the remaining fields, and tree branch support was computed with the aBayes method²⁰.

Molecular Cloning

RRE61 was generated by site directed mutagenesis of the pBluescript-5’ΔRRE plasmid (Chapter 2 – Table 1) using a single primer (5’-GTCTGGGGCATCAAGCAGCTCCAAGCAA-GAATCCTGGCTGTGGAAAGATACCTAAGG-3’) according to the methods in chapter two.

Rev sequences identified from a phylogenetic analysis of different HIV and SIV sequences within the Los Alamos Database were purchased as synthetic genes (IDT DNA) with codons optimized for expression in *E. coli* expression and with flanking *NcoI* and *HindIII* restriction sites (Table 1). The synthetic genes were cloned in between the *NcoI* and *HindIII* restriction sites of the pHG-Rev plasmid using the same procedure as described in chapter two.

In-vitro RNA transcription and purification

The RRE was transcribed from a linearized plasmid using T7 RNA polymerase and then purified by denaturing urea-polyacrylamide gel electrophoresis as described in chapter two.

Protein expression and purification

Rev, Crm1, and Ran were expressed in the *Escherichia coli* BL21(DE3) strain according to the methods in chapter two. Crm1 and Ran were purified by Ni-NTA chromatography followed by size-exclusion chromatography for Crm1 or cation exchange chromatography for Ran according to the procedures detailed in chapter two. Rev was also purified by Ni-NTA chromatography followed by cation exchange chromatography as previously described in chapter two but with the following modifications: The buffer for Ni-NTA chromatography was replaced with buffer A [100 mM $\text{K}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$, pH 7.5 @ 20°C; 200 mM NaCl; 2 mM 2-mercaptoethanol; 0-500 mM imidazole]. Following cleavage of the GB1 tag by tobacco etch virus protease, the purified fractions from Ni-NTA chromatography were directly loaded onto a 5 ml, HiTrap SP Fast Flow column (GE Lifesciences) equilibrated in buffer A. The column was washed with buffer A, and the protein was eluted with a linear gradient into buffer B [800 mM $\text{K}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$, pH 7.5 @ 20°C; 500 mM KCl; 2 mM 2-mercaptoethanol]. Pure fractions were pooled, supplemented with glycerol to final concentration of 10% (v/v), and concentrated using a 3.5 or 5 kD MWCO Amicon Ultra Free Centrifugal Concentrator (Millipore). Although HIV-2 Rev and HXB2 Rev were the only variants used for reconstituting complexes, we also purified all of the other Rev clones except from HIV-1 O. The purification expression and purification procedure was robust for all of the clones, but different versions of Rev can vary as much as ten-fold in their solubility.

Native Gel Electrophoresis

The RRE was radioactively labeled as according to chapter two. Protein concentrations were determined by Bradford assays using standards for each protein that had been quantified using amino-acid analysis (UC-Davis Proteomics Facility). Assembly reactions were performed according to the procedures in chapter two except that Rev was combined with the RRE to final concentrations of 40 nM and 2000 cpm/ μ M, respectively. An equal volume of Rev-RRE was then combined with Crm1 and Ran•GTP for the final concentrations as indicated. After reactions were incubated at 37°C for 15 min, they were loaded onto native gels and then dried, exposed, and quantified according to the methods in chapter two.

Negative-Stain Electron Microscopy

Complexes were assembled by combining 2 μ M RRE with 20 μ M Rev, 4 μ M Crm1, and 4 μ M Ran•GTP in buffer C [50 mM HEPES-KOH, pH 7.5 @ 20°C, 100 mM KCl, 2 mM $\text{Mg}(\text{CH}_3\text{CO}_2)_2$, 0.5 mM EDTA, 2 mM 2-mercaptoethanol, 2% (v/v) glycerol] with a 15 min incubation at 37°C. The assembled complexes were rapidly diluted ten-fold into buffer C immediately before adsorbing 2.5 μ l of the diluted reaction to glow-discharged, carbon-coated 400 mesh grids. Grids were stained with 0.75% (w/v) uranyl formate as previously described²¹. Images were acquired at 1.5 μ m defocus using low dose procedures on a T12 microscope (FEI) operating at 120 kV and recorded on a 4096 x 4096 CCD camera (Ultrascan 4000, Gatan) with a 52k magnification corresponding to a pixel size of 2.21 Å.

