Evolutionary strategies for MxA antiviral proteins to overcome breadth-specificity tradeoffs in *Orthomyxovirus* restriction

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A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2024

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Program Authorized to Offer Degree:

Molecular and Cellular Biology

University of Washington

Abstract

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Antiviral restriction factors such as MxA (myxovirus resistance protein A) inhibit a broad range of viruses. However, they face the challenge of maintaining this breadth as viruses evolve to escape their defense. Viral escape drives restriction factors to evolve rapidly, selecting for amino acid changes at their virus-binding interfaces to regain defense. The human innate immune protein MxA has been well-characterized as a restriction factor of orthomyxoviruses. MxA restriction of orthomyxoviruses depends on its binding to viral nucleoproteins (NPs). However, other biochemical details of its restriction mechanism are still poorly understood. Previously, positive selection analyses helped narrow down rapidly evolving residues in MxA loop L4 as being critical for NP binding and viral restriction. Subsequently, combinatorial mutagenesis in human MxA loop L4 identified amino acid variations within the mutational landscape of MxA

rapidly evolving residues that conferred enhanced ‘super-restriction’ of the Thogotovirus (THOV) *orthomyxovirus*. However, MxA’s gain of THOV restriction appeared to correlate with the loss of restriction of another orthomyxovirus, H5N1 avian influenza virus (IAV). This suggested a tradeoff between MxA’s antiviral specificity and breadth. Here, I employed a modified combinatorial mutagenesis strategy to readily identify super-restrictor MxA variants with over ten-fold enhanced restriction of the avian IAV strain H5N1. Consistent with previous studies, a gain of H5N1 restriction led to reduced THOV restriction. However, I discovered two evolutionary strategies that enable restriction factors, such as MxA, to increase their restriction of diverse viruses to overcome breadth-specificity tradeoffs. First, I found rare ‘generalist’ super-restrictors with enhanced restriction of both viruses. Second, a heterozygous combination of ‘specialist’ super-restrictors, one against THOV and the other against H5N1, could simultaneously enhance restriction against two viruses. The findings presented here contribute to our understanding of the evolutionary arms race of the MxA-NP interaction, lend insights into the structure and biochemistry of their arms race interface, and reveal strategies to overcome breath-specificity tradeoffs that may be pervasive in host-virus conflicts.

Acknowledgments

I would first like to express gratitude to the funding institutions that supported my PhD dissertation work – the UW MSTP (T32 GM 007266), NHGRI UW Genome Training Grant (T32 HG 000035), the HARC collaborative, and HHMI. I am greatly appreciative to all the administrators who supported me for every step of this process. Thank you to Sara Carlson, Kacee French, Alex Moreno, Dominique Soldato, Jasmine Gonzalez, Hannah Snider, Denise Barnes, Maia Lowe, Alice Ven, Carolina Chambers, Andrea Brocato, Maura Do, and Mel Leavens. Additionally, thank you to the program directors who have helped me many times after panicked emails and Zoom meeting requests. Thank you so much to Marshall Horwitz, Mary-Claire King, Stephen Tapscott, Heather Cheng, Celeste Berg, and Nina Salama.

Thank you to the scientists who contributed to my growth as an independent researcher. This work would not have been possible without you. Thank you to my thesis advisory committee, Julie Overbaugh, Alison Feder, Melody Campbell, Marshall Horwitz, Jesse Bloom, Michael Emerman, and Harmit Malik. I am deeply grateful to my collaborators at the University of Freiburg, Georg Kochs and Laura Graf, who provided essential guidance and support throughout this work and who graciously hosted me for an enriching and productive visit to Freiburg. Thank you, also, to Damini Khera and Megan Levy, two wonderful scientists and humans whose work was vital to the data presented in Chapters 2 and 3 of this work.

To my friends from Memphis, Duke, UW, and Seattle – how lucky I am to have you all to laugh and cry and hike and ski and craft and watch *Survivor* together. I know I don't say it enough, but I am so thankful to my family – to my extended family for reminding me of the brilliant legacy I belong to, to my brothers for keeping me grounded and sane, and to my parents for their unwavering love and support and quite literally everything else. I believe my greatest

strength is this beautiful community of people who surround me, and I am endlessly grateful for that.

When I joined the Malik and Emerman labs, I knew I was joining a supportive and vibrant scientific community, but I had no idea how important the members of these labs would become to me throughout my PhD. The friends I have made here are some of the closest and dearest in my life. From late nights in lab together to sharing homecooked meals, weekly trivia and cabin getaways, and even just quick breaks for a laugh outside the lab or at lunch, it has been such an honor to spend these years with you all. I am so fortunate to call you my friends and family.

And finally, I must express the deepest gratitude to my advisors, Harmit and Michael. Everyone around me knows how fortunate I am to have such supportive mentors because it's difficult for me not to constantly rave about you both. In that last four years, you have helped me grow as an experimentalist, creative thinker, writer, and communicator. More importantly, though, you have both taught me through your mentorship that the most important thing in science is supporting the human beings doing it. When you do that, high-quality work is sure to follow. I will forever aspire to be like you both as a scientist and mentor. Thank you for all you do.

Dedication

This thesis is dedicated to Michael Howse, Jr. To me, you will always be the sweet kid playing saxophone in his green football jersey. It is at each of these big life stages that I wish I could have seen all of the wonderful things you would have accomplished.

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Chapter 1 Introduction

1.1 Evolution of host-virus interactions

Viruses have existed at every stage of cellular life, and the evolutionary histories of most organisms are intertwined with those of their viral parasites. Since viruses cannot replicate independently of host cells, they must constantly evolve to maintain their ability to hijack host cell machinery for propagation. Host cells counter this parasitism of viral infection by evolving means to avoid or directly antagonize virus activity.

Innate immune pathways encode molecules for the detection and removal of pathogens. These pathways allow organisms to respond to insults from foreign material, including viruses and bacteria, in minutes to days. The innate immune system of eukaryotes is an evolutionarily ancient tool employed during antiviral defense for distinguishing self from non-self, (*1*, *2*). Unlike the adaptive immune system, which jawed vertebrates possess in addition to innate immunity, the innate immune system does not genetically adapt to its pathogenic targets through the course of infection. Instead, innate immune proteins are translated from genes encoded in the germline. As a result, innate immunity is less evolutionarily plastic than adaptive immunity and is thought to be shaped by the evolutionary pressures that pathogens have placed on organisms at a population level.

Over many millions of years of evolution, eukaryotes have evolved an expansive innate immune system that recognizes and responds to pathogenic threats. Viruses have evolved over this same timespan to take advantage of host cell biology to infect and replicate. Each stage in the viral lifecycle exposes viral components to host cellular processes. These exposures allow the host to mount an immune response that targets an essential viral component. These responses

include one of two antiviral defense strategies. The first strategy involves detecting the virus and initiating antiviral protein signaling and transcriptional programs. Initial detection of the virus occurs when molecular features of the viruses themselves (known as Pathogen Associated Molecular Patterns, or PAMPs) are recognized by host Pattern Recognition Receptors (PRRs), which activate downstream antiviral programs, including inflammatory cell death and expression of pro-inflammatory cytokines for paracrine signaling, which involves signaling to neighboring cells.

The second strategy involves directly antagonizing the virus using antiviral proteins, also known as restriction factors. After initial viral detection through PAMP-PRR interactions, infected cells produce interferon (IFN) cytokines. IFN produced by an infected cell binds to IFN receptors on the same cell (autocrine signaling) or neighboring cells (paracrine signaling), following which these cells then activate a new transcriptional program of hundreds of interferon-stimulated genes, or ISGs, whose protein products include antiviral restriction factors. Expression of these ISGs poises cells to antagonize viruses directly, protecting the uninfected cells from infection and helping the infected cells clear the virus. There are several classes of IFN. The response to viruses is primarily driven by Type I (IFN-α/β) and Type III (IFN-λ) IFNs. Each IFN upregulates a consistent panel of hundreds of genes and proteins in every cell it activates even though only a small subset of these is required or effective for restricting any specific virus (*3*, *4*).

In theory, the evolution of each component of the innate immune system, including each ISG, can be tied to the pathogen(s) that drove its evolution. Conversely, viral targets of innate immunity have evolved to evade host defense systems either through structural changes to avoid recognition or by developing novel host-antagonistic functions (Figure 1-1) (*5*). As a result of

this tit-for-tat relationship, genes involved in host-pathogen interactions must undergo positive selection, in which new mutations are constantly selected to keep pace with the rapidly changing evolutionary pressures they face from pathogens. This rapid evolution can be identified in antiviral host innate immune genes through analysis of rates of nucleotide substitutions in these genes. At hotspots of virus (or other pathogen) interaction, referred to as the molecular arms race interface, nonsynonymous nucleotide changes leading to amino acid changes are expected to occur more frequently than synonymous mutations. By comparing the frequency of nonsynonymous mutations (dN) to synonymous mutations (dS) from multiple species, it can be inferred whether genes or even specific sites in these genes are undergoing positive selection ($dN/dS > 1$), indicating their potential importance in engaging with their viral targets. Analyses to identify rapid evolution in antiviral proteins can help drive hypotheses about important interaction domains, sometimes called host-virus molecular arms race interfaces, which can be validated by biochemical and cell biological testing. For example, such studies have shown in cell culture models that positive selection in the antiviral protein APOBEC3G corresponds to amino acids that determine specificity for antagonism by primate lentiviral Vif proteins as well as physical interaction at the evolutionarily-predicted arms-race interface (*6*, *7*).

MxA was one of the first innate antiviral proteins identified in mammals (*8*, *9*), first in mice (where the gene product is referred to as Mx1) and subsequently in most other mammals, including humans and other animals. Subsequent analysis of its rapid evolution has been vital to its biochemical characterization, as evolutionary analysis to identify rapidly evolving residues in MxA opened the door to mapping interaction domains between MxA and its viral targets (*10*). However, these prior studies examined MxA evolutionary potential against single targets, while my thesis addresses how the antiviral protein MxA could adapt in the face of multiple nascent

viral threats, which is more likely to reflect the situation in a human host. By applying forward evolution to MxA, guided by its evolutionary history, we hope to gain a deeper understanding of the evolutionary potential and constraints of the MxA protein to determine how changes to the arms race interface impact protein-protein interactions at a structural and biochemical level against multiple viral threats.

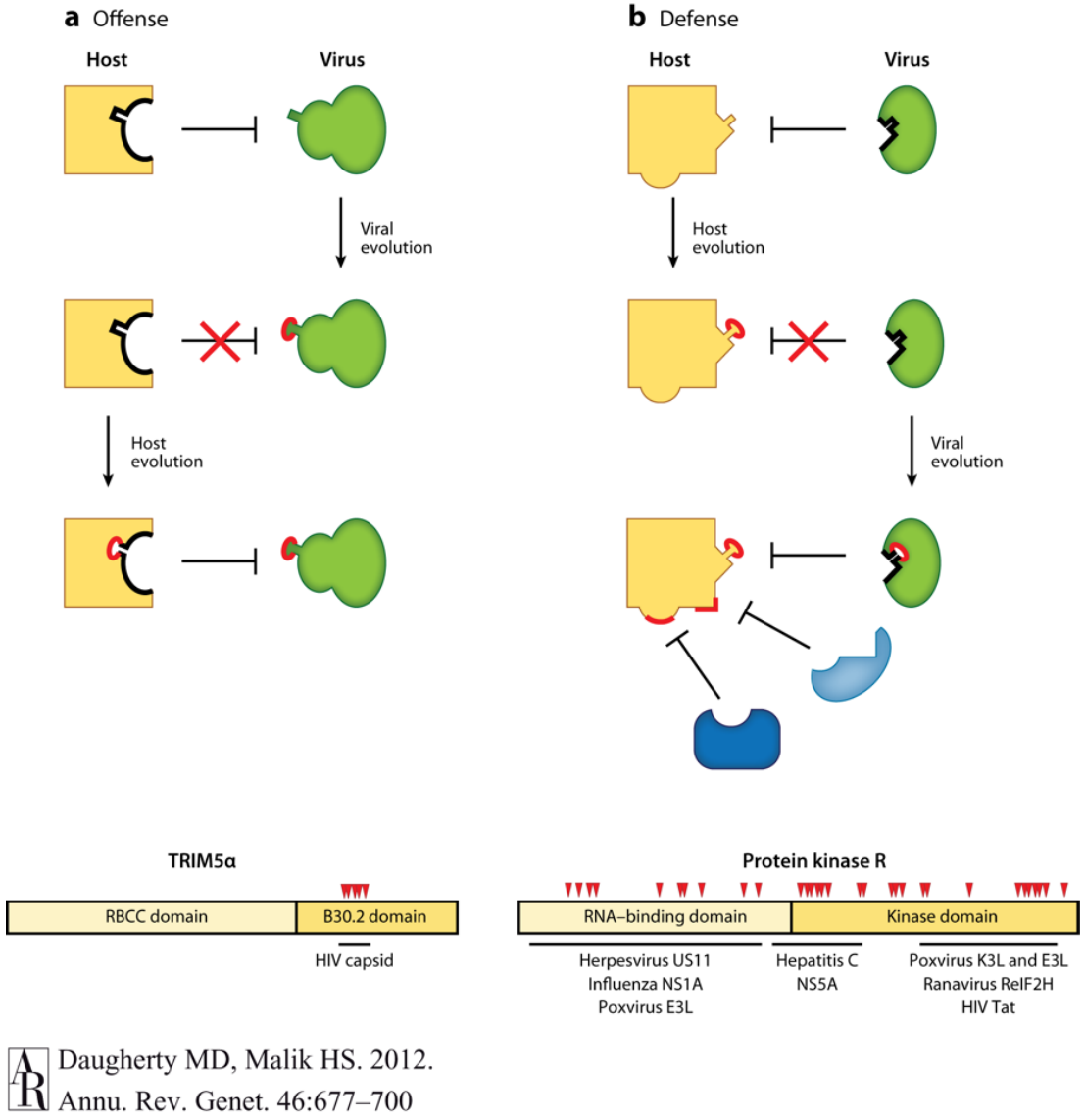

Figure 1-1. Examples of innate immune genes engaged in genetic conflict that act offensively against viruses (e.g. restriction factors) (A) and defensively (e.g. immune signaling proteins) (B). In each case, the "winning" protein places selective pressure on the other to rapidly evolve. From Daugherty, M. D., & Malik, H. S. (2012). Rules of engagement: molecular insights from host-virus arms races. *Annual review of genetics*, 46, 677–700. https://doi.org/10.1146/annurev-genet-110711-155522. CC license: 15423-1.

1.2 *Orthomyxoviruses* and mammalian innate antiviral defenses

The virus family studied in this thesis is the *Orthomyxoviridae*, which includes influenza viruses, thogotoviruses, and several other genera (*11*, *12*). Orthomyxoviruses have significantly impacted human society over much of the last century. For example, the 1918 H1N1 influenza pandemic killed around 50 million people and had lasting economic and political impacts. In 1957, an H2N2 influenza pandemic caused over 1 million deaths worldwide. As recently as 2009, a pandemic of H1N1 influenza led to up to half a million deaths globally. The H1N1 pandemic virus was so successful in its spread that it replaced other H1N1 strains circulating in the human population.

While annual vaccinations and antiviral treatments can curb some of the risks of infection from endemic or potential pandemic influenza infection, several critical features of these viruses make predicting and protecting from influenza and related orthomyxoviruses challenging. First, orthomyxoviruses circulate predominantly in non-human hosts, such as influenza (IAV) in waterfowl and livestock and thogotovirus (THOV) in ticks. Therefore, viral evolution occurs without human immune pressures, and spillover events can cause severe human disease. This is of particular concern with the continuing spread of H5N1 in waterfowl that has led to over 250 cases and 141 deaths in humans between 2003 and 2024, with additional cases rising in both humans and other mammals recently, but has not yet become competent for sustained human-to-human transmission (*13*). Second, the structure of orthomyxovirus genomes allows for genetic reassortment in multiply-infected hosts, expanding strain variability and the potential for severe outbreaks. These genetic reassortments have been responsible for the 1957, 1968, and 2009 influenza pandemics in humans.

Some orthomyxoviruses, such as thogotoviruses, replicate primarily in insect hosts and only occasionally spill over into mammals and birds. In contrast, influenza viruses have been identified in a broader range of hosts, including domesticated animals such as livestock, ferrets, cats, and dogs and wild animals such as seals, sea lions, whales, dolphins, and bats, which humans can be exposed to as human populations encroach into these animals' natural habitats (*14*). It is, therefore, imperative to understand how these viruses can evolve and spill over into human populations and how host immune defenses evolve to maintain restriction of rapidly changing viral challenges. Evaluating this host-pathogen conflict from the lens of viral evolution may allow us to predict spillover events. In contrast, studies examining the evolution of host proteins could provide an understanding of the mechanisms by which viruses can be restricted and how they might be targeted.

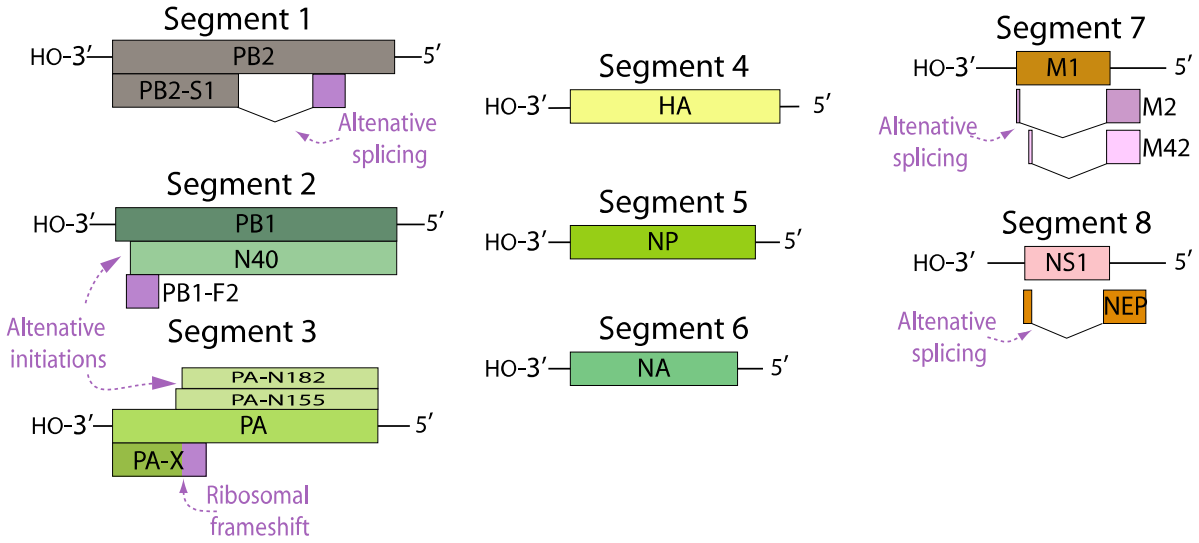

Figure 1-2. Representative structure of the linear, negative-sense, single-stranded RNA genome of orthomyxoviruses. Figure from ViralZone, Orthomyxoviridae, DOI:/10.1093/nar/gkq901.

It is important to understand the genetics and viral processes essential to successful viral infection replication to understand how orthomyxoviruses engage with host immune systems. Orthomyxovirus genomes comprise between 6 and 8 strands of negative-sense single-stranded

RNA (ssRNA) (Figure 1-2) encoding between 7 and 13 proteins depending on virus-specific alternative splicing. Proteins include surface proteins (hemagglutinin (HA) and neuraminidase (NA) or glycoprotein (GP)), matrix (M) proteins, polymerase subunits (PB2, PB1, PA), nucleoprotein (NP), and other non-structural (NS) proteins. Orthomyxoviruses are enveloped and package their segmented genome in viral ribonucleoprotein complexes (vRNPs); each RNA strand is wrapped around NP oligomers and complexed with an RdRp (Figure 1-3).

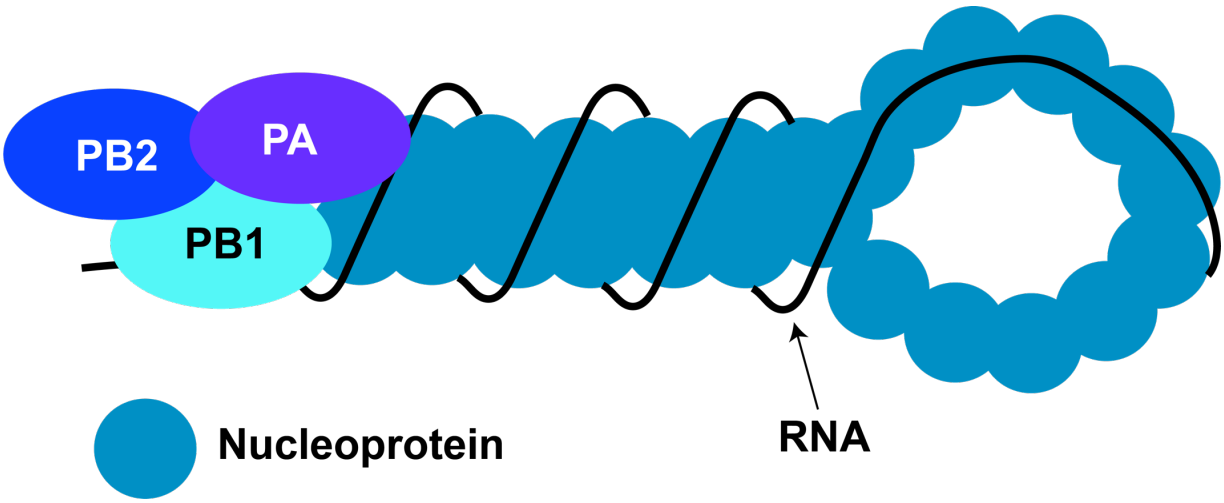

Figure 1-3. Cartoon representation of an orthomyxovirus vRNP, showing the interaction between RNA, nucleoproteins, and the trimeric polymerase (PB2, PB1, and PA). The nucleoprotein (NP) represented by blue circles is wrapped around the viral RNA.

The surface hemagglutinin proteins of orthomyxoviruses attach to host cell-surface sialic acids to initiate the viral entry steps (Figure 1-4 for viral lifecycle). The virus is then endocytosed and released into the cytoplasm upon acidification of the endosome. After this release, vRNPs traffic to the nucleus via nuclear localization signals (NLS) in PB2, PB1, PA, and NP. Once in the nucleus, pre-formed RdRp units begin primary transcription, during which viral mRNAs are produced from the viral genome (*15*). Viral proteins are translated in the cytoplasm, and newly formed vRNPs are transported back into the nucleus, where viral genome replication eventually begins. Finally, viral particle assembly occurs at the plasma membrane, and new virions are

released from the cell surface protrusions when neuraminidase cleaves sialic acids at host cell surfaces.

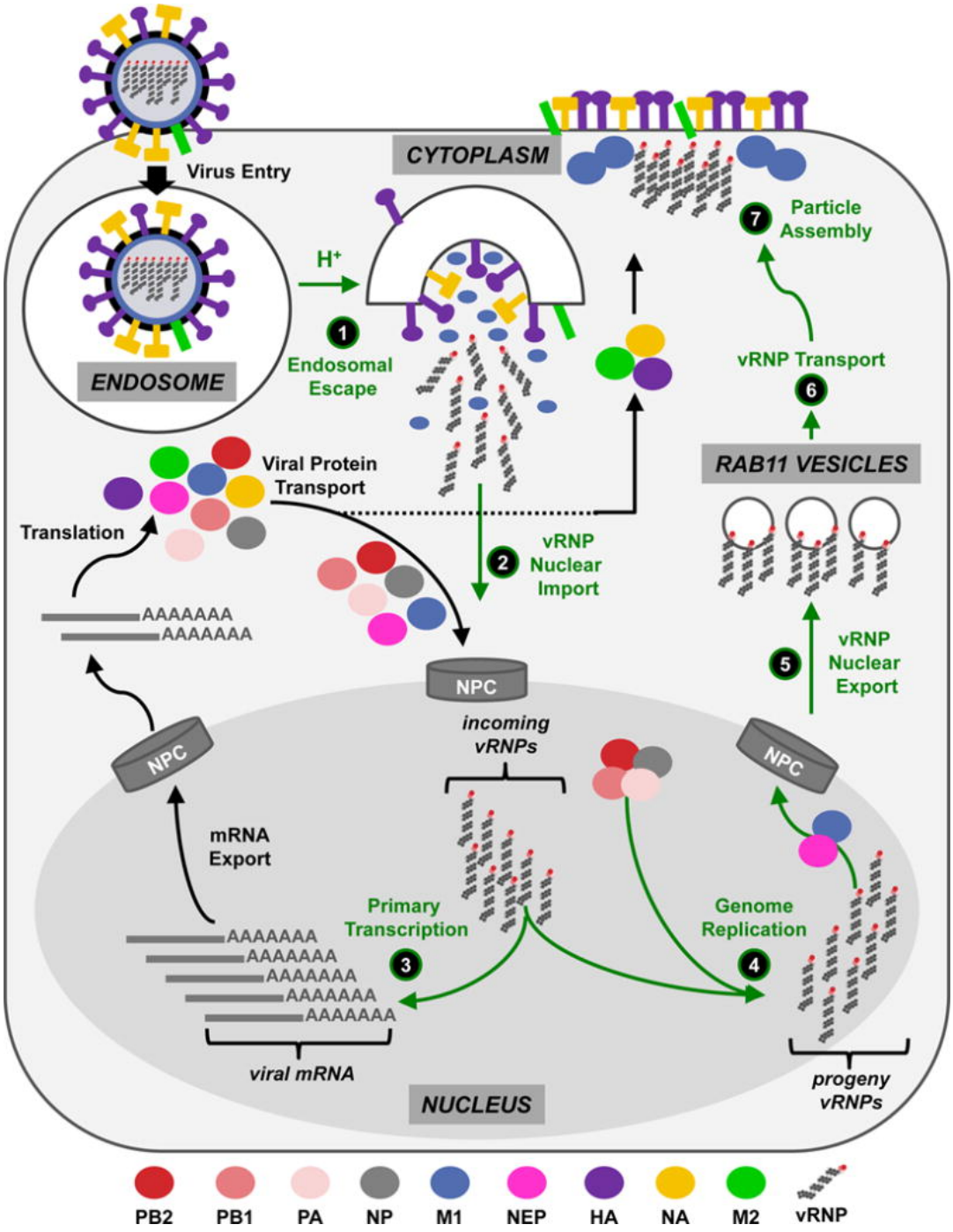

Figure 1-4. Diagram of the orthomyxovirus lifecycle, from viral entry to endosomal escape and vRNP nuclear trafficking; transcription, translation, and genome replication; and finally particle assembly and export. Figure from: Eisfeld AJ, Neumann G, Kawaoka Y. At the centre: influenza A virus ribonucleoproteins. *Nat Rev Microbiol*. 2015 Jan;13(1):28-41. doi: 10.1038/nrmicro3367. Epub 2014 Nov 24. PMID: 25417656; PMCID: PMC5619696. CC License: 5910930761619.

The genetic material of orthomyxovirus is recognized as foreign material (a PAMP) at multiple viral stages. Host PRRs called Toll-Like Receptors (TLRs) recognize early-stage viral ssRNA in endosomes upon viral entry. As the virus begins transcription and genome replication, nascent RNAs are recognized by the PRRs retinoic acid-inducible gene-I protein (RIG-I) and RIG-I-like receptors (RLRs). Recognition of these viral RNA PAMPs by TLRs or RLRs leads to the activation of transcription factors, which induce the expression of antiviral cytokines including IFN, leading to the induction of ISGs.

Table 1-1. Interferon-stimulated genes involved in cellular defense from influenza virus.

ISG	Intracellular location	Mode of action	References
MXA	Cytosol	Binds to viral nucleocapsid and prevents nuclear import of the virus	(*16–18*)
MX1	Nucleus	Blocks viral transcription into the nucleus	(*19–22*)
OAS and RNase L	Cytosol	Produces 2′-5′-oligoadenylate from ATP, which activates RNase L. RNase L can cleave viral RNA	(*23*)
PKR	Cytosol	Phosphorylates the α-subunit of EIF2α and blocks translation, activates the NF-κB pathway and stabilizes IFNA and IFNB mRNA	(*24–27*)
IFITM3 and other IFITM proteins	Endosomes	Block virus–host cell membrane fusion following viral attachment and endocytosis	(*28–31*)
Viperin	Lipid droplets and the cytosolic face of the endoplasmic reticulum	Blocks influenza virus release by interfering with the formation of the lipid raft from which the virus buds	(*32–34*)
CH25H	Cytosol	Converts cholesterol to a soluble 25-hydroxycholesterol, which is broadly antiviral by blocking viral fusion	(*35*, *36*)
TRIM22	Nucleus	Targets nucleocapsid for proteasomal degradation	(*37*)
ISG15	Cytosol	Ubiquitin-like protein that targets newly translated viral proteins for modification	(*38*, *39*)

CH25H, cholesterol 25-hydroxylase; EIF2α, eukaryotic translation initiation factor 2α; IFN, interferon; ISG, IFN-stimulated gene; IFITM, IFN-inducible transmembrane; MX, IFN-induced GTP-binding protein MX; NF-κB, nuclear factor-κB; OAS, 2′-5′-oligoadenylate synthase; PKR, protein kinase R; TRIM22, tripartite motif-containing protein 22. Table from Iwasaki A, Pillai PS. Innate immunity to influenza virus infection. Nat Rev Immunol. 2014 May;14(5):315-28. doi: 10.1038/nri3665. PMID: 24762827; PMCID: PMC4104278. CC License: 5910960830789.

Several ISGs have been well-characterized for their role in restricting influenza virus (Table 1.1) (*40*). For example, IFITMI proteins and cholesterol-25-hydroxylase prevent viral endocytosis and entry. In the cytosol, ISGs such as PKR, OAS/RNase L, TRIM22, and ISG15 target various viral components and function to block or degrade viral components. Finally, viperin serves to block newly formed viral particles from budding. Table 1.1 details anti-IAV ISGs, their mechanisms, and their location of action.

MxA is one of the most highly upregulated genes upon IFN stimulation (*41*). It has long been known as a potent anti-orthomyxovirus restriction factor in many infection models (reviewed in (*42*)). The long history of research into the MxA protein and its activity against orthomyxoviruses began in 1962 with a chance finding by Swiss virologist Jean Lindenmann, who had only several years earlier discovered IFN with colleague Alick Isaacs. Lindenmann reported that the A2G strain of laboratory mice was unusually resistant to influenza infection (*43*). Three decades after this first report of this resistance to influenza, the mouse Mx1 gene (gene product Mx1 in mice, MxA in humans) was identified as responsible for this resistance phenotype and linked to the alpha- and beta-IFN responses (*9*, *44*). Subsequently, Mx genes were identified in other organisms, including humans, fish, and poultry, and their roles and mechanisms for orthomyxoviruses were further characterized (*45*–*47*).

MxA, or myxovirus resistance 1, is a dynamin-related GTPase upregulated by IFN expression. Years of work have established MxA as an important firewall for many animal species against diverse orthomyxoviruses, serving as a potential barrier to interspecies viral transmission (*48*–*50*). In particular, human MxA has potent antiviral activity against some orthomyxoviruses, such as THOV and avian strains of IAV, while having little to no activity

against strains such as human endemic and pandemic IAV strains. The antiviral activity of MxA towards orthomyxoviruses depends on its interaction with nucleoproteins (NPs), which was established by comparing MxA activity against vRNPs from influenza strains with differing sensitivity to MxA (*48*, *51*). Experiments in which vRNP components from different influenza strains were reassorted in a viral polymerase reconstitution reporter assay (also known as a minireplicon assay) revealed that a vRNP's sensitivity or resistance to MxA depended on the NP protein (*48*, *51*). This was also validated to be the case in viral infection assays in the presence and absence of MxA with H5N1 (susceptible to human MxA) and H1N1 (resistant to MxA) and recombinant viruses with exchanged NP (*48*). Subsequent co-immunoprecipitation studies have shown that mouse Mx1 interacts with influenza NP and human MxA interacts with THOV NP (*52*–*54*). It is, therefore, understood that the arms race interface exists at the still somewhat undefined binding interface between MxA and orthomyxovirus NPs.

While human MxA has been most extensively studied for its restriction of orthomyxoviruses, it can also restrict several other families of viruses (*55*). These include the negative-sense ssRNA rhabdoviruses and bunyaviruses, positive-sense ssRNA flaviviruses and togaviruses, and DNA viruses, including hepatitis B virus and reovirus (reviewed in (*55*)). These viruses differ in the subcellular compartments in which they replicate and have very few conserved protein structures in common. Typically, broadly antiviral proteins would be expected to act on shared host cellular pathways. For example, broadly acting ISGs such as viperin and tetherin act by preventing host cell features such as nucleotide chain elongation (viperin) and plasma membrane composition (viperin and tetherin) (*56*, *57*). In contrast, restriction factors that target key features of viruses would be expected to be effective against only a small set of closely related viral proteins. For example, TRIM5alpha acts primarily on retroviruses by recognizing

their capsid architecture. Unlike these other examples of broad versus specific restriction factors, MxA has been shown to target distinct viral components directly while maintaining restrictive activity against diverse viruses (reviewed in (*58*)). It still remains a mystery how human MxA possesses such broad but potent restrictive activity against diverse viral families.

1.3 Antiviral mechanisms of MxA

Despite its long history of being studied as a critical antiviral innate immune factor, the exact mechanisms of MxA restriction of orthomyxoviruses remain poorly understood. It is, therefore, of interest to know how MxA has evolved and continues to evolve against orthomyxoviruses to gain insight into the molecular underpinnings of its interactions with viral targets and to understand the capacity and limitations for MxA to evolve improved restriction in the face of new and changing viral threats.

Structural analyses have revealed critical characteristics of MxA proteins for viral restriction: oligomerization, GTPase activity, and target binding. Crystal structures of MxA revealed important contact sites in the protein stalk domain that allowed for homo-oligomerization and proposed models of MxA restriction based on its oligomerization (*59*, *60*). Based on these x-ray crystallography studies, several amino acids in the stalk domain were identified as critical for contact between adjacent MxA molecules in antiviral activity. Later work revealed different requirements for MxA oligomerization when testing viral nucleoprotein interaction versus viral restriction. For example, the formation of MxA dimers or higher-order oligomers are required to co-immunoprecipitate with IAV NP.

One surprising result suggested that oligomerization in the stalk domain did not seem necessary for antiviral restriction (*61*). However, work characterizing the GTPase activity of

MxA nonetheless suggested that monomeric stalk mutants may achieve the transient oligomerization necessary for viral restriction. For example, it was long known that a functional GTPase domain was required for MxA to have activity against most viral targets (*59*, *60*, *62*, *63*). A study combining X-ray crystallography and enzyme kinetics assays determined the structural changes induced by MxA's GTPase domain upon binding, hydrolyzing, and releasing GTP/GDP in a "stalk-less" mutant of MxA and found that even without the oligomerization sites of the stalk domain present, transient dimerization was observed that allowed for GTPase activity and a subsequent power stroke involving a change in the position of the protein's bundle-signaling element (BSE) (*64*). These observations suggest that MxA's antiviral activity relies on GTPase-dependent conformational changes that disrupt viral structures or processes, presumably facilitated by transient stalk domain oligomerization and direct interaction with viral structures (*59*, *60*, *65*).

In addition to the structural basis of the interaction between MxA and its viral targets, there also remain questions about the timing and location of MxA activity during infection and in host cells. Mx gene paralogs and orthologs often differ in their subcellular localization and antiviral specificity. For example, human MxB, which shares 63% sequence identity with MxA, localizes to the nuclear periphery and possesses anti-HIV activity, whereas MxA is cytoplasmic and has not been shown to have activity against any lentiviruses (*66*–*70*). The murine ortholog of human MxA, mouse Mx1, is nuclear-localized and can restrict certain influenza viruses that human MxA cannot. This provides natural examples to study how subcellular localization of Mx proteins can directly impact the mechanism of viral restriction, although sequence differences may also contribute.

An early study compared the accumulation of primary viral transcripts in cells expressing the nuclear-localized mouse Mx1 to those in cells expressing cytoplasmic human MxA and showed that murine Mx1 inhibited early-stage primary transcription of viral RNA, whereas human MxA did not inhibit viral RNA production until the later viral genome replication stage (*71*). Similar experiments with THOV showed that human MxA restricts vRNP entry into the nucleus after initial viral entry to the cell (*72*). Thus, Mx proteins with different subcellular localizations may utilize different mechanisms of viral restriction. Several decades later, two more studies bolstered this conclusion by showing that controlling the timing of mouse Mx1 expression using a chemical activator, Mx1 can only restrict influenza virus replication at early stages of infection (*73*). More recently, a report used immunofluorescence to localize murine Mx1 and human MxA to viral proteins at various stages of infection. This report showed that Mx1 contributes to nuclear sequestration of viral proteins while MxA may inhibit viral protein transport at stages just before viral egress (*74*).

There have been several other instances of reported Mx activity against viruses that localize and replicate in varying subcellular localizations (*55*, *75*, *76*). However, the direct relationship between viral localization and the mechanism of MxA antiviral activity is not understood in these contexts. It remains to be determined how the subcellular localization of both viruses and MxA homologs impacts the mechanism MxA recognizes and restricts viral proteins. Taken together, the current understanding of the mechanism of MxA includes recognition of viral targets in the cytoplasm, oligomerization through the stalk and GTPase domains, and subsequent GTPase activity that facilitates viral restriction.

1.4 Evolution of MxA

The MxA protein domain responsible for direct viral targeting in orthomyxovirus restriction was only formally determined when evolutionary analyses were applied. Positive selection analysis compared MxA sequences across primate species, revealing twelve amino acid residues in the MxA protein undergoing positive selection (*10*). Notably, five of these were clustered in unstructured loop L4 of the stalk domain (Figure 1-5). This loop is in the same location as the pleckstrin homology domain (PH domain) in dynamin, which is required for dynamin to bind to lipid membranes, their functional target. Although Loop L4 is not homologous to the PH domain, it was previously shown to be able to bind and tubulate lipid membranes, which may have been merely a remnant of its evolutionary relationship to dynamins and unrelated to its evolution as an antiviral restriction factor. It was alternatively hypothesized that the loop L4 region of MxA evolved from an ancestral membrane-binding function to a viral targeting domain (*77*).

Loop L4 was subsequently genetically validated as the target interaction domain of MxA through chimeras of primate MxA proteins possessing loop L4 domains from different species. The species of origin of the loop L4 in MxA chimeras was sufficient to determine the antiviral specificity of the MxA variant (*10*). Additionally, alanine scanning of loop L4 identified critical residues for restricting both IAV and THOV (*54*). After these genetic validations were made, co-immunoprecipitation of the THOV NP with MxA was also shown to be dependent on the sequence of loop L4, providing biochemical evidence of loop L4 as the interaction domain (*54*). Further, the differential antiviral activity of Mx1 in two mouse species could be mapped to differences in their loop L4 regions (*54*, *78*). Thus, despite its lack of structure, loop L4 has been

shown to be critically important for MxA restriction of orthomyxoviruses via a combination of evolutionary analyses and genetic and biochemical experimental techniques.

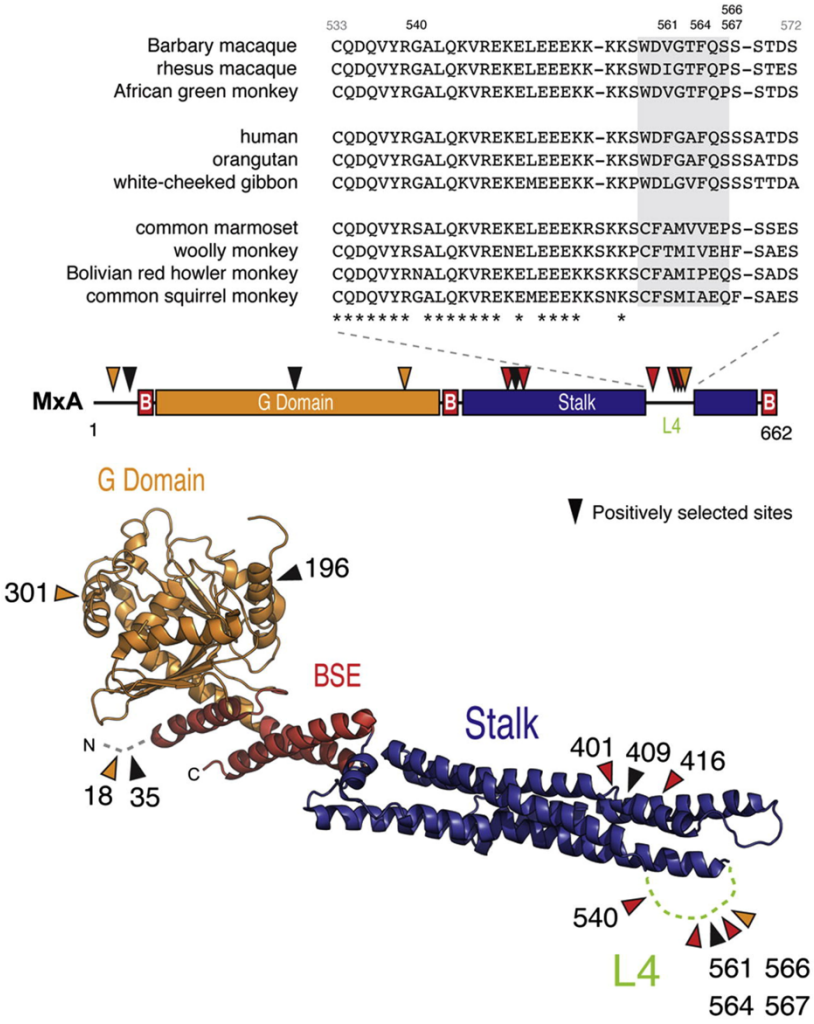

Figure 1-5. Rapid evolution of the MxA gene in primates. The loop L4 (amino acids 533-572) protein sequence alignment is shown from representative primates (top). Specific codons found to be evolving under positive selection in primate MxA are indicated in a linear schematic (middle) or a model of the MxA crystal structure (bottom) (Gao et al., 2011), where sites with a posterior probability greater than 0.95 and 0.99 are highlighted in orange and red, respectively. In the linear structure "B" depicts the three parts of MxA that form the bundle-signaling element (BSE). Figure from: Mitchell PS, Patzina C, Emerman M, Haller O, Malik HS, Kochs G. Evolution-guided identification of antiviral specificity determinants in the broadly acting interferon-induced innate immunity factor MxA. *Cell Host Microbe*. 2012 Oct 18;12(4):598-604. doi: 10.1016/j.chom.2012.09.005. PMID: 23084925; PMCID: PMC3540999. CC License: 5910940548284.

Although MxA's loop L4 has been functionally validated to be involved in the interaction with orthomyxovirus protein targets, it is improbable that extant orthomyxoviruses were the

driving forces of the evolution of Mx genes as they exist across primates today. This is believed to be true because of the 5 residues under positive selection in primate MxA, only one of them is necessary for determining antiviral activity against *orthomyxoviruses* (*10*). Beyond loop L4, there remain eight other positively selected residues whose evolutionary drivers remain a complete mystery. Other evolution-guided studies of Mx gene family members have also revealed that their modern viral targets are not placing selective pressure on these genes (*79*). For example, none of the amino acids required for MxB antiviral activities against lentiviruses and herpesviruses have been shown to overlap with rapidly evolving residues in the MxB gene (*79–81*). Given its broad antiviral range, it is likely that other viruses have driven the majority of rapid evolution in MxA, including in the L4 loop; nevertheless, these changes do have a profound consequence on human MxA's present-day restriction of orthomyxoviruses.