Image processing and 3-D Reconstruction

Negative-stain images were binned two-fold and processed according to the procedures described in chapter two. Particles were automatically selected using a cross-correlation based method implemented in SPIDER^{22, 23} using class averages from chapter two as references. The

chosen particles were screened using Samviewer²³ to eliminate particles too close to aggregates or to the edges of micrographs. The selected particles were then CTF-corrected and class averages were generated as in chapter two. Particles from heterogeneous classes or incompletely assembled complexes were eliminated from the particle stack, and the remaining particles were used to calculate a 3-D reconstruction using FREALIGN²⁴. A random-conical tilt reconstruction from chapter two served as the initial model 3-D reconstructions, followed by iterative refinement of Euler angles and in-plane shifts using a frequency-limited range between 240 Å and 34 Å. The reconstructions of complexes containing HIV-2 Rev with SF-2 RRE or RRE61 were determined from 2076 particles or 1947 particles, respectively.

Table 1 – HIV and SIV Rev sequences optimized for E. coli expression

Strain	Sequence (5'-<i>NcoI</i>-ORF-<i>HindIII</i>-3')
HIV-1 M HXB2	GCGCCATGGCGGGTCGCTCTGGTGACTCTGACGAAGAACTGATCCGTAC CGTTCGTCTGATCAAACCTGCTGTACCAGTCTAACCCGCCGCCGAACCCGG AAGGTACCCGTCAGGCGCGTCGTAACCGTCGTCGTCGTTGGCGTGAACGT CAGCGTCAGATCCACTCTATCTCTGAACGTATCCTGGGTACCTACCTGGG TCGCTCTGCGGAACCGGTTCCGCTGCAGCTGCCGCCGCTGGAACGTCTGA CCCTGGACTGCAACGAAGACTGCGGTACCTCTGGTACCCAGGGTGTTGGT TCTCCGCAGATCCTGGTTGAATCTCCGACCGTTCTGGAATCTGGTACCAA AGAATAACTCGAGGCGC
HIV-1 O	GCGCCATGGCGGGTCGTTCTGACGACGACCAGCTGCTGCAGGCGATCCG TATCATCAAAATCCTGTACCAGTCTAACCCGCACCCGTCTCCGATCGGTTT TCGTTCTGCGCGTCGTAACCGTCGTCGTCGTTGGCGTCGTCGTCAGGCGC AGGTTGACACCATCGCGGCGCGTATCCTGGCGACCGTTGTTACGGTCCG CAGAACAACAACCTGGTTGACCTGCCGCCGCTGGAACAGCTGTCTATCCG TGACCCGGAATCTGACCAGCCGTCTGGTAACTGGACCGTTGACCCGCGTG CGGAAGACAATAACTCGAGGCGC
SIV _{CPZ}	GCGCCATGGCGGGTCGTGAAGAAGACGCGAACCTGCTGCAGACCATCCG TGTTATCAAAATCCTGTACGACTCTAACCCGTACCCGTCTGGTGCGGGTTC TCGTGCGGCGCGTCGTAACCGTCGTCGTCGTTGGCGTCGTCGTCAGGCGC AGGTTGACGCGCTGGCGTCTCGTATCCTGCACTACCGTCTGGGTGGTCCG CAGAAACCGCCGCCGCTGGACATCCCGGACCTGTCTAAACTGCACCTGGA CCCGGTTGAACACCCGGCGTCTCCGGAACCGGTGACAACAGCCGTCTC GTCAGCCGTCTAACAACACCTAACTCGAGGCGC
SIV _{MAC}	GCGCCATGGCGAACCACGAACGTGAAGAAGAAGAACTGCGTAAACGTCTGCG TCTGATCCACCTGCTGCACCAGACCAACCCGTACCCGACCGGTCCGGGTA CCGCGAACCAGCGTCGTCAGCGTAAACGTGTCGTTGGCGTCGTCGTTGGCAG CAGCTGCTGGCGCTGGCGGACCGTATCTACTCTTTCCCGGACCCGCCGAC CGACACCCCGCTGGACCTGGCGATCCAGCAGCTGCAGAACCTGGCGATC GAATCTATCCCGGACCCGCCGACCAACACCCCGGAAGCGCTGTGCGACCC GACCGAAGACTCTCGTTCTCCGCAGGACTAACTCGAGGCGC
HIV-2	GCGCCATGGCGGAACGTGCGGAAGAAGAAGAACTGCGTCGTAAACTGC GTCTGATCCGTCTGCTGCACCAGACCAACCCGTACCCGCAGGGTCCGGGT ACCGCGTCTCAGCGTCGTAACCGTCGTCGTCGTTGGAAACAGCGTTGGCG TCAGCTGGTTGCGCTGGCGGACAAAATCTACACCTTCCCGGACCCGCCGG CGGACTCTCCGCTGGAACAGACCATCCAGCACCTGCAGGGTCTGACCATC CAGGAACTGCCGGACCCGCCGACCAACCTGCCGGAATCTTCTGAATCTAT CGACTCTTCTCAGCGTCTGGCGGAAATCTAACTCGAGGCGC
SIV _{DEN}	GCGCCATGGCGGGTCTGCAGGAACGAGACCAGGAAGTGGTTAAAGCGGT TCGCCTGATCAAAAACTGTACCAGTCTAACCCGTACCCGACCCCGGAAG GTTCTAAATCTGCGCGCCGCCGTCGACGCCGGAATGGCGTCGACAGCAG GCGCAGGTTGTTGCGATCTCTGAACGAATCCTGGAATACCACCTGGGTCTG CTCTCTGACCGCGGCGGACGTTGAACTGCCGGACCTGGAAAAACTGTCTG TTTCTGACTAACTCGAGGCGC