Further clarifying Mx genes' evolutionary history, a recent evolutionary analysis challenges the previously held belief that Mx genes arose after the IFN response developed in the ancestor to jawed vertebrates (*82*). This phylogenetic analysis suggests that Mx genes predate IFN and exist across domains of life, including animals, fungi, and plants (*83*). This suggests a vast evolutionary playing field on which Mx genes may have evolved antiviral responses to ancient and modern viruses over hundreds of millions of years.

1.5 Viral evolution and MxA escape

MxA serves as a critical barrier to the emergence of new viral threats. The evolutionary pressure it places on viral proteins can be seen in the variable susceptibility of IAV strains to MxA (*51*). Because NP is the target of MxA's antiviral activity in this host-virus evolutionary arms race, it would be expected that MxA places selective pressure on orthomyxovirus NP to

escape restriction. This has indeed been shown to be the case in orthomyxovirus evolution through experimental evolution studies and by sampling natural variants of influenza and thogotoviruses (*84*–*87*) . Identifying naturally occurring MxA-resistant NP variants in potentially pathogenic orthomyxoviruses highlights the importance of understanding how viral NPs can evolve and escape MxA. This understanding could reveal the constraints on NP evolution and identify druggable targets within orthomyxovirus.

One strategy to identify NP amino acid mutations involved in viral adaptation to MxA restriction is to compare NP protein sequences between IAV strains with different susceptibilities to MxA. By creating chimeras or point mutants of differing amino acids and assessing for resulting MxA restriction, key residues for NP escape can be determined. This strategy led to the finding that three out of 14 amino acid differences between the avian H5N1 NP and the 1918 pandemic H1N1 NP were critical for allowing the avian NP to overcome MxA restriction and contribute to devastating human disease (*85*). MxA-escape mutations were identified in another swine H7N7 strain with avian flu-like features in distinct amino acid residues in a similar region of the NP protein (*84*). These two studies were particularly important in highlighting the risk of swine flu spillover events. Because pigs possess a mildly restrictive MxA, they serve as an intermediate host in which IAV strains can pre-adapt resistance to human MxA (*84*).

Because of the significant correlation between orthomyxovirus strain pathogenicity in humans and their escape from MxA restriction in minireplicon, cell infection, and mouse studies, other studies have assayed new and emerging orthomyxoviruses for their MxA susceptibility in the lab (*86*). This has led to the determination of several orthomyxoviruses of zoonotic spillover concern. For example, in a sampling of thogotoviruses isolated from wild ticks around the world, a strain called Jos virus (JOSV) isolated from cattle-fed ticks in Nigeria was found to be uniquely

and potently capable of escaping human MxA restriction by mutations in only two amino acids in the NP relative to MxA-sensitive strains of thogotoviruses (*87*). Additionally, the ability of bat Mx1 to restrict avian influenza to similarly modest levels as does human MxA has raised concerns that bats could serve as an intermediate host in which influenza strains can pre-adapt to escape MxA, as with swine (*50*, *88*). While the H18N11 strain of bat-derived influenza was shown to be susceptible to human MxA in minireplicon, cell infection, and mouse infection studies (*89*). Of more significant concern, the H9N2 strain, which originated in bats and has since become endemic in some poultry populations, was shown to be deadly in ferrets, even though it appears to be well-inhibited by human MxA in primary human cell and transgenic mouse models (*50*).

In addition to surveillance for zoonotic orthomyxoviruses with the potential to cause human outbreaks, studying NP evolution in the face of MxA selective pressures provides an understanding of the constraints on NP evolution, which could help identify druggable features. Several studies have addressed this by performing experimental evolution to determine which NP mutations arise in the face of MxA restriction and how such mutations impact overall fitness. A deep mutational scanning screen tested the viral fitness in a cell infection model using influenza viruses containing all possible single amino acid substitutions in the NP of a human-adapted H3N2 IAV strain (*90*). While most amino acid substitutions across NP increased MxA susceptibility, those that increased MxA resistance came at a cost to viral fitness.

Serial passaging of viruses in cells or mice also provides data on how resistance mutations can arise stably in infectious viruses. One study found that introducing MxA mutations from H1N1 into H5N1 attenuated viral ribonucleoprotein trafficking efficiency. Serial passaging of these mutant viruses in cell cultures led to the fixation of compensatory mutation that

improved vRNP trafficking (*86*). In a complementary study, an H1N1 strain with mutations increasing MxA susceptibility was serially passaged in mice. A new mutation in the same protein region as other resistance mutations arose in these viruses. Intriguingly, a newly emerging H7N9 avian influenza strain already possessed this compensatory mutation in its NP (*91*). By mapping evolutionary pathways to MxA susceptibility and resistance in both natural and lab-evolved orthomyxovirus, emerging viruses can be better understood for their potential to cause significant human disease and, more ideally, for their weaknesses in the face of human MxA.

1.6 The breadth-specificity tradeoff for antiviral proteins

The broad range of activity that MxA possesses against orthomyxoviruses and the presence of amino acids undergoing diversifying selection inspired a forward evolution study to understand the evolutionary potential of MxA restrictive ability with mutations to rapidly evolving residues of loop L4 (*92*). In this study, a minireplicon of *orthomyxoviruses* was used as a means to experimentally test the activity MxA variants against the viral polymerase and NP without requiring full length viruses and viral infection, which can require prohibitive levels of biosafety clearance (Figure 1-6). A combinatorial mutagenesis library of human MxA variants was made, in which all five rapidly evolving residues of loop L4 were allowed to vary to any of the 20 proteinogenic amino acids and then used to screen for restrictive ability against a THOV minireplicon. It was found that variants with greatly improved restriction of THOV relative to wildtype, deemed “super-restrictors,” were readily identifiable.

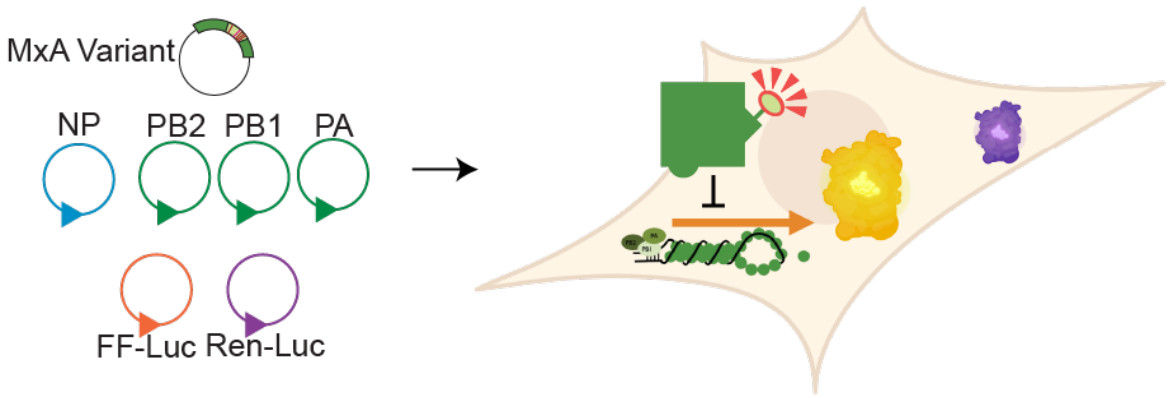

Figure 1-6 Design of minireplicon screening assay. HEK293T cells are transfected with an MxA variant, each component of the FLUAV polymerase complex (PB1, PB2, PA, and NP), a firefly luciferase (FF-Luc) viral replication reporter gene, and a constitutively expressed Renilla luciferase (Ren-Luc) control gene. A reconstituted polymerase complex allows expression of FF-Luc, whose levels are measured and normalized to Ren-Luc. Transfection of different MxA variants inhibits NP-dependent expression of FF-Luc to different extents, allowing measurement of restriction.

Biochemical patterns of these super-restrictors supported previous findings that an aromatic amino acid at residue 561 is required for orthomyxoviruses (Figure 1-7A) (*92*). Most intriguingly, super-restrictors of THOV could not improve restriction of the avian H5N1 influenza strain. Instead, highest levels of anti-THOV super-restriction was anti-correlated with anti-IAV activity (Figure 1-6B) (*92*). These studies showed that within the sequence space of the positively selected residues in Loop L4, there existed the potential for increased antiviral activity against a target that was already readily restricted, but that increased restriction often came at a cost to restriction of another target, called the "breadth-specificity tradeoff" here.

The tradeoff to H5N1 restriction described with THOV super-restrictors suggested that improvement against other viruses may be more difficult or even impossible to achieve. However, the ability to improve MxA restriction of an already potently restricted virus and the modest to potent restrictive ability of MxA to restrict other orthomyxoviruses also suggested that, within the sequence space already sampled during primate evolution, MxA may possess the ability to fine-tune its restrictive power against multiple nascent viral threats. Chapter 2 of my

thesis describes the identification of super-restrictors of H5N1 IAV, which is modestly restriction by human MxA. I also show how super-restriction of both H5N1 IAV and THOV simultaneously can be achieved by either rare mutations in MxA loop L4 or by combining virus-specific super-restrictors as heterozygous alleles. In Chapter 3, I extend this work to the study of a virus fully escaped from human MxA restriction, 1918 H1N1 IAV, and show that evolution of super-restriction of H1N1 IAV requires both adaptations in loop L4 and in MxA subcellular localization. The work described in my thesis contributes to mapping the evolutionary landscape within which MxA can gain restriction against multiple orthomyxoviruses to understand better how the breadth-specificity tradeoff might be overcome in the evolution of variants of MxA

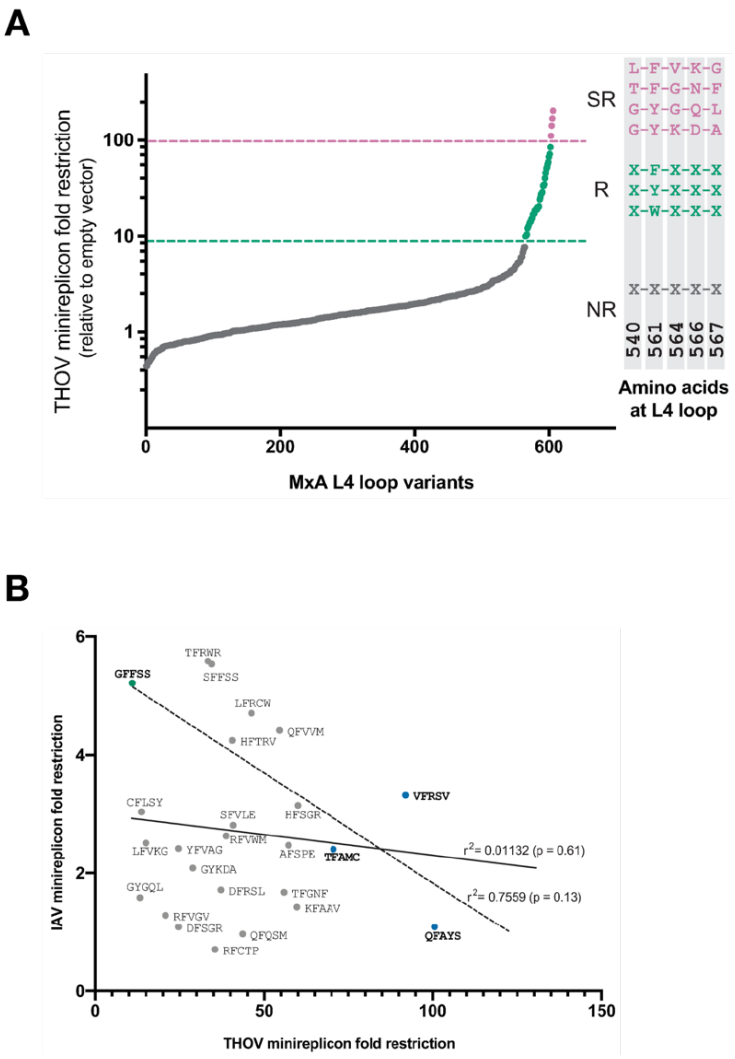

Figure 1-7. THOV super-restrictors require an aromatic residue at position 561 and are virus-specific. (A) Waterfall plot showing THOV minireplicon restriction profiles of 523 MxA loop L4 variants and the amino acids present at mutated sites for non-restrictors (NR, gray), restrictors (R, green), and super-restrictors (SR, fuschia). (B) Correlation of THOV (x-axis) and H5N1 IAV (y-axis) minireplicon fold restriction by a selection of MxA loop L4 variants. Dotted line represents correlation of strongest THOV super-restrictors while solid line represents correlation of all THOV super-restrictors. Figures from: Colón-Thillet R, Hsieh E, Graf L, McLaughlin RN Jr, Young JM, Kochs G, Emerman M, Malik HS. Combinatorial mutagenesis of rapidly evolving residues yields super-restrictor antiviral proteins. *PLoS Biol.* 2019 Oct 1;17(10):e3000181. doi: 10.1371/journal.pbio.3000181. PMID: 31574080; PMCID: PMC6772013. CC License: Open Access under Attribution 4.0 International.

Chapter 2 Heterozygous and generalist MxA super-restrictors overcome breadth-specificity tradeoffs in antiviral restriction

This chapter is under peer review. A version of it can be found on bioRxiv (https://doi.org/10.1101/2024.10.10.617484).

2.1 Abstract

Antiviral restriction factors such as MxA (myxovirus resistance protein A) inhibit many viruses. Viral escape drives restriction factors to evolve rapidly at virus-binding interfaces to regain defense. How do antiviral proteins balance restricting many viruses with evolving specificity against individual viruses? We explored this question in human MxA, which uses its rapidly evolving loop L4 as the specificity determinant for orthomyxoviruses such as thogotovirus (THOV) and influenza (IAV). Previous combinatorial mutagenesis of rapidly evolving residues in human MxA loop L4 identified THOV 'super-restrictors' and suggested an antiviral breadth-specificity tradeoff. Using a modified combinatorial mutagenesis strategy, we find super-restrictor MxA variants specific to H5N1 IAV and a single residue that underlies the MxA breadth-specificity tradeoff. However, rare 'generalist' super-restrictors or a heterozygous combination of more common 'specialist' super-restrictors can overcome the breadth-specificity tradeoff. Thus, two strategies enable restriction factors such as MxA to increase their restriction of diverse viruses to overcome breadth-specificity tradeoffs that may be pervasive in host-virus conflicts.

2.2 Introduction

Pathogenic viruses pose persistent, ever-changing challenges to their hosts. To combat them, hosts encode various germline-encoded antiviral proteins, also called restriction factors, which form a critical component of innate immunity. Successful restriction by antiviral proteins often relies on their recognition and inhibition of viral targets in a cell-autonomous manner. However, these defenses must adapt to keep pace with viral target evolution and new viral challenges. Unlike adaptive immunity, which can adapt almost contemporaneously with viral evolution, the evolution of restriction factors is limited to rates of germline evolution (*93*). Nonetheless, such evolutionary arms races drive rapid, recurrent amino acid changes (positive selection) at host-virus interaction interfaces, with hosts evolving to establish recognition of viral targets and viruses adapting to evade interaction with host restriction factors. Understanding the adaptive potential of rapid evolution in restriction factors is critical for understanding the biochemical and evolutionary constraints on viral restriction.

Because restriction factors are part of the genetically constrained innate immune system, they are often responsible for defending against a broad range of viruses with common molecular features (*57*). MxA has one of the broadest antiviral ranges of any mammalian restriction factor; it can restrict multiple families of RNA and DNA viruses (*55*, *57*). MxA is a dynamin-like, IFN-inducible GTPase whose antiviral activity, in most cases, depends on direct interactions with different viral targets (*54*, *59*, *60*). MxA has been most extensively characterized for its restriction of orthomyxoviruses such as influenza A virus (IAV) and Thogotovirus (THOV) (*53*, *75*, *94*–*97*). This antiviral activity requires GTP binding and hydrolysis by the globular G domain, oligomerization through sites in the stalk domain, and direct interactions with viral nucleoproteins (NP) (*54*, *60*, *61*, *64*, *98*).

A growing body of evidence suggests that human MxA is a critical barrier to crossover events of animal-borne IAVs to humans (*84–87*, *89*, *91*, *99*). Recently, it was shown that individuals infected with the avian IAV subtype H7N9 are more likely to have a dominant loss-of-function mutant MxA allele than healthy control groups (*99*). Moreover, human-endemic IAV strains have acquired escape mutations in their NP proteins to resist human MxA restriction (*48*, *51*, *85*), a prerequisite for continuous circulation in the human population (*85*, *86*, *100*). For example, distinct mutations in the viral NP allowed the 1918 H1N1 pandemic IAV strain to evade human MxA and may have facilitated one of the most devastating IAV pandemics in humans (*85*). In contrast, human MxA can restrict avian-derived influenza viruses such as the waterfowl-endemic H5N1 IAV, which is pathogenic in human individuals but has not acquired the capability for human-to-human transmission (*48*). However, the recent rampant spread of an avian H5N1 IAV strain in domestic cattle populations in the US has raised renewed concerns about the possibility of zoonotic spillover events (*101–103*). To better understand the ongoing arms race between MxA and human pathogenic orthomyxoviruses, we analyzed the evolutionary constraints that shape MxA restriction of orthomyxoviruses.

We previously identified the unstructured loop L4 of MxA, especially an aromatic residue (phenylalanine, tyrosine, or tryptophan) at amino acid position 561, as a critical determinant of THOV and IAV NP binding and restriction (*10*). Furthermore, using combinatorial mutagenesis of five rapidly evolving residues in loop L4, we identified super-restrictor human MxA variants that could augment wildtype human MxA's (hereafter referred to as wtMxA) already potent restriction of THOV (*92*). In some cases, altering only a few positively selected residues in MxA L4 led to a ten-fold increase in potency. However, increased THOV restriction correlated with loss of restriction of the H5N1 strain of IAV (hereafter referred to as H5N1). These findings

suggested a ‘breadth-specificity’ tradeoff in MxA restriction of orthomyxoviruses, wherein variants with increased potency against one virus lost specificity against another (*92*).

Understanding the basis of such breadth-specificity tradeoffs and identifying strategies by which restriction factors like MxA can overcome them is critical to understanding their role in the human species’ barrier to potentially zoonotic viruses. In the present study, we modified our combinatorial mutagenesis strategy to identify human MxA super-restrictor variants with over 10-fold higher restriction of H5N1 IAV than wtMxA. By comparing H5N1-specific versus THOV-specific super-restrictors, we showed that the identity of the aromatic residue at residue 561 explained most of the breadth-specificity tradeoff; phenylalanine or tyrosine favored THOV restriction, whereas tryptophan favored H5N1 restriction. Despite this strong bias, we were nevertheless able to identify rare ‘generalist’ super-restrictor MxA variants that can simultaneously restrict both THOV and H5N1 more potently than wtMxA, thereby providing an intrinsic means to overcome the breadth-specificity tradeoff. Moreover, combining two ‘specialist’ restrictors of THOV and H5N1 in heterozygous combinations provided an extrinsic means to improve potency against both viruses, allowing the host to benefit from each allele without suffering from dominant-negative interference. Our study reveals the basis of breadth-specificity tradeoffs that constrain the evolution of host restriction factors like MxA against multiple pathogenic viruses and two strategies that may help to overcome them.

2.3 Combinatorial mutagenesis of loop L4 reveals MxA variants with enhanced H5N1 restriction

To understand how an antiviral protein can improve defense against a specific viral target through changes to rapidly evolving residues, we previously carried out two combinatorial mutagenesis screens to identify potential MxA super-restrictors with greater than ten-fold

improved restriction of THOV (*92*). However, the MxA variants with the highest levels of THOV restriction were impaired in their restriction activity against H5N1 IAV, revealing a breadth-specificity tradeoff. Here, we sought to identify H5N1 super-restrictor variants of MxA and to identify pathways through which the host might evolve around this breadth-specificity tradeoff.

Like with THOV restriction (*92*), we first confirmed that non-aromatic residues at residue 561 were incompatible with H5N1 restriction (Figure 2-S1). Our previous study revealed that W561 variants maintained potency against H5N1 despite losing THOV restriction (*92*). Therefore, we hypothesized that we could select H5N1 super-restrictors more successfully if we allowed residue 561 to be either F, Y, or W instead of restricting it to F alone (*92*). We generated a new combinatorial mutagenesis library in which residue 561 sampled only the aromatic amino acids while allowing the other four rapidly evolving residues (540, 564, 566, and 567) to encode any amino acid (see Methods) (Figure 2-1A). We randomly selected nearly 200 unique MxA variants from this combinatorial mutagenesis library, only discarding variants with stop codons introduced during NNS mutagenesis or variants with missense mutations outside loop L4. We then tested these MxA variants for their ability to restrict H5N1 (A/Vietnam/1203/04) using a previously described minireplicon assay (*51*) (see Methods), which assesses the effects of MxA restriction on viral transcription and genome replication (*10*, *51*, *92*, *104*). Briefly, we co-expressed all components of the viral ribonucleoprotein (vRNP), including the polymerase complex, the nucleoprotein (NP), an artificial, viral RNA genome segment encoding a reporter firefly luciferase gene that is transcribed and replicated by the viral polymerase in an NP-dependent manner, and a transfection control Renilla luciferase reporter. NP-dependent polymerase activity is measured as a ratio of firefly-to-Renilla luciferase. We calculated the fold

restriction as the decrease of luciferase activity of the H5N1 minireplicon in the presence of co-transfected MxA. We used catalytically dead human MxA (T103A) as a negative control for restriction and multiple replicates of wtMxA to define the wildtype range of H5N1 restriction.

We found that wtMxA has a relatively modest restriction of H5N1 (average restriction of 6.85-fold relative to empty vector control, Figure 2-1B), consistent with previous studies (*85*, *92*). However, 51 out of 194 MxA combinatorial variants showed higher H5N1 restriction than the highest level of restriction observed among multiple wtMxA replicates – in some cases up to 15-fold (we refer to variants with this improved restriction as 'super-restrictors') (Figure 2-1B). To validate our hits, we retested the H5N1 restriction activity of ten of the top super-restrictor variants obtained in our initial screen using an independently performed minireplicon assay (Figure 2-1C). This reanalysis reconfirmed the improved H5N1 restriction activity of 7 out of 10 MxA variants. All variants tested express MxA protein at similar levels (Figure 2-1C). Therefore, increased restriction of the top super-restrictors does not result from higher expression or stability. We retested the dose-dependent restriction for four validated super-restrictors – SWSTR, MWSRR, TWTRR, and YWHKR, where each letter refers to the positions of the positively selected residues in L4 of human MxA – 540, 561, 564, 566, and 567, respectively. The titration confirmed the enhanced H5N1 restriction activity of the super-restrictor variants compared to wtMxA at multiple levels of plasmid input (Figure 2-1D).

Since nuclear-localized MxA has enhanced IAV restriction (*17*, *105*, *106*), we tested whether altered subcellular localization could underlie the enhanced activity of these top four variants. We visualized FLAG-tagged versions of these MxA variants in HeLa cells (see Methods). We found that the super-restrictors localized diffusely to the cytoplasm, like wtMxA (Figure 2-1E).

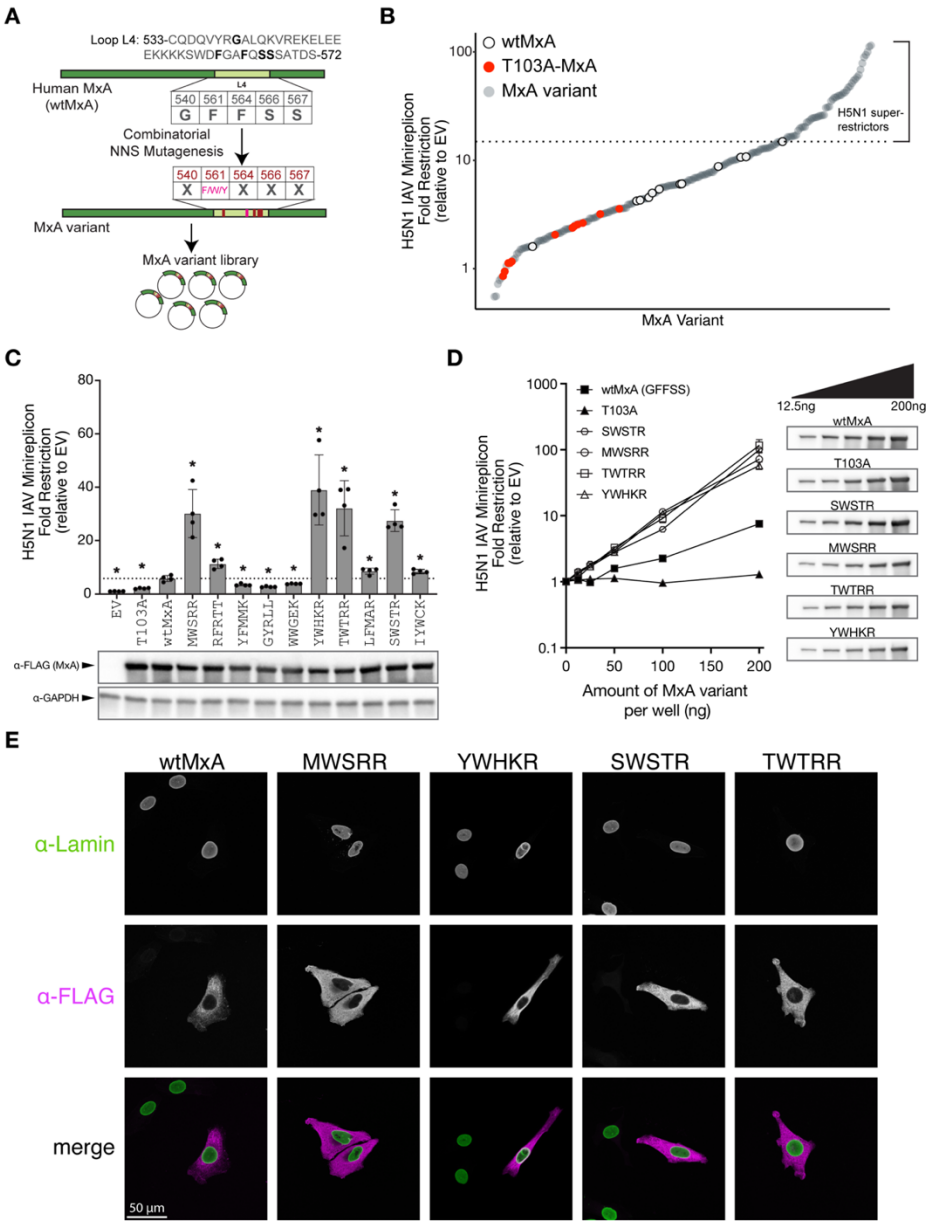

Figure 2-1. Combinatorial mutagenesis of human MxA identifies H5N1 super-restrictors. (A) An Mx variant library was constructed using combinatorial mutagenesis. Amino acid residues 540, 564, 566, and 567 (top, bolded) from loop L4 of wildtype human MxA were allowed to mutate to any amino acid residue using NNS mutagenesis, whereas residue 561 was varied to encode either F, W, or Y. (B) Restriction profile of 194 unique MxA combinatorial variants in L4 (gray circles) against the H5N1 minireplicon activity relative to empty vector control (on a log10 scale). For comparison, we also include multiple replicates of a catalytically inactive GTPase MxA variant, T103A (red circles), and several independent replicates of wtMxA (open circles) to define the wildtype range of H5N1 restriction. All analyses were performed in biological triplicates, and average restriction is represented. We used the highest level of wtMxA restriction to define the threshold level of H5N1 restriction (dotted line), above which the variants were tentatively classified as 'super-restrictors.' (C) Ten of the top super-restrictors were re-evaluated for H5N1 restriction as a fold difference to the empty vector control using an independently performed minireplicon assay restriction in biological quadruplicates (on a linear scale). Variants are identified based on their amino acid identities at the five variable sites in MxA L4, i.e., 540, 561, 564, 566, and 567. We used unpaired Welch's t-tests between each variant

and wtMxA to evaluate statistical significance (*p-value < 0.05). Protein expression levels of each variant were monitored by Western blotting. (D) Four validated super-restrictors were tested for their H5N1 restriction relative to empty vector (on a log10 scale) at increasing doses of MxA expression. The total amount of transfected DNA was equalized in all experimental conditions by supplementing empty vector plasmid DNA. Protein expression levels of each variant at each dosage were monitored by Western blotting. (E) Immunofluorescence analysis of the subcellular localization of wtMxA and four validated MxA super-restrictor variants using FLAG epitope-tagging in transiently transfected HeLa cells, which were also stained for lamin (which localizes to the nuclear membrane).

Moreover, in all cases, appending an SV40 NLS to their N-termini (Methods) could drive their nuclear localization (Figure 2-S2A) and enhance H5N1 restriction (Figure 2-S2B) without altering their expression levels. In contrast, the nuclear re-localization of a non-super-restrictor (WWGEK) was insufficient to enhance its potency over NLS-wtMxA. Therefore, we conclude that the H5N1 MxA super-restrictors act in their native cytoplasmic location without altered subcellular localization. These findings confirm that the sequence space of the rapidly evolving residues in loop L4 includes MxA variants with super-restriction of diverse orthomyxoviruses.

2.4 Positive epistasis between L4 residues underlies H5N1 super-restriction in MxA variants

Next, we aimed to understand the amino acid patterns underlying H5N1 super-restriction. Using a DiffLogo plot, we compared the frequencies of MxA L4 residues found in H5N1 super-restrictors from Figure 2-1B versus all other tested variants (Figure 2-2A). This comparison revealed a clear preference for tryptophan at position 561 (W561) among H5N1 super-restrictors, whereas Y561 was strongly disfavored. F561 (the residue in wtMxA) showed no strong preference, which is why it is barely visible in the DiffLogo plot. This pattern is even more apparent when the H5N1 restriction activities of all tested MxA variants are grouped based on residue 561 (Figure 2-2B). These findings sharply contrast with our previous analysis of THOV super-restrictors, which favored either Y561 or F561 and disfavored W561 (*92*). The DiffLogo plot also highlighted a striking preference for positively charged residues (arginine [R] or lysine [K]) at positions 564, 566, and 567 (Figure 2-2A).

Next, we investigated the contribution of the single residues at the five variable positions of a selected super-restrictor for enhanced H5N1 restriction. The SWSTR variant was one of the strongest H5N1 super-restrictors in our initial and revalidated screen (Figure 2-1), with ~5-fold higher H5N1 restriction than wtMxA (GFFSS) (Figure 2-2C, far left). We found that individually reverting residues 540, 561, 566, and 567 in SWSTR to wtMxA (i.e., S540G, W561F, T566S, or R567S) led to a significant reduction in H5N1 restriction (Figure 2-2C). W561F and R567S reversions showed the most dramatic decrease in restriction, consistent with the preference for both 561W and 567R among super-restrictor variants (Figure 2-2A). In contrast, we found that reversion of residue 564 (i.e., S564F) increased H5N1 restriction, consistent with 564S being disfavored among super-restrictors (Figure 2-2A). The resulting SWFTR variant is 10-fold better than wtMxA at restricting H5N1 (Figure 2-2C). Thus, multiple residues in the SWSTR variant, most notably W561 and R567, are necessary for the increased H5N1 activity of super-restrictor variants.

We also tested which residues of SWSTR were sufficient to confer H5N1 super-restriction to wtMxA (Figure 2-2C). Several single residue changes from SWSTR into the wtMxA backbone led to statistically significant increases in restriction. However, these increases were modest, with S567R providing the largest 2.4-fold increase of H5N1 restriction over wtMxA (Figure 2-2C, right). Thus, we conclude that robust H5N1 super-restriction by SWSTR requires multiple changes from wtMxA. Therefore, we tested whether two changes might be sufficient to confer SWSTR-like levels of super-restriction onto wtMxA (Figure 2-2D). Since the DiffLogo plot indicated a preference for W561 and basic residues at positions 564, 566, and 567 among H5N1

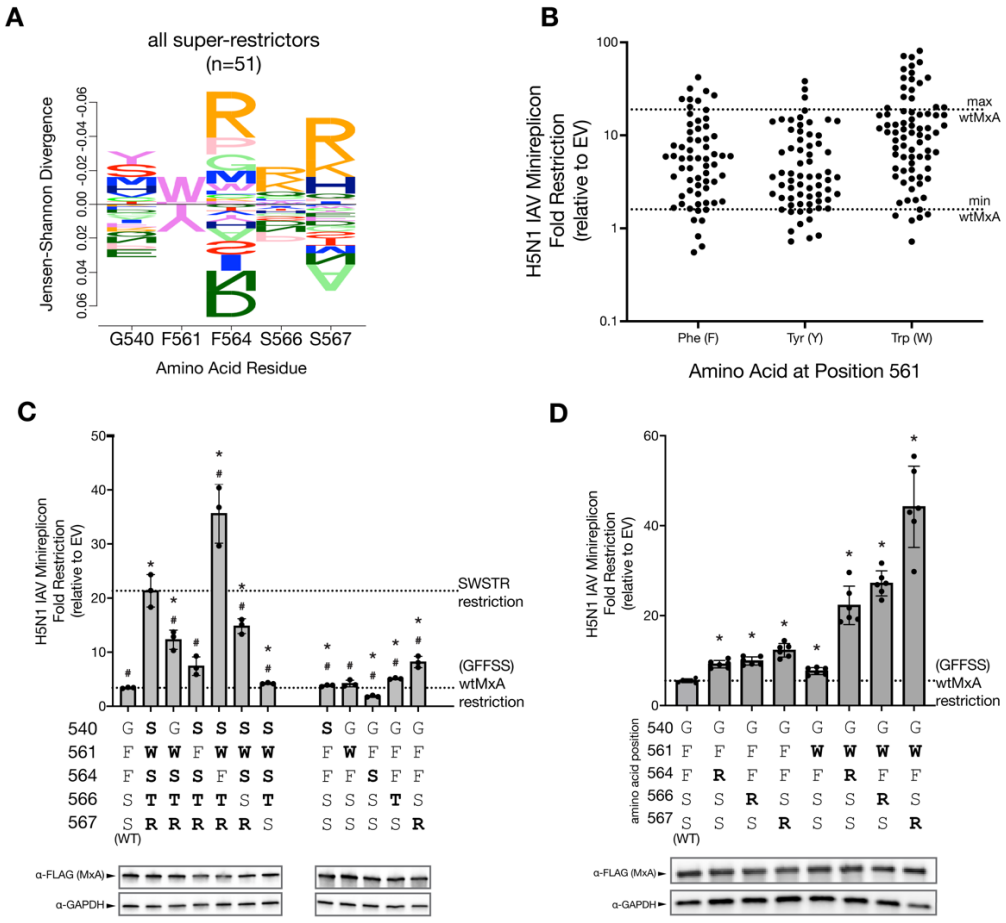

Figure 2-2 Necessity and sufficiency of L4 residues reveal positive epistasis underlies MxA super-restriction of H5N1. (A) DiffLogo plot compares residue frequencies among super-restrictors (variants with restriction greater than maximal wtMxA restriction) relative to all tested variants, indicating the amino acid preferences at each position for H5N1 super-restriction. Bar height is proportional to Jensen-Shannon divergence per site, and letter height is proportional to frequency. The wtMxA residues (GFFSS) are indicated at the bottom. (B) Dot plot showing the restriction profiles (as fold restriction relative to empty vector, log10 scale) of all 194 MxA variants, sorted according to the amino acid identity at residue 561. The dotted lines indicate the highest and lowest restriction levels of wtMxA (taken from Figure 2-1B). (C) We analyzed the contribution of each residue of super-restrictor variant SWSTR in conferring increased anti-H5N1 activity by reverting each position to the wtMxA residue, GFFSS (left). We also tested the sufficiency of individual changes in L4 residues from the SWSTR variant to confer increased H5N1 restriction to wtMxA (right). Protein expression levels of each variant were monitored by Western blotting. (D) We analyzed the ability of dual changes in L4 residues – F561W combined with either 564R, 566R, or 567R – to confer increased H5N1 super-restriction to wtMxA. Protein expression levels of each variant were monitored by Western blotting. The restriction is reported as a fold-change relative to an empty vector on a linear scale. For (C), we performed unpaired Welch's t-tests between each variant and wtMxA (asterisks, p-value < 0.05*) or SWSTR (hashes, p-value < 0.05 #). For (D), we performed unpaired Welch's t-tests between each mean and the wtMxA mean. Significant differences (p-value < 0.05) are noted as *.

super-restrictors (Figure 2-2A), we introduced W561 in conjunction with either 564R, 566R, or 567R in wtMxA. We found that each of these combinations conferred H5N1 super-restriction levels that were higher than the sum of individual mutations (Figure 2-2D), reiterating the

importance of the W561 residue and the interchangeability of the arginine residue at either residue 564, 566, or 567. These findings imply that positive epistatic interactions among at least two residues of loop L4 are required to achieve H5N1 super-restriction, as with THOV super-restriction (*92*).

2.5 'Generalist' MxA variants overcome breadth-specificity tradeoffs in viral restriction

Our previous study revealed that increased THOV restriction often weakened H5N1 restriction (*92*). We investigated this breadth-specificity tradeoff in more detail, aided by our identification of novel H5N1 super-restrictors. We selected 52 MxA combinatorial variants from our screen based on their H5N1 restriction (Figure 2-1B): 42 super-restrictor variants with better than wtMxA restriction, five variants with equivalent restriction as wtMxA, and five non-restrictors with lower than wtMxA activity. We assayed all 52 MxA variants for their ability to restrict THOV (x-axis) and re-assayed them against H5N1 (y-axis) using minireplicon assays (Figure 2-3A). By comparing their activity to the range of wtMxA values (using two standard deviations above mean wtMxA activity as a threshold), we classified variants as H5N1 specialist super-restrictors (above the horizontal shaded region, Figure 2-3A) or THOV super-restrictors (right of the vertical shaded region, Figure 2-3A).

Consistent with a breadth-versus-specificity trade-off, most MxA super-restrictors are 'specialists,' i.e., they have increased H5N1 but not THOV restriction or increased THOV but not H5N1 restriction. Seventeen of 22 H5N1 specialist super-restrictors encode W561 (Figure 2-3A, magenta circles). In contrast, none of the THOV specialist super-restrictors encoded W561; instead, they encoded F561 or Y561 (Figure 2-3A, blue and yellow circles). To further test the role of residue 561 on H5N1 versus THOV restriction, we tested the effects of swapping this residue with the other aromatic amino acids for three MxA super-restrictors containing either

W561 (SWSTR), F561 (LFMAR), or Y561 (IYWCK) (bold, Figure 2-3A) on their THOV or H5N1 restriction activity. For all three variants, we found that F561 and Y561 in all three variants produced robust THOV restriction, whereas W561 resulted in a dramatic loss of THOV restriction (Figure 2-3B), confirming earlier findings that W561 may be incompatible with THOV restriction (*92*) (Figure 2-3A). Furthermore, we found a clear hierarchy (W561 > F561 >Y561) in H5N1 restriction for the SWSTR and LFMAR backgrounds (Figure 2-3B), consistent with our DiffLogo analyses (Figure 2-2A). Together, these results bolster our previous findings. Despite a few exceptions, we conclude that most of the breadth-specificity trade-off in THOV versus H5N1 super-restriction by MxA may stem from the identity of the aromatic amino acid found at MxA's critical residue 561.

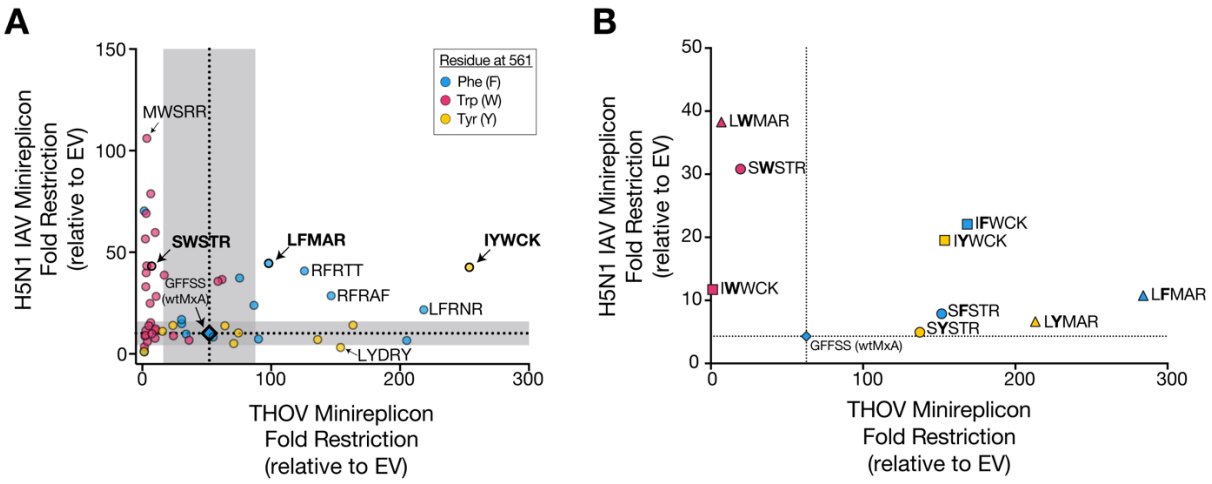

Figure 2-3 'Generalist' MxA variants can restrict both H5N1 and THOV. (A) We screened 52 MxA variants for antiviral activity in H5N1 and THOV minireplicon assays. Each point represents an average of three independent replicates. The mean wild-type restriction of H5N1 and THOV are represented by horizontal and vertical dotted lines, respectively, with gray shaded areas representing two standard deviations among three replicate wtMxA measurements. We classified MxA variants based on residue 561 – either phenylalanine (F, cyan dot), tyrosine (Y, yellow dot), or tryptophan (W, magenta dot). (B) We replaced the amino acid at residue 561 in selected super-restrictors with other aromatic residues. All points represent the average of three replicate experiments. X- and y-axes in linear scale.

Unexpectedly, our analyses also revealed five 'generalist' super-restrictor MxA variants, which had much improved (greater than two standard deviations above mean wtMxA) restriction

against both THOV and H5N1 (circles above and to the right of gray shaded areas, Figure 2-3A). Intriguingly, four of these MxA variants encoded F561, while one encodes Y561. The IYWCK generalist super-restrictors also had an atypical sequence preference for H5N1 restriction, with IYWCK and IFWCK outperforming IWWCK (Figure 2-3B). Although generalist super-restrictors constitute ~10% of the 52 MxA variants tested for both viruses, this is likely an overestimate of the frequency of generalist super-restrictors among MxA combinatorial variants since we biased our selection towards H5N1 super-restrictors. Indeed, our previous analysis of 24 THOV super-restrictors found that most of them lost H5N1 restriction (*92*). Our findings suggest that MxA's breadth-specificity trade-off in H5N1 and THOV restriction is not insurmountable; generalist super-restrictor variants can simultaneously improve their intrinsic restriction of multiple divergent orthomyxoviruses such as THOV and H5N1.