Table 1-continued

SIV _{RCM}	GCGCCATGGCGCTGGGTGAAGAAGACCAGGAAATCCGACGCCGGCTGCG TCTGATCAACTTCCTGCTGCAGTCTAACCCGTACCCGCAGCCGGGTGGTA CCGCGAACCAGCGACGCCGGCGTCGACGCCTGTGGCGGCGTCGATGGCT GCAGATCGCGCAGCTGGCGGAACGGATCTTCCTGCTGCCGGACACCCCGC CGGACCGCGACCTGATCGAAGGTGCGGTTGAACAGCTGCAGCAGCTGGC GATCTCTGACCTGCCGGAACCGCCGGTTAACCCGTTCTCTAACCCGGCGG ACTCTGCGGTTGACAACTCTTA ACTCGAGGCGC
SIV _{SYK}	GCGCCATGGCGGGTCGTGAACGTGAAGACACCCAGGAACAGCTGCTGCG TACCCTGCTGCGTATCGCGCAGCAGCTGGAAGCGGCGGCCGTACCCGC TGCCGCAGGGTCCGTCTCGTGCGCGTCGTCGTAACCGTAACCGTTACCGT CAGCTGCAGGCGCAGCGTCTGTACGTTTACGAGCGTATCTTCGAAACCAT CGCGCGTCGTTCTGCGGAAACCCTGGAACAGTCTTTCGGTGAACTGCAGA TCACCGACTAA CTCGAGGCGC

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CHAPTER 4: Future Directions

The most significant advance from the previous chapters is detailing a structural framework to understand how Rev-RRE recruits Crm1 with Ran to elicit nuclear export of HIV RNA, but this development is only a starting point for mechanistically understanding how Rev interacts with Crm1 to transition the viral lifecycle from an early phase where no new virions are produced to an actively replicating phase. In this last chapter, I relate insights gained from the work described in the previous chapters to outstanding questions about this critical host-virus interaction.

How does the RRE assemble the Rev oligomer to interact with Crm1?

To fully understand how Rev-RRE interacts with Crm1 and how adaptations in Rev or the RRE augment nuclear export, we still need a structure of the Rev-RRE complex. While crystal structures of Rev free of and bound to RNA have provided interesting insights into RNA recognition and the variety of shapes the Rev oligomer may adopt¹⁻³, we do not know which of the conformations are active, if the RRE can also rearrange the oligomeric interface between Rev dimers to form alternate conformations, and how Rev recognizes remaining sites of the RRE. Moreover, the mutations in the RRE that allow HIV to tune nuclear export activity lie outside of the region that was co-crystallized with the Rev dimer⁴⁻⁶.

The only structural model for the RRE currently available is a low-resolution model derived from small-angle x-ray scattering (SAXS) that shows the overall fold as an A-shape⁷. How Rev forms an oligomer on this structure is unclear. The authors did propose a model for the Rev oligomerization on this RNA structure, but their data are inconsistent with work Yun Bai

has done in the Doudna Lab that details the pathway for assembling the Rev-RRE complex⁸ and functional data corroborating the crystal structure of the Rev dimer bound to stem II³. Clearly, there are many outstanding questions and inconsistencies that could be answered by a high-resolution Rev-RRE structure.

The work in chapter three has the greatest prospect for solving the high-resolution structure of Rev-RRE bound to its host cofactor. Using a variant of the RRE and of Rev, we were able to obtain structures with a stronger density for Rev-RRE when bound to the Crm1-Ran•GTP dimer. These complexes show promise for structure determination by electron cryo-electron microscopy, taking full advantage of advances in direct detector cameras⁹ and reconstruction methods¹⁰ in order to obtain an atomic resolution structure of the host-virus complex¹¹.

What is the function of Crm1 dimerization for the host?

The dimer interface between each copy of Crm1 shows an important feature from the host that helps HIV replication. The evolutionary analysis from the Malim Lab shows that this interface is conserved in simian primates¹². It is unlikely that this feature of Crm1 evolved to help the virus, so the presence of the interface in this lineage begs the question “What does this interface do for the host?”