2.6 Heterozygous MxA 'specialist' variants combine to yield 'generalist' super-restriction

Generalist super-restrictor variants are rare in the evolutionary landscape compared to specialist super-restrictors (Figure 2-3), whose enhanced antiviral activity can be achieved by just two amino acid changes (Figure 2-2). We hypothesized that generalist super-restriction might still be achieved by combining two distinct specialist super-restrictors as heterozygous alleles. Two observations inspired this hypothesis. First, previous studies have shown that loss-of-function variants found in the human population can have a dominant-negative effect on wtMxA restriction of IAV, by 'poisoning' MxA oligomers required for antiviral activity (*99*, *107*). Second, recurrent signatures of positive selection in MxA (*10*) suggest that any de novo gain-of-function MxA variant must have been able to provide increased function even as a heterozygous allele with wtMxA.

To test this hypothesis, we first investigated whether a super-restrictor MxA variant could provide enhanced restriction in the presence of wtMxA, mimicking its origin as a heterozygous allele. We selected two H5N1 specialists (MWSRR, SWSTR), two THOV specialists (QFAYS, LYDRY), and one generalist (IYWCK). We re-tested them against H5N1 and THOV to confirm their specialist and generalist restriction activities (Figure 2-S3). We then tested the H5N1 and THOV restriction activity of each MxA super-restrictor variant in a 1:1 ratio with wtMxA, mimicking equal amounts of heterozygous alleles (Figure 2-4A,B). As dosage controls, we tested our wtMxA in a 1:1 ratio with either an empty vector (1X wtMxA) or another wtMxA three other variants in a 1:1 ratio with wtMxA for restriction: catalytically inactive MxA (T103A) previously reported to be dominant-negative to wtMxA (*62*), an oligomerization-defective MxA variant (M527D) (*59*, *61*), and a dominant-negative MxA variant identified in a human patient (L542S) (*99*).

As expected, we found that the dominant-negative MxA variants (T103A and L542S) abrogated both H5N1 and THOV restriction compared to wtMxA (Figure 2-4A and B). In contrast, the oligomerization-defective M527D variant only modestly impaired H5N1 and THOV restriction (Figure 2-4A and B). Both specialist (MWSRR, SWSTR) and generalist (IYWCK) H5N1 super-restrictors continued to enhance H5N1 restriction despite their half dosage (Figure 2-4A). In the THOV minireplicon, only the generalist (IYWCK) super-restrictor significantly enhanced THOV restriction at half dosage (Figure 2-4B), but all other super-restrictors maintained at least wtMxA levels of restriction (Figure 2-4B). Thus, in H5N1 restriction, a wtMxA allele does not interfere with super-restrictor variants. Moreover, in all cases, specialist super-restrictors do not impair wtMxA activity against the non-targeted virus, suggesting they do not act in a co-dominant or dominant-negative manner. For example, THOV-specialist super-

restrictors (QFAYS, LYDRY) did not substantially lower H5N1 restriction compared to 1X wtMxA levels (Figure 2-4A). Instead, we found that H5N1 specialist super-restrictors modestly enhanced THOV restriction relative to 1X wtMxA levels (Figure 2-4B), despite having weak to no activity against THOV (Figure 2-S3B). These data indicate that MxA super-restrictors can cooperate with wtMxA to enhance antiviral restriction without impairing existing antiviral functions.

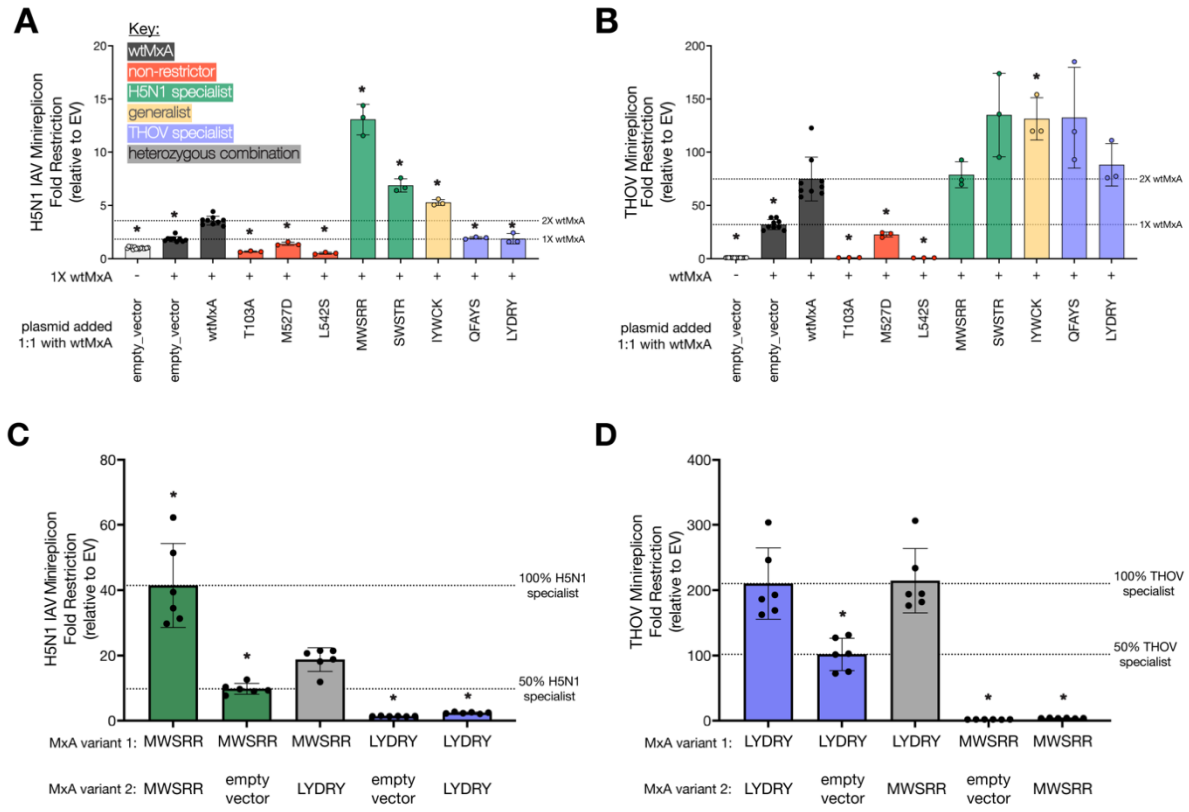

Figure 2-4 Heterozygous MxA 'specialist' variants combine to yield 'generalist' super-restriction of H5N1 and THOV. (A) Using minireplicon assays, we tested 1X wtMxA (100ng per well for H5N1 assay and 50ng per well for THOV assay) and equimolar ratios of different MxA variants, including wtMxA, for (A) H5N1 restriction and (B) THOV restriction. Variants tested in equimolar ratios include empty vector controls, wtMxA (total 2X wtMxA) (black bars, dosage control), inactive variants (red bars), H5N1 specialists (green bars), a generalist (yellow bar), or THOV specialists (purple bars). Using minireplicon assays, we also measured (C) H5N1 restriction and (D) THOV restriction of H5N1 specialist MWSRR, THOV specialist LYDRY, or their equimolar combination. All assays contained the same amount of transfected plasmid DNA. Minigenome restriction is reported as fold-change relative to an empty vector on a linear scale. For (A) and (B) we performed unpaired Welch's t-tests between each variant and the restriction level of 2X wtMxA. For (C) and (D) we performed unpaired Welch's t-tests between the combined specialists with each other combination. Significant differences are noted as * for p-value < 0.05. Protein expression levels were monitored for each condition by Western blotting.

Finally, we tested whether combining an H5N1 specialist super-restrictor (MWSRR, with low THOV restriction) with a THOV specialist super-restrictor (LYDRY, with low H5N1 restriction) in heterozygous allelic combinations might achieve high restriction of both viruses. Indeed, we found this to be the case (Figure 2-4C, D). A 1:1 mixture of the MWSRR and LYDRY variants led to a modest increase of H5N1 restriction over the half-dosage of the MWSRR variant (Figure 2-4C). The same mixture also led to increased THOV restriction over the half-dosage of the LYDRY variant (Figure 2-4D). These results demonstrate that the specialist super-restrictors do not act as dominant-negative variants for antiviral restriction. Instead, two specialist super-restrictor alleles in the same cell lead to simultaneously increased antiviral activity against two different viral targets.

2.7 Discussion

Here, we demonstrate that amino acid variation within the positively selected residues in the L4 loop of MxA contains the potential to significantly enhance the restriction of the highly pathogenic avian H5N1 strain of IAV. We find that MxA super-restriction can be attributed to positive epistasis, where two different mutations in L4 combine to confer a significant gain of restriction above wtMxA levels. We also find that a critical determinant of the breadth-specificity trade-off is a single amino acid residue at position 561, with tryptophan favoring H5N1 restriction and phenylalanine or tyrosine favored in THOV restriction. Although W561 appears to prohibit THOV restriction, F561 and Y561 permit restriction of both THOV and H5N1.

Our finding that a single aromatic amino acid at position 561 determines MxA restriction activity against divergent orthomyxoviruses has important implications for its biochemical interactions with viral proteins and the likelihood of evolutionary transitions between different

antiviral states. Several studies have pointed to the viral NP as the target of MxA antiviral action (*48*, *84*, *86*). We speculate that the contrasting preference in THOV versus H5N1 super-restrictors (especially the inability of W561 to restrict THOV) results from the size or orientation of this amino acid, which might affect its interactions with THOV versus H5N1 NP. Incorporating these insights could help protein docking and modeling studies that advance our understanding of the MxA-NP interface, which is still poorly understood.

Previous evolutionary analyses have revealed that MxA genes in humans and other hominids (gorilla, chimpanzee, bonobo, orangutan) encode phenylalanine (F) at position 561. In contrast, other primate species encode a wide variety of amino acids at this critical site, including aliphatic (leucine, isoleucine, valine), sulfur-containing (cysteine, methionine), hydroxylic (serine), acidic (aspartic acid), and aromatic (tyrosine) (*10*). As a result, hominoid MxA alleles are better poised than the MxA of other primates to restrict THOV and IAV (*10*). Yet, despite their biochemical similarity, evolutionary transitions between F, Y, and W are not trivial. A single non-synonymous change can accomplish transitions between phenylalanine (F, encoded by codons TTT/TTC) and tyrosine (Y, encoded by codons TAT/TAC). However, transitions from phenylalanine or tyrosine to tryptophan (W, encoded by TGG) require two simultaneous transversion changes since all intermediate states (leucine, cystine, or a stop codon) would lose function against orthomyxoviruses (*10*). Moreover, the restriction phenotype of residue 561 is reliant on epistatic interactions with other rapidly evolving residues, which in turn influence the constraints acting on residue 561.

Our analyses reveal two strategies antiviral proteins like MxA can use to bypass breadth-specificity trade-offs for orthomyxovirus restriction (Figure 2-5). First, MxA variants capable of enhanced restriction of both H5N1 and THOV can act as ‘generalist’ super-restrictors. Based on

previous analyses, we anticipate this generalist super-restriction is achieved by increasing avidity to the NP proteins from both THOV and H5N1 (*61*, *92*). However, such generalist super-restrictors were rarely detected and not likely to be readily accessible in the MxA loop L4 mutational landscape.

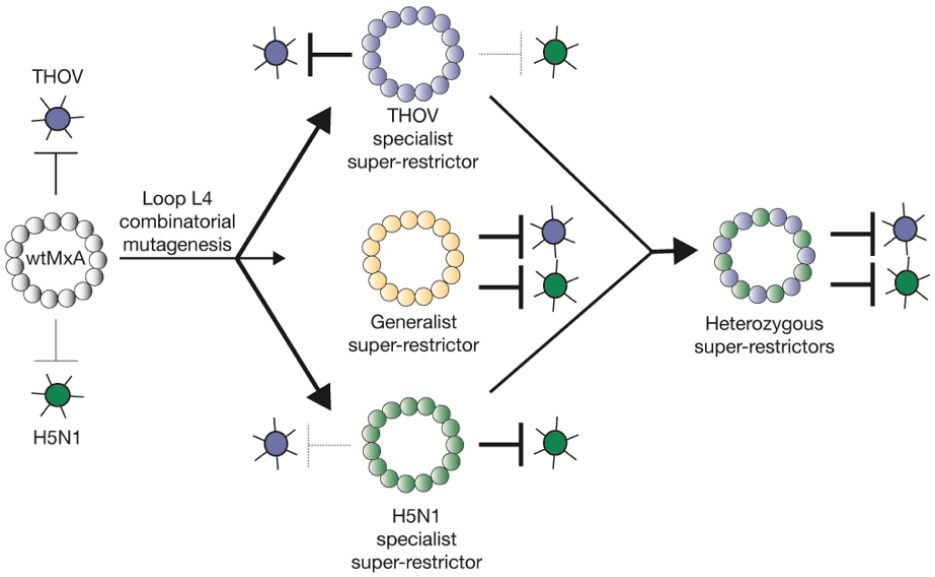

Figure 2-5 Two strategies enable MxA to overcome breadth-specificity tradeoffs. Wildtype human MxA (wtMxA), depicted as an oligomeric ring of individual gray MxA monomers, can robustly restrict THOV (red) and modestly restrict H5N1 IAV (purple). Combinatorial mutagenesis of just five rapidly evolving residues in Loop L4 readily yields 'specialist' super-restrictor variants with enhanced restriction of THOV but lower restriction of H5N1, or vice-versa. Our analyses also identified 'generalist' super-restrictors with enhanced restriction of both THOV and H5N1, but these variants were much rarer. However, combining two 'specialist' super-restrictors as heterozygous alleles can restrict both viruses. Based on their antiviral profile (Figure 2-4), we hypothesize this enhanced restriction of both viruses occurs via mixed oligomers combining monomers of both types.

We show that host genomes could also achieve broad enhanced restriction of divergent orthomyxoviruses without the requirement to evolve rare generalist super-restrictors but rather by combining two divergent alleles of MxA with distinct 'specialist' super-restriction activities. Since specialist super-restrictors are more common than generalist super-restrictors in MxA's mutational landscape (Figure 2-3A), we propose that heterozygous combinations of specialist

super-restrictor alleles are more likely to provide host populations with a facile means to overcome the breadth-specificity tradeoffs imposed by multiple pathogenic viruses.

In theory, heterozygous MxA restriction could rely on two distinct MxA oligomers, primarily comprised of the same monomer. Because MxA restricts THOV and H5N1 in a dose-dependent manner, we would expect this to reveal a co-dominant phenotype of two alleles. However, our analyses suggest that monomers of both MxA variants intermix in heterooligomers, in which the restrictive variants can manifest their enhanced restriction even while oligomerized with less-restrictive variants. Such variants contrast with previously studied MxA variants, in which missense mutations in the MxA 'backbone' resulted in dominant-negative loss-of-function oligomers with wtMxA (*99*, *107*). Such loss-of-function mutations effectively lead to loss of MxA restriction and are unlikely to propagate at high levels in populations. In contrast, gain-of-antiviral specificity variants of MxA, like those we have identified in loop L4, are expected to be positively selected, especially in the face of pathogenic viruses. They may even be co-propagated under balancing selection. Indeed, genes encoding restriction factors are often subject to diversifying and balancing selection, with heterozygote advantage maintaining multiple diverse protective alleles in host populations (*108*).

Our combinatorial mutagenesis strategy focusing on positively selected residues provides a general means to elicit super-restrictor variants of restriction factors and bypass inherent breadth-versus-specificity trade-offs in antiviral restriction. The insights we have gained in the present study of MxA variants will likely apply to other restriction factors that share three critical attributes with MxA. First, like MxA, many restriction factors evolve under positive selection at their viral interaction surfaces; amino acid changes at these positively selected interfaces can confer gain of antiviral specificity and successful host restriction. Second, they can also be

subject to balancing selection due to heterozygote advantages. Third, many restriction factors function as dimers or higher-order oligomers, enabling a strategy combining diverse monomeric units in oligomers to manifest a much broader antiviral restriction.

Given the immense selective pressures imposed on restriction factors like MxA, it is surprising that most mammalian genomes only encode two Mx-family proteins, one localizing to the cytoplasm and the other to the nucleus or nuclear periphery. Given similar selective pressures from pathogenic viruses, many other antiviral restriction factors have undergone dramatic expansions, like the APOBEC3 genes in primates or the TRIM5-like genes in rodents or carnivore genomes *(40, 41)*. Similarly, virus-specific alleles of the murine restriction factor Fv-1 arose and became distributed among subspecies based on varied exposure to cocirculating viruses (*111*, *112*). The fact that we don't see such rampant duplication and diversification among MxA paralogs in mammalian genomes suggests either that two paralogs are enough to provide broad protection or (more likely) that there is some hidden cost associated with rampant Mx gene expansion in mammals, either to host fitness or due to dominant-negative interference of Mx restriction. Understanding the nature of this hidden cost would help us design better strategies to identify only slightly altered human Mx variants that could provide significantly improved antiviral protection in the face of a new IAV variant, which we will inevitably encounter in the future.

2.8 Supplementary Figures

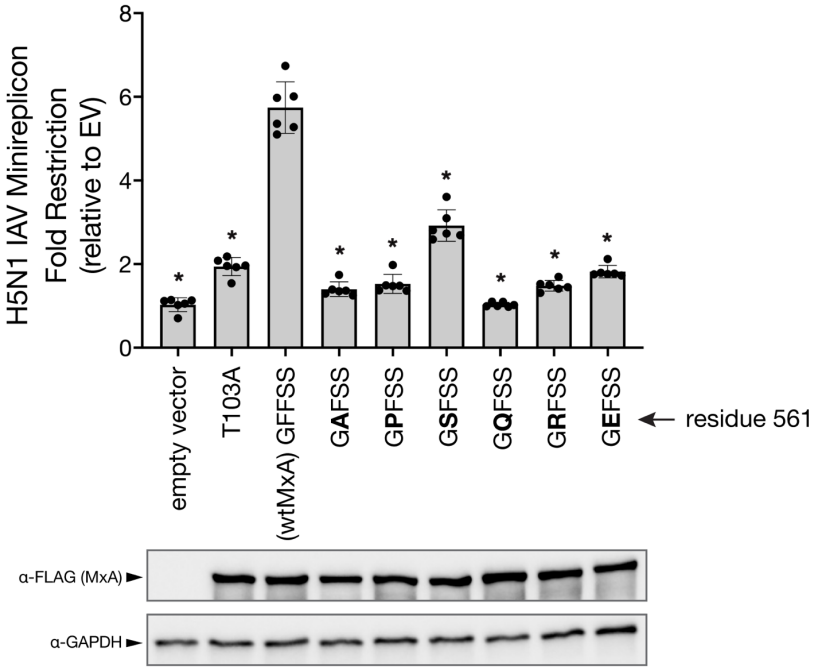

Figure 2-S1. Restriction profiles of MxA variants with various non-aromatic amino acid residues at position 561. Fold restriction is reported relative to an empty vector. Each variant is labeled using amino acid identities at the five variable sites. We used unpaired Welch's t-tests between each variant and wtMxA to evaluate statistical significance (*p-value < 0.05).

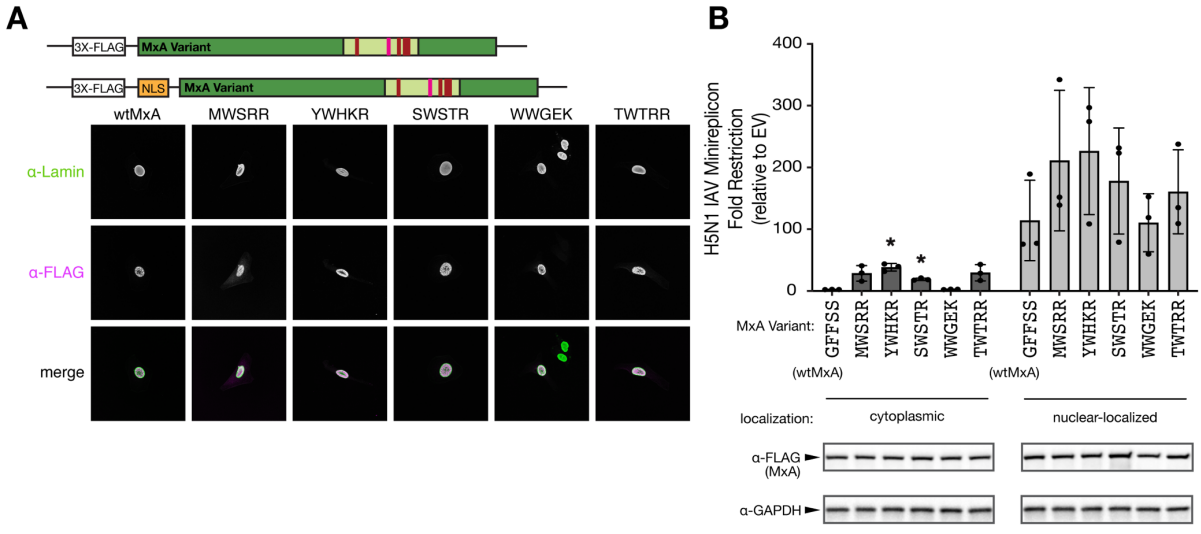

Figure 2-S2. Nuclear localization further enhances H5N1 super-restriction. (A) The SV40 large T antigen nuclear localization signal (NLS) PKKKRKV was cloned into the N-termini of MxA variants between a 3X-FLAG tag and the MxA gene. NLS-tagged variants were transfected into HeLa cells and imaged in the same manner as described in Figure 2-2A. (B) The five super-restrictor variants, as well as wtMxA, with and without an N-terminal NLS were assayed for their H5N1 restriction relative to an empty vector control in the minireplicon assay. Their expression levels were also tested by Western blotting. Unpaired Welch's t-tests were performed between restriction levels of cytoplasmic variants and wtMxA as well as between NLS-tagged variants and NLS-wtMxA (* p< 0.05).

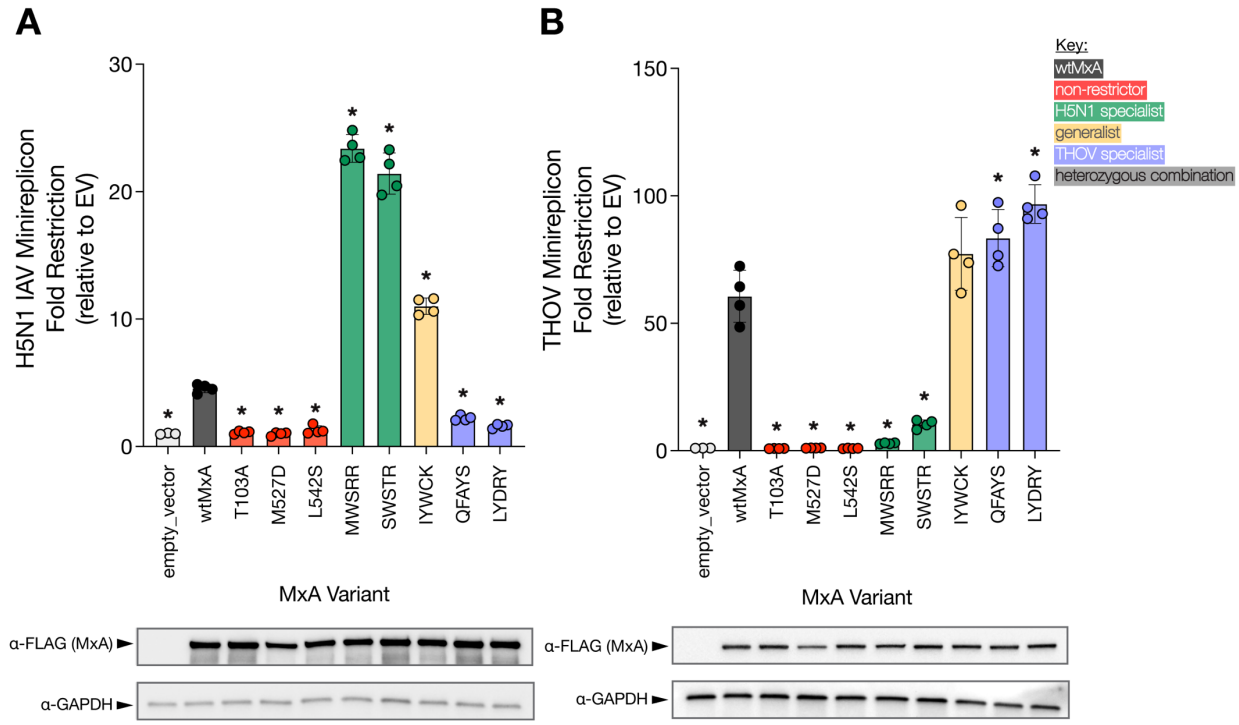

Figure 2-S3. 'Generalist' and 'specialist' MxA variants restriction of H5N1 and THOV. We retested H5N1 (A) or THOV (B) restriction by wtMxA, non-restricting controls, specialist, and generalist MxA variants relative to an empty vector control based on a minireplicon assay; data is represented on a linear scale. The total amount of empty vector or MxA variant per condition is 100ng per well for H5N1 assay and 50 ng per well for the THOV assay. Unpaired Welch's t-tests were performed between restriction levels of cytoplasmic variants and wtMxA as well as between NLS-tagged variants and NLS-wtMxA (* $p < 0.05$).

2.9 Materials and Methods

Library Construction and Plasmid Preparation

A library of 3X-FLAG-tagged human MxA variants was designed, in which codons 540, 564, 566, and 567 were randomized by NNS mutagenesis, and codon 561 was randomly allowed to a W, F, or Y. This library was synthesized and cloned into a pQCXIP plasmid backbone. The pooled library of plasmids was transformed into NEB 5-alpha competent E. coli (NEB #C2987) and plated sparsely to obtain single colonies. Single colonies were inoculated into 6mL of 100 µg/mL ampicillin LB broth, grown overnight at 37°C at 250rpm. After 16-18 hours of growth, 1mL of culture was added to 1mL of 50% glycerol for storage at -80°C. The remaining 5mL

were used for plasmid purification (Promega #A1223). The C-terminus of MxA, including the loop L4 region, of the purified plasmids was sequenced by Sanger sequencing using the following primer: 5-' CGT GGT AGA GAG CTG C -3'. We cloned and sequenced ~270 randomly selected variants from the pooled plasmid library. Of those, 194 did not contain stop codons or frameshift mutations. All variants used for follow-up assays after the initial screen were sequenced again to verify the remaining N-terminal sequence using a plasmid-specific primer upstream of MxA (5-' ACA CCG GGA CCG ATC CAG-3').

Cell Lines

HEK-293T/17 and HeLa cells were grown on treated tissue-culture plates in DMEM (Thermo Fisher #11965118) containing high-glucose and L-glutamine with 1x penicillin/streptomycin (Thermo Fisher #15140122) and 10% fetal bovine serum (Gibco #10437028). Cells were grown at 37°C, 5% CO_2 in humidified incubators, and passaged by digestion with 0.05% trypsin-EDTA (Thermo Fisher #25300120).

Minireplicon Assays

All minireplicon assays were performed in black, opaque, clear-bottomed 96-well plates by transfection of 50-80% confluent HEK293T/c-17 cells with minireplicon components using Mirus TransIT-293 reagent. For the H5N1 minireplicon system, 1 ng each of PB2, PB1, and PA, 0.5 ng of NP (all in a pCAGGS vector), 25 ng of pHH21-vNP-FF-Luc (firefly luciferase), 5 ng of pTK-Ren-Luc (Renilla luciferase), and 100 ng of pQCXIP-MxA were transfected. For the THOV minireplicon system, 4 ng each of PB2, PB1, and PA, 1 ng of NP (all in a pCAGGS vector), 20 ng of pHH21-vNP-FF-Luc (firefly luciferase), 50 ng of pTK-Ren-Luc (Renilla luciferase, constitutively expressed under the HSV TK promotor to serve as a transfection control), and 50

ng of pQCXIP-MxA were transfected. After 24 hours, firefly and Renilla luciferase luminescence were measured using the Promega Dual-Glo Luciferase Assay System. For the 96-well format, all but 20 µL of the medium was removed. To each well, 20 µL of Dual-Glo Luciferase Reagent was added for lysis and luciferase activation, incubated for 10 minutes at room temperature, and then luminescence was read on a Biotek Cytation3 plate reader. 20 µL of the Stop and Glo Reagent was added, incubated at room temperature for 10 minutes, and luminescence was re-read. Normalized minireplicon activity for each sample was calculated as the value of firefly luciferase luminescence divided by the Renilla luciferase luminescence. Each assay plate contained empty vector, wtMxA, and catalytically inactive MxA(T103A) controls, providing the range of control variant restriction reported in Figure 2-1B. Results are reported as "fold-restriction", calculated as the average minireplicon activity in the presence of empty pQCXIP vector for the paired assay plate divided by the minireplicon activity of the experimental sample. All samples were assayed by transfection of a master mix into triplicate wells.

Logo Plots

The DiffLogo plot was generated in R using the MotifStack and DiffLogo packages from the Bioconductor library. Frequencies of each amino acid were calculated at each site. The DiffLogo plot was generated by comparing amino acid frequencies at each site among super-restrictors to amino acid frequencies at each site across all assayed variants. The code and associated files can be found at https://github.com/rag125/h5n1_srs.

Western Blots

We used western blot analyses to assay protein expression after plasmid transfection in HEK293T cells for 24 hours in a 24-well format. Cells were collected and lysed on ice in RIPA

buffer (Invitrogen #89900). Cell debris was removed by centrifugation, after which protein concentration in all supernatants was measured using the Pierce™ BCA Protein Assay Kit and normalized to an equal concentration by diluting samples with RIPA buffer. Samples were reduced and denatured by adding Laemmli buffer (Bio-Rad #1610737) containing β-mercaptoethanol and heating to 95°C for 5 minutes. Samples were run by SDS-gel electrophoresis and blotted using the Bio-Rad Mini-PROTEAN TGX Gel system. Membranes were cut using a razor to separate MxA protein (~76kDa) and GAPDH (~37kDa). We used the following primary antibodies: Sigma F1804 monoclonal M2 Anti-FLAG and Genetex GTX100118 anti-GAPDH. Secondary antibodies conjugated to horseradish peroxidase (HRP) are from R&D systems (HAF007 and HAF008). HRP was detected using Supersignal West Pico Plus Chemiluminescent Substrate (Fisher #34577) on a Bio-Rad Gel Doc.

Immunofluorescence Microscopy

HeLa cells were seeded in a 24-well plate at a density of 10^5 cells/well. When cells were confluent about 24 hours later, they were transfected with the appropriate MxA variant equivalent to five times the volume delivered to their corresponding 96-well plates using Lipofectamine 3000 reagent (Invitrogen #L3000). Nineteen hours after transfection, cells were trypsinized and reseeded in Cellvis 24-well glass-like bottomed plates (Cellvis #P24-1.5P) at a density of 0.5-1x10^4 cells/well. About 12 hours after reseeding, cells were washed with PBS (+Mg^{2+} +Ca^{2+}) for 5 minutes, fixed in 4% paraformaldehyde for 15 minutes, rewashed, permeabilized with 0.25% Triton X-100 for five minutes, and washed again. Cells were blocked in 10% BSA at 37°C for 30 minutes. Primary antibody incubation included anti-lamin antibody (Sigma-Aldrich #L1293) at 1:500 and anti-FLAG (Sigma #F1804) at 1:2000 in 3% BSA for 1

hour at 37°C. After two PBS++ washes, cells were incubated with secondary antibodies Alexa-Fluor488 Anti-rabbit (Fisher #A21206) at 1:2000, Alexa Fluor633 Anti-mouse (Invitrogen #A21050) at 1:2000, Hoechst stain (Invitrogen #H1399) at 1:1000, and Alexa Fluor568 Phalloidin (Thermo Fisher #A12380) at 1:400 for 1 hour at 37°C. Cells were washed twice in PBS++ before imaging on a Leica DMi8 inverted microscope. Images were processed using LAS X software.

Statistical Analyses

Statistical analyses were performed in R. The code and associated files can be found at https://github.com/rag125/h5n1_srs.

Chapter 3 Super-restriction of 1918 H1N1 IAV through subcellular re-localization

3.1 Abstract

In this chapter, I extended my studies of the evolutionary potential of human MxA to gain enhanced restriction capabilities against the 1918 H1N1 IAV strain, which has evolved to escape MxA restriction. Building upon previous findings that identified super-restrictor MxA variants against THOV and H5N1 IAV through mutations in the rapidly evolving loop L4 region, I investigated whether similar strategies could be applied to regain restriction against the escaped 1918 H1N1 strain. I first introduced three key escape mutations (R100I, L283P, and F313Y) into H5N1 NP to recapitulate the 1918 H1N1 escape phenotype from wild-type MxA (wtMxA). Upon testing previously obtained H5N1 super-restrictor MxA variants against H5N1* (H5N1 with escape mutations), I found only modest improvements in restriction were observed, with a maximum improvement of about 10-fold. However, adding a nuclear localization signal (NLS) to MxA variants greatly enhanced their ability to restrict H5N1* and H1N1. Based on this finding, I created an NLS-tagged MxA loop L4 variant library, successfully identifying additional super-restrictors of 1918 H1N1 IAV. These findings reveal that MxA possesses the evolutionary potential to regain restriction ability even against escaped viruses like H1N1 IAV. However, my findings further suggest that H1N1 restriction requires both loop L4 specificity and nuclear localization. This work provides crucial insights into the evolutionary paths by which MxA can regain restriction against fully escaped viruses, potentially informing strategies to combat human-endemic and pandemic IAV strains.

3.2 Introduction

Chapter 2 of this thesis and previous work have established the loop L4 of MxA as the specificity determinant for species-specific restriction of *orthomyxoviruses* such as THOV and H5N1. Five amino acid sites in the loop L4 have rapidly evolved among primate species (*10*). Combinatorial mutagenesis of these five rapidly evolving residues reveals super-restrictor MxA variants with significantly increased restriction of both THOV and H5N1 (*92*). The initial identification of THOV MxA super-restrictors demonstrated that, within the mutational landscape of the already strongly restricting human MxA, there existed even stronger restrictors to a well-restricted virus (*92*). This paved the way to the next question: whether a virus less well restricted by human MxA – the avian-adapted H5N1 IAV strain – could be better restricted by exploring the MxA loop L4 mutational landscape. Indeed, I showed such improved restriction of H5N1 IAV to be quite readily attainable *(Chapter 2).* We next sought to challenge the limits of MxA super-restriction against a virus that completely escaped from wild-type human MxA restriction: the human-adapted 1918 H1N1 strain of IAV. Previous studies have meticulously determined that the 1918 H1N1 IAV strain has escaped from human MxA restriction through three key residues in its NP (*85*). Taking advantage of these critical insights into which NP residues are essential for differences in phenotypes between H5N1 and H1N1 restriction, we could screen for novel MxA super-restrictors of H1N1.

We first tested for restrictive ability of MxA L4 variants against a minireplicon proxy for the A/Brevig Mission/1/1918 H1N1 strain of influenza. This proxy is a mutated version of the H5N1 minireplicon genome with the three key escape mutations of 1918 H1N1 NP introduced (*85*), allowing for all phenotypic differences between the two minireplicons to be attributed to these mutations. We found that loop L4 variants that could confer enhanced restriction against

H5N1 only led to very modest increases in H1N1 restriction. This might suggest that there was little sequence overlap between H5N1 super-restrictors and (potential) H1N1 super-restrictors. Alternatively, we considered the possibility that H1N1 restriction might have more stringent requirements than H5N1 restriction. This motivated us to ask how other species' Mx genes can restrict H1N1 IAV. Mx genes such as murine Mx1 can restrict H1N1 and are localized to the nucleus, unlike the cytoplasmic human MxA (*20*). Inspired by this natural restrictor of H1N1, we added a nuclear localization signal to MxA variants and found that this enabled H5N1 super-restrictors to also become H1N1 super-restrictors. Thus, super-restriction of the fully escaped 1918 H1N1 IAV is possible but requires a combination of mutational solutions and nuclear localization of the MxA protein. It remains to be determined how easily accessible these mutational trajectories are for human MxA and how such mutations would impact the breadth-specificity tradeoffs faced by MxA genes.

3.3 Mutations that confer escape from wtMxA also confer escape from MxA loop L4 variants

The 1918 H1N1 NP sequence differs from that of H5N1 by only fourteen amino acids, but only three of those amino acids (I100, P283, and Y313) are sufficient to confer escape from wtMxA when introduced into the H5N1 NP (*85*). We hypothesized that, because of the similarities between H5N1 NP and 1918 H1N1 NP, H5N1 super-restrictors might also provide improved restriction of H1N1 relative to wtMxA. To test this, we first reconfirmed that introducing the three escape mutations – R100I, L283P, and F313Y – recapitulates the 1918 H1N1 escape from restriction by human MxA using the minireplicon system (Figure 3-1). Indeed, we found this to be the case with this triple mutant of H5N1 NP (referred to as H5N1* or H5N1-IPY). There we saw only a 1.5 -fold increase in restriction of both H1N1 and H5N1* by

wtMxA, compared to a 4.3-fold restriction of H5N1. H5N1 super-restrictors restricted the H5N1 minireplicon to levels of 30-fold and greater, while restriction levels of H1N1 and H5N1* remained below 10-fold.

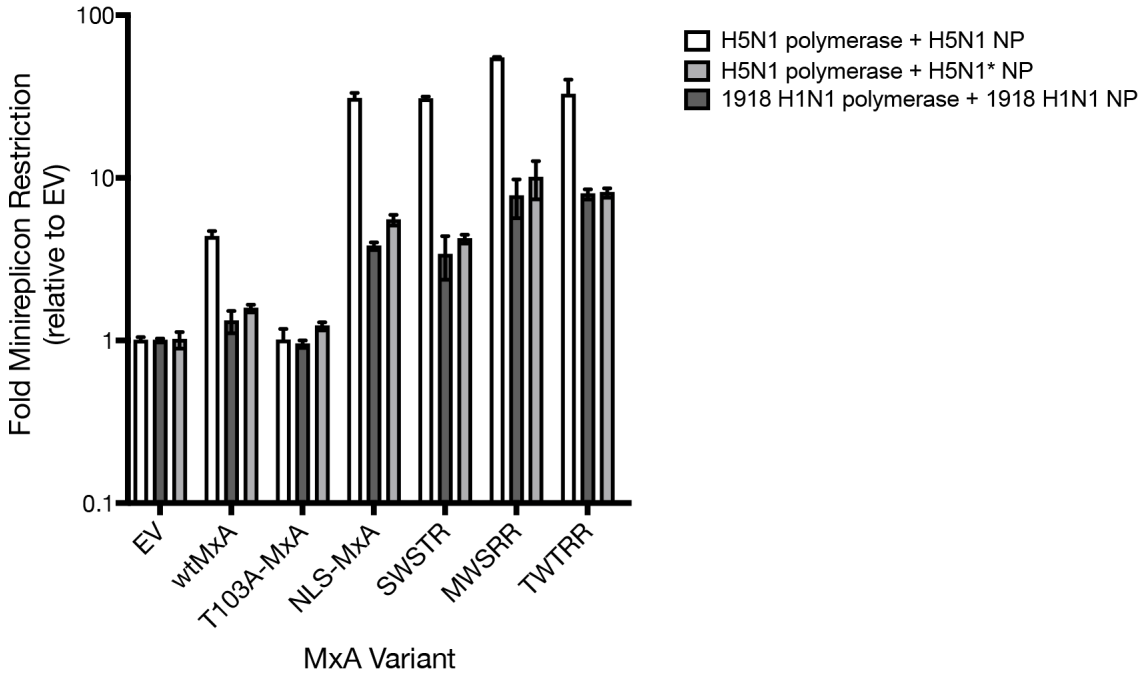

Figure 3-1. Introducing escape mutations into the H5N1 NP recapitulates the 1918 H1N1 escape phenotype from wtMxA and H5N1 super-restrictors. MxA variants were evaluated for H5N1 (white bars), H5N1* (light gray bars), and 1918 H1N1 (dark gray bars) restriction as a fold difference to the empty vector control using an independently performed minireplicon assay restriction in biological triplicates (on a log scale). Variants are identified based on their amino acid identities at the five variable sites in MxA L4, i.e., 540, 561, 564, 566, and 567.

We assayed three minireplicon systems to test for the role of these three mutations to confer an escape phenotype from MxA and MxA variant restriction. The H5N1 minireplicon, 1918 H1N1 minireplicon, and H5N1* in the H5N1 minireplicon background all showed no restriction in the presence of an empty vector or catalytically dead T103A-MxA (Figure 3-1). In the presence of wtMxA, only the H5N1 minireplicon was well-restricted, while 1918 H1N1 and H5N1* showed similar and low levels of restriction. Similarly, NLS-MxA and three representative H5N1 super-restrictors (Figure 2-1C) strongly restricted the H5N1 minireplicon.

However, they improved restriction of H5N1* and 1918 H1N1 minireplicons to similar levels that remained below 10-fold that of no MxA. Because the H5N1* NP in combination with H5N1 minireplicon components phenocopies the 1918 H1N1 minireplicon restriction profile, we infer that H5N1* serves as a reasonable proxy for MxA escape by 1918 H1N1 NP, allowing us to determine that any phenotypic differences between restriction of H5N1 and H5N1* could be attributed to the three amino acid changes introduced.

3.4 MxA loop L4 variants with increased H5N1 restriction confer only modest increases in H1N1 restriction

We next tested a panel of loop L4 variants (Figure 2-3B) for restriction of both H5N1 and H5N1* (Figure 3-2). We found that MxA super-restrictor variants with increased restriction of H5N1 only conferred weak restriction against H5N1*, with a maximum improvement of about 10-fold compared for H5N1* versus greater than 50-fold for H5N1 (all comparisons are relative to a no MxA, or empty vector, control). Meanwhile, wtMxA restricts H5N1 at a level of 6.85-fold compared to no MxA (Figure 2-1B), showing that super-restrictors of H5N1 can barely bring restriction levels of an escaped IAV NP variant to the modest levels of H5N1 restriction by wild-type MxA. This would suggest that the MxA variants tested here cannot confer robust restriction of H1N1, and therefore the MxA determinants for restriction of H5N1 are not the same as those for H1N1.

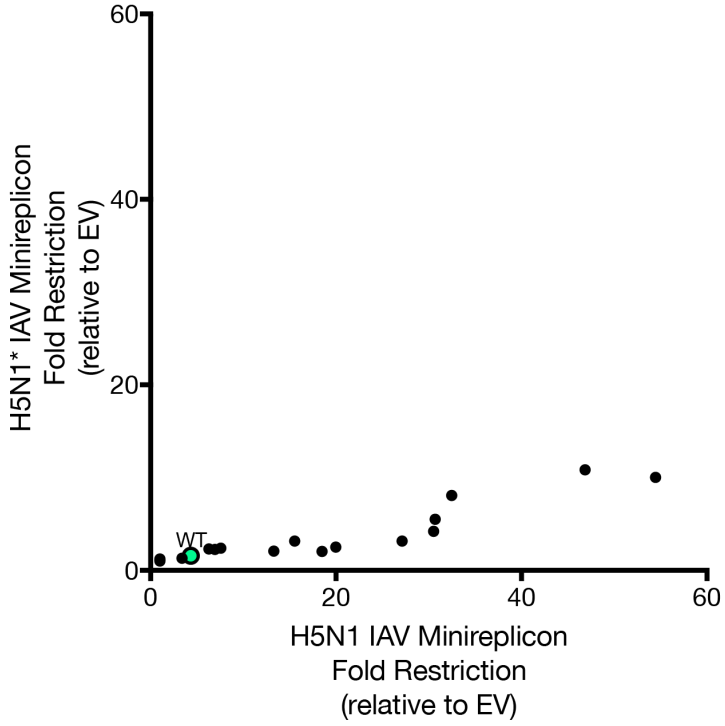

Figure 3-2. H5N1 super-restriction confers only modest improvements to H5N1* restriction. The panel of MxA variants from Figure 2-3B was screened for antiviral activity in H5N1 (x-axis) and H5N1* (y-axis) minireplicon assays. Fold-restriction of each minireplicon (relative to an empty vector control) is presented on a linear scale. Each point represents an average of three independent replicates. The mean wild-type restriction of H5N1 and H5N1* is indicated by the green dot.