There are many possibilities for the putative function of the interface. As discussed in chapter two, this interface could serve a role in nuclear transport as it does for HIV RNA. Additionally, Crm1 has important roles in cell cycle complexes, including associations with the kinetochore¹³ and the centrosome¹⁴. These additional functions are consistent with the original screen that identified Crm1 as important for chromosome integrity in *Schizosaccharomyces pombe*¹⁵. No matter the function, identifying what Crm1 does for an uninfected cell could reveal

the roles for Crm1 cellular homeostasis, such as how upregulation of Crm1 contributes to carcinogenesis^{16, 17}. For HIV, these alternate functions could reveal how Rev alters cellular processes for the purpose of viral replication in addition to its role the mediating nuclear export of viral RNAs. For example, the HIV protein Vif hijacks a transcription factor to form a complex that specifically degrades an antiviral factor¹⁸. A consequence of Vif preventing this transcription factor to associate with its regulatory elements is the downregulation of antiviral genes¹⁹. Likewise, Vpr, another accessory protein, targets the degradation of a protein to cause cell cycle arrest and to dampen the innate immune response²⁰. Analogously, Rev-RRE may have more complex physiological roles for supporting viral replication by sequestering Crm1 from its endogenous roles in addition to facilitating nuclear export of HIV RNA.

The difficulty with determining how the interface may act for normal cellular function is that Crm1 is an essential protein and the organisms where dimerization occurs have complicated genetics. It would be helpful to have a system expressing a version of Crm1 with potentially deleterious mutations while controllably eliminating the activity of endogenous Crm1. Fortunately, leptomycin B is a pharmacological tool that can inhibit Crm1 function²¹ and mutations resistant to leptomycin B have been isolated²². These tools could be adapted by expressing mutant versions of Crm1 that are insensitive to leptomycin B while inhibiting the activity of endogenous Crm1 through leptomycin B treatment. Naomi Yonis and I attempted to develop this system by transiently transfecting versions of Crm1 insensitive to leptomycin B into human embryonic kidney cells and testing for complementation of Crm1 function by assessing cell survival after leptomycin B treatment. The results of these experiments were noisy and ambiguous. A potentially better system would be generating stable cell lines with these mutations in Crm1 instead of using transient transfections. If this approach can complement

Crm1 function during leptomycin B treatment, then these cell lines could be used to determine how Crm1 dimerization impacts gene expression and cell cycle progression.

How does Crm1-mediated export influence subsequent events in the viral lifecycle?

Rev initiates a chain of events for producing new virions when it delivers the viral RNA to the cytoplasm. Proteins that constitute virions or prime the cell for assembling virions are translated from the viral RNAs exported by Rev. The unspliced RNA also serves as the genome for the next generation of viruses. Work from the Malim Lab suggests that export through the Crm1 pathway influences how virion biogenesis proceeds²³, and Rev has been implicated in both translation of viral RNAs and packaging the viral genome²⁴. The mechanisms by which Rev coordinates nuclear export with translation and RNA packaging are unknown.

An important step during nuclear export that occurs in the cytoplasm is the removal nuclear export receptors from their cargoes to generate vectorial transport. For export receptors using Ran as a cofactor, dedicated machinery associates with nuclear pore complexes that removes Ran from transported cargoes and then stimulates GTP-hydrolysis in order to prevent Ran from rebinding cargoes²⁵. Directional transport of mRNAs requires a different set of factors, including helicases located at the nuclear pore to remove transport receptors from mRNAs²⁶. Ran GTP-hydrolysis²⁷, the removal of receptors by helicases^{28, 29}, and other cofactors have been implicated in the transport of HIV RNA³⁰. Additionally, helicases have been proposed to interact with Rev-RRE during downstream events for packaging viral RNA into new virions^{31, 32}. Yet, how these factors remodel Rev-RRE bound to Crm1-Ran•GTP and how they may work together to facilitate translation and packaging are poorly understood.

The ability to reconstitute Rev-RRE with Crm and Ran•GTP provides a necessary tool to interrogate the mechanisms for disassembling this complex in the cytoplasm. By using native gel electrophoresis assays developed in chapter two or fluorescence based methods suggested in chapter three to monitor the assembly state of this complex, a classical biochemistry approach could be taken to naïvely identify cofactors that remodel the complex from cytoplasmic extracts or a candidate-based approach testing the proteins that have already been implicated in HIV replication. Mutations that prevent disassembly steps or hyper-stabilize the complex could further be used to identify when interacting partners recognize intermediate states of disassembly and facilitate in vivo assays to monitor the localization of HIV RNA and the progression of viral replication. In sum, the reconstitution of the host-virus nuclear export complex opens a new suite of experiments to mechanistically investigate how it is remodeled along the trajectory of HIV biogenesis.

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APPENDIX: List of publications

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A handwritten signature in black ink, reading "David S. Booth". The signature is written in a cursive style with a large, looped "D" and "B".

Date: September 15, 2014