3.5 Nuclear localization of MxA variants allows for H1N1 super-restriction

To gain further insight into H1N1 restriction, we examined other naturally occurring Mx restrictors of H1N1. In contrast to human MxA, murine Mx1 can robustly restrict H1N1. Human MxA is cytoplasmic whereas mouse Mx1 is nuclear. Moreover, previous studies have shown that the nuclear localization of mouse Mx1 is critical for its H1N1 restriction. Based on these findings, we hypothesized that re-localizing human MxA variants could bring them closer to their nuclear-localized viral targets and enhance restriction. We added an N-terminal SV40 T antigen nuclear localization signal (NLS) to five of the top H5N1 super-restrictors to test this hypothesis. When tested for restriction of H5N1*, we found that nuclear localization of variants

greatly enhanced restriction to super-restricting levels (Figure 3-3). The four most potent H5N1 super-restrictors were able to confer nearly 500-fold higher protection than wild-type MxA after nuclear localization. In contrast, the WWGEK super-restrictor, which had close to wild-type levels of H5N1 restriction, also remained at the same level as wild-type even after adding an NLS. These findings show that the H5N1 super-restrictors appear to correlate with the H1N1 super-restrictors, with the important difference being that H5N1 super-restrictors could still function in the cytoplasm, whereas the H1N1 super-restrictors cannot.

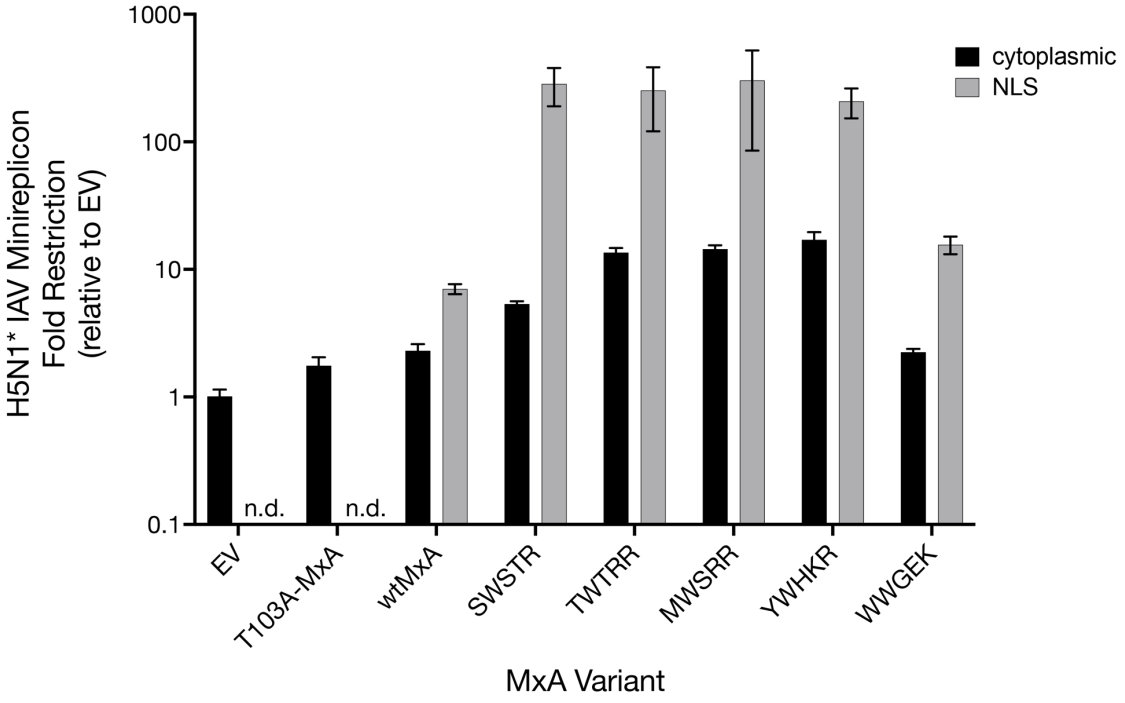

Figure 3-3. MxA variants with low levels of cytoplasmic restriction become super-restrictors upon nuclear localization. MxA variants localized to the cytoplasm (black bars) and with an N-terminal SV40 T antigen NLS tag (gray bars) were evaluated for H5N1* restriction as a fold difference to the empty vector control using an independently performed minireplicon assay restriction in biological triplicates (on a log scale). Variants are identified based on their amino acid identities at the five variable sites in MxA L4, i.e., 540, 561, 564, 566, and 567. n.d. – no data.

The vast improvements to H5N1* restriction seen from nuclear localization of MxA variants led us to hypothesize that combining two strategies – the addition of an NLS and combinatorial mutagenesis of rapidly evolving loop L4 residues – in an MxA variant library might allow for the identification of additional 1918 H1N1 IAV super-restricting MxA variants (Figure 3-3). To test this idea, we screened a preliminary library of 114 NLS-tagged MxA loop L4 variants (randomly mutagenized as in Chapter 2) for their ability to restrict the 1918 H1N1 minireplicon (Figure 3-4).

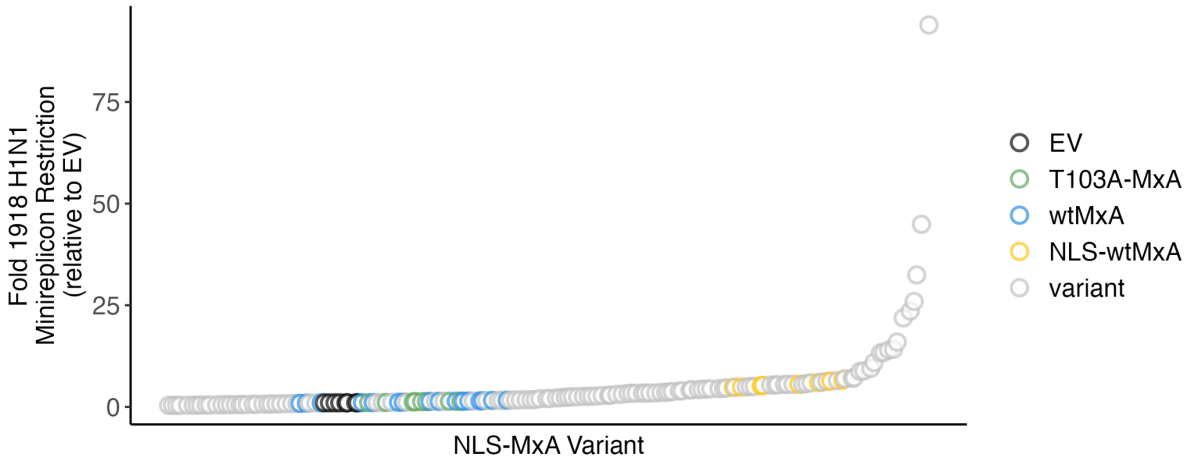

Figure 3-4. NLS-tagged MxA loop L4 variants can super-restrict 1918 H1N1 IAV. An NLS-tagged MxA variant library was constructed using combinatorial mutagenesis. Amino acid residues 540, 564, 566, and 567 from loop L4 of wildtype human MxA with an N-terminal SV40 large T antigen NLS were allowed to mutate to any amino acid residue using NNS mutagenesis, whereas residue 561 was varied to encode either F, W, or Y. Restriction profile of 114 unique NLS-MxA combinatorial L4 variants (gray circles) against the 1918 H1N1 minireplicon relative to empty vector control (on a log10 scale). For comparison, we also include multiple replicates of a catalytically inactive GTPase MxA variant, T103A (green circles), several independent replicates of wtMxA (blue circles), and NLS-tagged wtMxA (yellow cirlces) to define the wildtype range of 1918 H1N1 restriction. All analyses were performed in biological triplicates, and average restriction is represented.

We found several variants with increased H1N1 restriction, with the most potent among them being several orders of magnitude more restrictive than even NLS-wtMxA. These findings demonstrate that mutations in rapidly evolving L4 residues similarly contribute to the interaction between MxA and escaped IAV NPs such as H1N1 or H5N1*. However, these improvements are not sufficient to allow for H1N1 super-restriction because the MxA-NP protein-protein

interactions appear to be localization-specific. Nuclear localization of these MxA variants thus gives the restriction factor the ability to overcome these limitations and achieve super-restriction through a combination of increased NP avidity and correct localization.

3.6 Discussion

This work deepens our understanding of the evolutionary potential of the human antiviral restriction factor MxA to evolve gains in restriction of extant viruses which cause severe human disease. It was previously known that by exploring the evolutionary landscape of rapidly evolving residues in the loop L4, MxA super-restrictors could be identified against THOV, an *orthomyxovirus* well-restricted by human MxA, and against H5N1 IAV, an *orthomyxovirus* only modestly restricted by human MxA (*62, Chapter 2*). However, it was unclear whether MxA possessed the evolutionary potential to regain restriction of viruses such as H1N1 IAV that had evolved mutations to their NPs, allowing them to escape from MxA restriction (*85*). The data presented in this chapter show that MxA indeed possesses the evolutionary potential to regain the upper hand over H1N1 IAV. However, to do so may require more elaborate evolutionary steps than merely sampling amino acid changes in the rapidly evolving loop L4. Further, future work will be needed to reveal how changes to subcellular localization in combination with loop L4 mutations affect super-restriction of H5N1 IAV and THOV. This work lays the foundation to begin understanding the evolutionary paths by which human MxA can gain restriction of human-endemic and -pandemic IAV strains and whether such MxA super-restrictors will be able to overcome breadth-specificity challenges related to maintaining restriction of other viral targets.

A surprising finding is the extent to which the H5N1* NP has escaped from MxA restriction. Because cytoplasmic human MxA is not able to restrict 1918 H1N1 but nuclear mouse Mx1 is, it was previously predicted that the difference in restrictive ability was largely

due to differences in subcellular trafficking of H1N1 vRNPs. Past reports hypothesized this was due to different possibilities, including activating protein partners of MxA in different cellular compartments (*20*), small changes in MxA susceptibility having large impacts on NP efficiency in genome replication (*48*), or other strain-specific viral components contributing to MxA sensitivity (*53*). Another study showed that, even in two nuclear-localized mouse Mx1 proteins, the loop L4 sequence still contributed to antiviral specific (*78*). The data presented in this chapter confirms that changes to both loop L4 specificity and nuclear localization are required to gain H1N1 super-restriction by this forward evolution method. Simply localizing the non-restrictive cytoplasmic human wtMxA to the nucleus is insufficient to gain a large magnitude of increase to restriction of H1N1 (Figure 3-3). However, the potency of the escape phenotype conferred by just three mutations to H5N1 NP (Figure 3-1) still begs the question of how such minimal changes to the NP sequence contribute to a phenotype that requires entirely new subcellular localization of the MxA protein to overcome. It is possible – and remains to be tested – that these mutations to NP cause allosteric changes to the protein that impact interactions with other vRNP components, impact its subcellular trafficking, or somehow alter its accessibility to cytoplasmic host proteins.

It is intriguing that wtMxA is able to restrict the H5N1 and THOV minireplicons, and that NLS-wtMxA also greatly improves their restriction (*Chapter 2, 58*). This supports two important hypotheses regarding the unique escape phenotypes observed with regards to H1N1 restriction by MxA. First, it is possible that within the mutational landscape of loop L4 variants, exist versions that have greatly improved restriction of H1N1 IAV even in the cytoplasm. Just as identification of H5N1 super-restrictors required an altered mutagenesis approach relative to THOV super-restrictors, more data on the amino acid changes in loop L4 that improve H1N1

restriction may allow for a more fine-tuned, evidence-based variant library design to increase the proportion of H1N1 super-restrictors. Second, the mechanisms of vRNP trafficking of H1N1 may differ from H5N1 and THOV vastly, despite being relatively closely related to H5N1. This second hypothesis is further supported by the differing viral lifecycle stages that seem to be impacted by MxA or Mx1 for different *orthomyxoviruses* (*71*, *75*). Future experiments on the sequence-structure-function relationships for each of these viral NPs will help to distinguish how such small sequence changes so strongly impact MxA sensitivity.

While much work remains to understand how easily accessible the super-restriction of H1N1 IAV is by MxA, and how human-endemic and pandemic strains of IAV can robustly escape MxA restriction, this work nonetheless provides critical insights into the evolutionary paths by which MxA can regain restriction to a fully escaped virus. These data demonstrate that, while MxA exists in both winning and losing (and intermediate) positions in the molecular evolutionary arms race (Figure 1-1) with *orthomyxoviruses*, we can use forward evolution studies guided by historical evolution of MxA (*10*, *20*, *58*, *79*, *92*) to readily move MxA into and winning position for improved viral restriction.

3.7 Materials and Methods

Library Construction and Plasmid Preparation

A library of variants of 3X-FLAG-tagged human MxA with an N-terminal SV40 large T antigen nuclear localization signal (PKKKRKV) was designed, in which codons 540, 564, 566, and 567 were randomized by NNS mutagenesis, and codon 561 was randomly allowed to a W, F, or Y. This library was synthesized and cloned into a pQCXIP plasmid backbone. The pooled library of plasmids was transformed into NEB 5-alpha competent E. coli (NEB #C2987) and plated sparsely to obtain single colonies. Single colonies were inoculated into 6mL of 100 μg/mL

ampicillin LB broth, grown overnight at 37°C at 250rpm. After 16-18 hours of growth, 1mL of culture was added to 1mL of 50% glycerol for storage at -80°C. The remaining 5mL were used for plasmid purification (Promega #A1223). The C-terminus of MxA, including the loop L4 region, of the purified plasmids was sequenced by Sanger sequencing using the following primer: 5-' CGT GGT AGA GAG CTG C -3'. We cloned and sequenced ~115 randomly selected variants from the pooled plasmid library. Of those, 194 did not contain stop codons or frameshift mutations. All variants used for follow-up assays after the initial screen were sequenced again by whole-plasmid sequencing. Whole-plasmid sequencing was performed by Plasmidsaurus using Oxford Nanopore Technology with custom analysis and annotation.

Cell Lines

HEK-293T/17 were grown on treated tissue-culture plates in DMEM (Thermo Fisher #11965118) containing high-glucose and L-glutamine with 1x penicillin/streptomycin (Thermo Fisher #15140122) and 10% fetal bovine serum (Gibco #10437028). Cells were grown at 37°C, 5% CO_2 in humidified incubators, and passaged by digestion with 0.05% trypsin-EDTA (Thermo Fisher #25300120).

Minireplicon Assays

All minireplicon assays were performed in black, opaque, clear-bottomed 96-well plates by transfection of 50-80% confluent HEK293T/c-17 cells with minireplicon components using Mirus TransIT-293 reagent. For the H5N1 and H5N1* minireplicon system, 1 ng each of PB2, PB1, and PA, 0.5 ng of NP (all in a pCAGGS vector), 25 ng of pHH21-vNP-FF-Luc (firefly luciferase), 5 ng of pTK-Ren-Luc (Renilla luciferase), and 100 ng of pQCXIP-MxA were

transfected. For the 1918 H1N1 minireplicon system, 1 ng each of PB2, PB1, and PA, 0.5 ng of NP (all in a pCAGGS vector), 20 ng of pHH21-vNP-FF-Luc (firefly luciferase), 10 ng of pTK-Ren-Luc (Renilla luciferase, constitutively expressed under the HSV TK promotor to serve as a transfection control), and 100 ng of pQCXIP-MxA were transfected. After 24 hours, firefly and Renilla luciferase luminescence were measured using the Promega Dual-Glo Luciferase Assay System. For the 96-well format, all but 20 µL of the medium was removed. To each well, 20 µL of Dual-Glo Luciferase Reagent was added for lysis and luciferase activation, incubated for 10 minutes at room temperature, and then luminescence was read on a Biotek Cytation3 plate reader. 20 µL of the Stop and Glo Reagent was added, incubated at room temperature for 10 minutes, and luminescence was re-read. Normalized minireplicon activity for each sample was calculated as the value of firefly luciferase luminescence divided by the Renilla luciferase luminescence. Each assay plate contained empty vector, wtMxA, and catalytically inactive MxA(T103A) controls. Results are reported as "fold-restriction", calculated as the average minireplicon activity in the presence of empty pQCXIP vector for the paired assay plate divided by the minireplicon activity of the experimental sample. All samples were assayed by transfection of a master mix into triplicate wells.

Chapter 4 Perspectives and future directions

4.1 The evolutionary arms race between MxA and NP

Previous work has shown that MxA is one of the most versatile antiviral proteins in the mammalian innate immune repertoire. It can bind multiple, distinct viral targets to restrict an unusually broad range of viruses. As a result, MxA is also locked in a simultaneous arms race with many different viruses. How MxA maintains antiviral breadth even in the face of multiple viruses evolving to escape MxA restriction is a question of considerable biomedical and biological interest because of this protein's importance in restricting zoonotic threats. This thesis addresses this important question by exploring the evolutionary potential of human MxA through combinatorial mutagenesis of residues in its loop L4 that are evolving under positive selection.

Chapter 2 expands on previous findings that loop L4 is the protein domain necessary for interaction with its viral targets – *orthomyxovirus* NP proteins – and that the forward evolution of rapidly evolving L4 residues can reveal super-restrictors of the THOV (*10*, *54*, *77*, *92*). Although THOV is already well-restricted by wild-type human MxA, demonstrating that such a strategy could further enhance THOV restriction was an important validation of this approach. However, wild-type human MxA only modestly restricts H5N1 IAV. Moreover, previously identified THOV super-restrictors were not improved in restricting avian H5N1 IAV. Thus, it was not known whether human MxA possesses the ability to gain restriction against any strains of IAV, which are less well restricted by MxA and which cause more disease burden in humans than THOV. Here, by modifying the combinatorial mutagenesis strategy, I have shown that it is indeed possible to gain restriction against H5N1 through changes in rapidly evolving residues of human MxA loop L4. I also demonstrated that such enhanced super-restriction often requires just two or three mutations in critical L4 positions due to positive epistasis. Thus, it might be possible

to engineer and select MxA variants against several IAV strains of high public health interest. Indeed, I showed that such a combinatorial mutagenesis approach (combined with nuclear localization) could also elicit enhanced restriction of H1N1 strains of IAV, which have adapted to escape human MxA restriction.

Gaining super-restriction against one virus most often comes with tradeoffs to restriction to another. However, I have shown that this tradeoff is not insurmountable. I have found two evolutionary strategies that MxA proteins might use to overcome such breadth-specificity tradeoffs. First, I found that rare combinations of mutations in L4 allow for the 'generalist super-restriction' of two viruses simultaneously. Second, I discovered that heterozygous combinations of more commonly found 'specialist super-restrictors' could enhance the restriction of divergent viruses. These findings also suggest that supplementing wild-type human MxA with L4 variants is unlikely to interfere with endogenous antiviral activities, which is an important consideration for any future interventions. My work reveals insights about MxA evolutionary potential that might apply to several antiviral factors that evolve under positive selection and act as dimers or oligomers.

4.2 The breadth-specificity challenge of MxA evolution

The evolutionary history of positive selection in MxA and its ability to restriction many families of viruses shows that this protein has been able to gain and maintain restriction specificity to multiple viruses (*10*, *58*, *113*). However, the escape of human-endemic and pandemic-causing strains of influenza as well as other *orthomyxovirus* strains with zoonotic spillover potential from MxA restriction raises the concern that there are limits to MxA's ability to maintain breadth and specificity (*87*, *114–116*). It remains to be determined whether the

evolution of MxA will allow for the maintenance of potent antiviral activity with broad specificity for viruses. This has been seen with TRIMCyp and utilization of its unstructured binding domain (*117*) and TRIM5α, which is mutationally tolerant to most single amino acid changes to its protein sequence (*118*). It may also be the case that the evolutionary landscape of MxA is perilous, and few mutations in the course of adapting to new viruses can be tolerated to maintain antiviral functionality, as has been suggested by the deep mutational scanning of the MxA protein in the context of H3N2 infection (*57*).

In summary, this thesis work addresses how the evolution of broad antiviral restriction factor MxA can evolve to overcome present viral challenges while maintaining its functional breadth and specificity. Several pieces of evidence suggest MxA may be able to maintain breadth and specificity as it adapts to new viral threats. However, other experimental data can be used to test the hypothesis that the MxA mutational landscape will primarily only allow evolutionary paths toward specialized restriction of individual viruses or closely related viruses. Ultimately, only a continuation of experimentally testing the limits of MxA antiviral activity and combining experimental restriction data with structural modeling will allow the potentials and constraints to MxA evolution to be thoroughly defined. If successful, such engineered antiviral variant approaches could help jumpstart intervention strategies using engineered innate immune factors such as MxA, which, although extremely important to protect against zoonoses, evolve too slowly to be helpful in cases such as H1N1, after viruses have already successfully crossed the species barrier.

4.3 Evolution of human MxA towards seasonal influenzas

Evolutionary arms races between antiviral proteins and their viral targets are often simplified to two proteins placing selective pressures on each other. However, this is rarely the case. Viruses encounter tens to hundreds of antiviral proteins upon infection of a host, and hosts face the risk of infection by multiple viruses at any time. This is particularly relevant in the case of human MxA, as humans presently face the threat of infection by many diverged *orthomyxoviruses.* In addition to *orthomyxoviruses* that pose zoonotic spillover threats, such as avian IAVs (*91*, *100*, *114*, *115*, *119*), tick-borne thogotoviruses (*87*), and bat-derived influenza-like viruses (*50*, *89*), seasonal influenza viruses are a pertinent threat that could theoretically be overcome by MxA evolution (*53*, *100*). Thus far, studies evaluating susceptibility of both zoonotic *orthomyxoviruses* and endemic human IAVs show a correlation between ability to cause high rates of human disease and escape from human MxA restriction. For example, the H5N1 strain of highly pathogenic avian influenza (HPAI) circulating in dairy cows in the US is of great concern because of its ability to spill over into humans and cause severe disease (*120*). These HPAI strains have not been shown to spread from human to human (*121*), and have yet to show signatures of MxA escape in their NP sequences (Figure 4-1). However, they are now circulating widely among dairy cows (*120*, *122*). This raises concern for reassortment and adaptation in a mammalian host that may make them more adaptive to cause sustained spread and disease in humans. Therefore, it is important to continue to surveil zoonotic *orthomyxoviruses* for their MxA susceptibility and spillover potential. Loss of susceptibility to human MxA could indicate an increase in escape from immune mechanisms and therefore increased pathogenicity.

Figure 4-1. Alignment of NPs from 1918 H1N1, VN04 H5N1, and 2024 dairy cattle H5N1. Alignment of the NPs of the 1918 H1N1 strain (A/Brevig Mission/1/1918) and avian H5N1 (A/Viet Nam/1203/2004) used in minireplicons in Chapters 2 and 3 with recently isolated avian H5N1 found to be circulating among North American dairy cattle (A/cattle/Texas/24-009028-002/2024). Green bar (top) indicates percent identity for each amino acid site across the three sequences. Orange boxes indicate the three residues (I100, P283, and Y313) that confer MxA escape in 1918 H1N1, which are identical between VN04 H5N1 and Texas 2024 H5N1.

Strains of IAV that currently circulate among humans include derivatives of the 2009 pandemic H1N1 strain and an H3N2 strain. Although the ability to reassort afforded by the multi-segmented *orthomyxoviruses* genome makes phylogeny building complex, it appears that the NPs of the currently circulating H1N1 and H3N2 IAVs are directly descended from the 1918 pandemic H1N1 strain (Figure 4-2). The 1918 H1N1 influenza strain came from an avian source and underwent several reassortments with other avian-derived HA, NA, and PB1 segments (*123*). This eventually resulted in current endemic seasonal H3N2 influenza strains. In parallel, the 1918 H1N1 influenza also entered circulation in swine, which ultimately led to the reassortment of its HA, NP, M, and NS segments with other avian-derived segments, leading to the 2009 H1N1 pandemic strain (*123*).

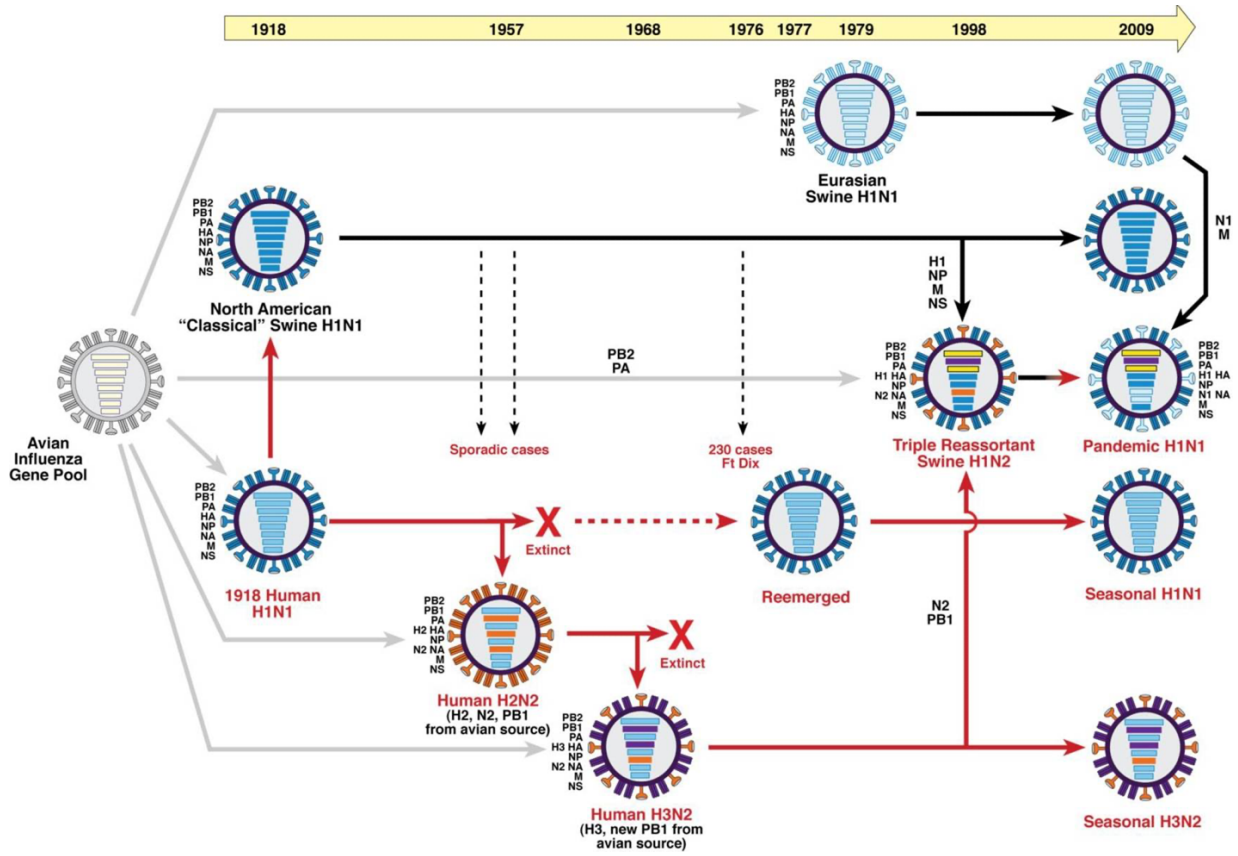

Figure 4-2 Genetic Relationships between Human and Relevant Swine Influenza Viruses, 1918–2009. Gray arrows reflect derivation of one or more gene segments from the avian influenza A virus gene pool (although the timing and mechanism of emergence in each case remains unknown). The dashed red arrow indicates a period without circulation of H1N1 in humans. Solid red arrows indicate the evolutionary paths of human influenza A virus lineages; solid black arrows, of swine influenza A virus lineages; and the black-to-red arrow, of the swine-origin 2009 human H1N1 pandemic influenza A virus. The dashed black descending black arrows reflect human zoonotic infections with swine influenza A viruses. Figure from: Taubenberger JK, Kash JC. Influenza virus evolution, host adaptation, and pandemic formation. *Cell Host Microbe*. 2010 Jun 25;7(6):440-51. doi: 10.1016/j.chom.2010.05.009. PMID: 20542248; PMCID: PMC2892379. CC License: 5913261098607.

While each genomic segment contributes to the host specificity and pathogenicity of these IAVs, it is notable that each of the human-endemic and pandemic strains possesses a closely related NP. The 1918 H1N1 NP is remarkable for its escape from human MxA restriction through only three amino acid mutations from the susceptible avian H5N1 NP (*116*). This makes it desirable to understand the consequences of NP evolution to escape MxA restriction and the potential for human MxA to evolve restrictive ability against these viruses that cause devastating

human disease. NP evolution can be studied by comparing sequences of NPs with differential susceptibility to human MxA and performing genetics and biochemical-based tests; the residues necessary for MxA sensitivity in each NP can be determined. This was what enabled the identification of the three escape residues in 1918 H1N1 NP and the ten escape residues in 2009 H1N1 NP (*116*). Similarly, the combinatorial mutagenesis techniques presented here and previously (*92*) provide a means to study MxA evolution in the face of these differing NPs. These studies indicate that MxA can evolve improved restriction of *orthomyxoviruses* through mutations in the loop L4 residues that have undergone positive selection in the past due to previous host-virus arms races. However, improvement through changes to loop L4 residues alone may constrain MxA's ability to maintain antiviral breadth. Therefore, additional evolutionary solutions may be required to maintain both the breadth and specificity of MxA antiviral activity.

The understanding of MxA evolutionary potential provided in this thesis work, as well as past work (*92*), can be employed to understand how the restriction of seasonal strains of IAV could be achieved. Chapter 3 explores the evolutionary potential to gain super-restriction against diverse IAV strains. I show that super-restriction of H5N1 IAV is correlated with small improvements to restriction of 1918 H1N1. However, these improvements are modest at best and would be unlikely to provide levels of antiviral protection seen with anti-THOV or anti-H5N1 activity from wild-type human MxA. A deeper exploration of the mutational landscape of rapidly evolving residues in MxA loop L4 might reveal rare combinations of mutations that confer super-restriction of H1N1 IAV. Another likelihood is that the escape mutations in H1N1 NP constrain MxA's ability to regain restriction through only mutations in the rapidly evolving residues of loop L4 and that nuclear localization of these variants is a requirement. There

remains much research to be done to fully understand the evolutionary landscape in which human MxA can adapt to combat extant viral threats.

Comparison of human MxA, which is incapable of restricting 1918 H1N1 IAV, to Mx genes that can restrict H1N1 IAV provides hints to evolutionary paths that may lead to H1N1 restriction. Murine Mx1 is a known restrictor of 1918 H1N1 IAV, and one of its key differences from human MxA is its nuclear localization (*20*, *22*, *71*, *106*). The nuclear localization signal of Mx1 is in the C-terminus of the protein and can be abolished by a single amino acid mutation at residue 614 (*22*). This led to the hypothesis that nuclear localization of human MxA may allow for H1N1 restriction. While nuclear localization of wild-type MxA did not improve H1N1 restriction, the nuclear localization of L4 variants with small improvements to H1N1 restriction led to substantial boosts in restriction. This suggests that nuclear localization allows for MxA to re-engage with the NP of H1N1 only if there is some small baseline interaction at the loop L4-NP interface. These findings could be validated and extended by confirming that improvements to H1N1 restriction found with loop L4 mutations do indeed correlate with increased interaction between MxA and NP through methods such as co-immunoprecipitation, yeast two-hybrid systems, proximity ligation, or biolayer interferometry (BLI) ((*52*, *124–126*). The magnitude of increased protein-protein interaction should reflect the small magnitude of restriction improvement relative to super-restriction of H5N1 IAV. If direct loop L4-NP interaction does not match restriction phenotypes, it is possible that mutations in these NPs impact the structural mechanism by which MxA engages them, and subcellular re-localization required to overcome this viral escape.

Beyond understanding how these different NPs affect the mechanism of MxA restriction, the evolutionary potential of MxA to achieve super-restriction can be explored beyond rapidly

evolving residues in loop L4. Rapidly evolving residues outside of L4 (Figure 1-5) could be assayed for super-restriction through combinatorial mutagenesis. However, this would require a significantly higher throughput to analyze the sheer number of combinatorial variants needed to sample the large sequence space; I discuss one such approach below. In parallel, mutations in conserved residues in MxA that improve H1N1 could be identified through deep mutational scanning of the MxA gene and assaying for H1N1 restriction, as was previously performed for H3N2 restriction (*90*). Finally, evolutionary analyses of Mx genes that have gained nuclear localization could be used to suggest what minimal mutations may be required to achieve this re-localization in human MxA.

In addition to H3N2 and H1N1 IAV, annual circulation of influenza strains also includes influenza B virus (IBV). Epidemiologic reports suggest that IBV causes about a quarter of the disease burden of IAV (*127*). While it appears to be less of a concern for human disease and pandemic potential, it remains understudied relative to IAV. It may have caused up to 80% of influenza infections in some seasons (*128*). No studies to date have tested whether MxA can directly restrict IBV infection or IBV vRNP activity.

To expand our understanding of how MxA can combat currently circulating strains of disease-causing influenza, it should be assessed whether human MxA can restrict IBV and whether MxA restriction of IBV strains correlates with disease burden in humans. Because of its close phylogenetic relationship to IAV and greater sequence identify of NPs between IAV and IBV than IAV and THOV, we might expect that IBV restriction by MxA could be studied using similar methods to IAV studies. It would be predicted that the NP of IBV, like THOV and IAV, is the target of MxA activity, making minireplicon systems a reasonable model for studying restriction. Viral infection studies in infection-relevant cell culture systems with wild-type

endogenous human MxA, knocked out MxA, and complemented exogenous MxA would also help determine the relevance to IBV infection beyond merely being upregulated because of IFN induction. IBV is distinguished from *orthomyxoviruses* such as IAV and THOV because its only known host is humans (*128*). Therefore, all NP adaptations resulting from the evolutionary pressure of MxA restriction would be human-specific. Studying how MxA can improve restriction to a human-specific virus could reveal insights into MxA's adaptive potential against a highly specialized virus.

4.4 Structurally defining the MxA loop L4 and NP interaction

Structural studies of the mammalian MxA protein thus far have included X-ray crystallography, electron microscopy, and fluorescence resonance energy transfer (FRET) (*59*, *60*, *65*, *77*, *129*). While it is difficult to discern the exact limitations to achieving a full-length structure of the MxA protein or a structure of MxA and NP in complex without specific reports of negative data, several features of these proteins likely create barriers to solving these structures. The first of these is the unstructured nature of loop L4 (*59*). Unstructured or intrinsically disordered proteins and protein domains have always challenged structural studies using classical techniques such as X-ray crystallography (*130–132*). This is a likely explanation for the inability to obtain crystal structures of MxA with loop L4. However, a structural study of the antiviral protein TRIMCyp, which uses an unstructured loop to bind multiple viral targets using different conformations, provides evidence that crystallization of MxA in complex with its viral target may be possible (*133*). This study determined crystal structures of TRIMCyp in complex with viral targets, performed nuclear magnetic resonance (NMR) spectroscopy to assess relaxation states of the unstructured loops, and engineered conformationally fixed TRIMCyp

mutants to confirm the phenotypes caused by conformational changes. Similar studies have not been shown with MxA and its NP targets. It could be reasonably hypothesized, because of its unstructured nature and broad antiviral activity against diverse *orthomyxoviruses*, that MxA loop L4 similarly adopts unique conformations upon engagement with different viral targets. Co-crystallization studies and molecular dynamics studies with techniques such as NMR are thus imperative to determine whether conformational flexibility is a key feature of MxA antiviral breadth.

Lipid tubulation, in which spherical liposomes conglomerated into tube structures, induced by full-length MxA and mediated by loop L4 has been imaged by negative stain electron microscopy, suggesting that electron microscopy could also be a promising technique for obtaining full-length structures of MxA (*77*). Further, cryogenic electron microscopy (cryoEM) was recently used to solve the structure of MxB oligomers and identify the critical interface for HIV-1 interaction (*134*). Beyond these instances of Mx-specific uses for electron microscopy, cryoEM shows promise as a technique for solving the structure of MxA and the MxA-NP interface because it uniquely maintains proteins' native conformations, unlike other structural biology techniques such as X-ray crystallography or BLI (*135*).

One of the likely challenges to solving structures of MxA in complex with NP is the high-order oligomerization status of both proteins. MxA can exist in several different oligomeric states (*60*, *61*). The uncertainty of the dominant oligomeric states that MxA occupies and the cellular conditions that contribute to determining preferred oligomerization status make optimizing protein purification conditions difficult (*61*, *129*, *136*). In addition to difficulties with the purification of oligomerized proteins, oligomerization status, especially if there is no single dominant oligomerization state, can make the computational analysis of oligomer structures

difficult (*136*). Thus, purification of the MxA protein should be attempted with monomeric versions of MxA, such as the M527D mutant (*61*). The dynamic states of the monomer structures could then be analyzed with cryoEM or NMR.

The oligomerization of the viral NP may pose an even greater problem than MxA oligomers. NP oligomers exist on the order of trimers to 9-mers, which then further oligomerize in their final vRNP structure (Figure 1-3) (*137*). Beyond its ambiguous and high-order oligomerization, NP also binds RNA strongly, a feature that can also increase the difficulty of protein purification due to non-specific binding mediated by the presence of RNA (*138*). This produces a conundrum in which stringent RNase treatment may be necessary to properly observe interactions between MxA and NP through either X-ray crystallography or electron microscopy. However, RNA might be a biologically relevant component of the interaction interface between MxA and NP, as was shown to be the case between the antiviral protein APOBEC3G and its viral binding partner, HIV-1 Vif (*7*).

To fully determine the structure of the MxA-NP interface, a series of cryoEM experiments could provide the conditions necessary to define this structure. Purifications could be performed in which monomers of each protein are stringently purified and analyzed in conditions in each other's presence or absence and in the presence or absence of RNA. This will aid in determining how single MxA and NP units interact and the interface involved in MxA restriction of *orthomyxovirus* vRNPs, in combination with previous data on MxA and NP oligomerization (*60*, *137*).

4.5 A higher-throughput approach to identify MxA super-restrictors

This work describes the basis of a system through which thousands of MxA variants can be screened for their ability to restrict many different viruses. As the number of MxA variants

and viruses screened increases, we can better understand the mutations that allow for generalizable super-restriction by MxA and how specific viruses may be refractory to super-restriction. However, the low throughput of the minireplicon assays used in this thesis limit the extent to which this can be achieved. A solution to this would be the introduction of a pool of MxA variants into a cell population. A pooled variant library of the restriction factor TRIM5α was recently used to assay for HIV-1 viral infection across thousands of cells, each encoding a single TRIM5α variant. This study thoroughly described the mutational landscape of TRIM5α as being permissive to mutations without breaking antiviral activity (*118*). Increasing the throughput of screening MxA variants against many viruses would allow for a deep mapping of the MxA mutational landscape and antiviral activity against its many viral targets.

To increase the throughput of screening MxA variants for super-restriction, a well-validated landing pad technology could be employed to create cell lines in which barcoded MxA variants and a barcoded GFP gene (instead of FF-luciferase), flanked by viral 5'- and 3'-UTRs, are stably integrated into the genome of HEK293T cells (*139–141*) (Figure 3-2). The landing pad strategy ensures that only a single MxA variant and reporter pair is expressed per cell. The remaining components of the minigenome system will be transiently transfected into these cells. The vRNP activity can then be measured by GFP gene expression via RNA-seq, with MxA variant-associated barcodes revealing which MxA variant is associated with high versus low GFP expression. This approach would lead to a highly quantitative readout of MxA super-restrictor activity. In theory, this pooled cell assay approach could be employed in any number of viral infection assays compatible with this cell type.

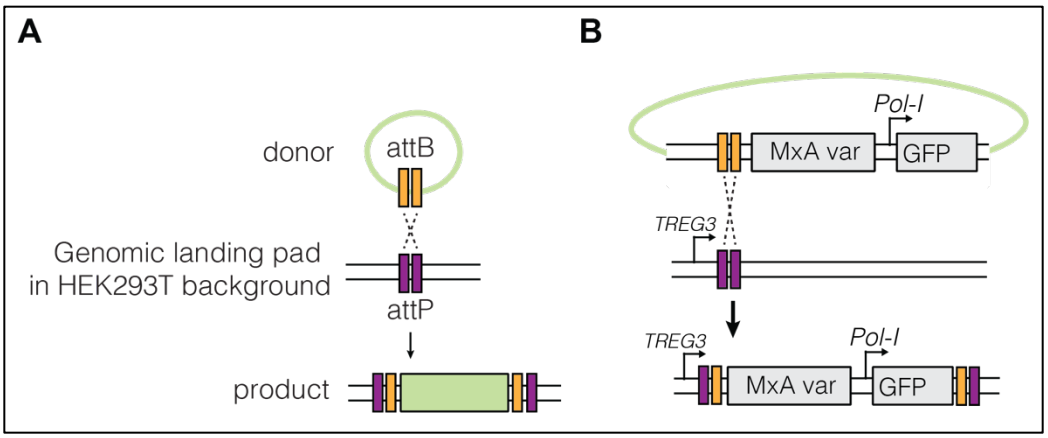

Figure 4-3 Landing pad strategy of introducing MxA library and reporter gene. A) The landing pad strategy involves introducing genes of interest via a plasmid with a Bxb1 attB recombination site. Transduction of this construct into HEK293T cells with a Bxb1 attP recombinase site downstream of a known promotor with Bxb1 recombinase leads to introduction of the gene in a known location of the genome. B) I will use this strategy to introduce constructs of MxA variant-GFP reporter pairs into a previously described landing pad site under a TREG3 promoter.

In addition to increasing the throughput of these MxA variant screens, the relevance of these variants must also be tested in the context of whole viral infections. Viral infection assays with constitutively expressed MxA have been well-established (*86*, *99*). Because of the time constraints involved in developing stable cell lines as well as the biosafety concerns of testing IAV strains such as HPAI H5N1, viral infection assays in stable cell lines would best be used for follow-up studies on interesting MxA variants rather than screening purposes. As promising super-restrictors and generalist super-restrictors are identified through high throughput screening methods and validated in viral infection assays, the most promising variants can be tested in transgenic mice (*18*). Developing transgenic mouse lines with MxA variants would be far costlier and time-consuming than using minireplicon assays or viral infection assays in cell culture but such work could show great promise for the biological relevance of MxA super-restrictors in *orthomyxovirus* infection. New technologies to introduce specific MxA variants via messenger RNAs of protein therapeutics delivered by lipid nanoparticles could greatly accelerate this process and reduce both costs and time associated with such screening (*142*).

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