TRIM2 is Essential for the Proper Neurological Functioning and it is Antiviral Against Arenaviruses

By

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THESIS

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I dedicate this study to everyone who believed in me. To each person who stuck with me during the hardships, the setbacks, disadvantages and throughout it all, never lost hope in my vision for the person I know I can become, I say thank you. I dedicate this to those who have nurtured an open mind and have been consistent in their words and actions.

I thank my family who always worked diligently to provide me with the best they could and who raised me to be a respectful, hardworking, intelligent and faithful person regardless of any setbacks we encountered together along the way.

I thank those who seek the truth and see science as a means to that evolutionary end instead of the end result. I thank those who have dared to try and are willing to be wrong for the sake of finding future answers.

Contribution of Authors Statement

Guliz Otkiran Clare, Dr. Nicolas Sarute, and Dr. Susan Ross conceptualized the work presented in this thesis, developed the methodologies used, and acquired funding and resources used to complete the work. Guliz Otkiran Clare, Dr. Nicolas Sarute, and Dr. Susan Ross contributed to writing, reviewing, and the editing of data from this thesis that has been published. Dr. Susan Ross supervised the work presented in this thesis and is responsible for project administration. Under the Materials and Methods and the Results chapters, a part of previously published work, by the authors listed above, was presented. Figures were cited properly with attribution to the individuals who performed curation and analyzation of the data wherever applicable. In the results sections where data was presented without a citation, the curation and analyses were completed by the writer of this thesis, Guliz Otkiran Clare.

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α2δ2 subunit, a polyprotein which is cleaved to make a single subunit with a glycosylated α2 domain disulfide-bonded to the δ2 membrane domain, a cytosolic β signaling subunit and γ, another multi-membrane spanning protein

ACE-2, angiotensin converting enzyme 2

AHF, Argentinian hemorrhagic fever

BMDM, bone marrow derived macrophages

BSL, biosafety level

CACNA2D2, voltage-dependent calcium channel subunit α2δ2

CLDN-, claudin-

CMT, Charcot-Marie-Tooth

CNS, central nervous system

CTD, C-terminal domain

ECL, extracellular loop

GM-CSF, granulocyte-macrophage colony-stimulating factor

GP, glycoprotein

GP1, glycoprotein 1

GP2, glycoprotein 2

GPC, glycoprotein precursor

HCV, hepatitis C virus

HET, heterozygous

HCMV, human cytomegalovirus

HIV-1, human immunodeficiency virus type 1

IFN, interferon

IL, interleukin

JUNV C1, attenuated vaccine strain of Junín virus, Candid-1, Candid#1

JUNV, Junín virus

KO, knock-out

LCMV, lymphocytic choriomeningitis virus

MACV, Machupo virus

MERS, Middle East respiratory syndrome

MLV, murine leukemiavirus

MYO5A, myosin VA

NF-L, neurofilament light-chain

NWA, New World arenavirus

OCLN, occludin

OWA, Old World arenavirus

PICALM, phosphatidylinositol binding clathrin assembly protein

PC, proprotein convertase

RT-qPCR, reverse transcription quantitative real-time PCR

S1P, site-1 protease

SARS-CoV, severe acute respiratory syndrome coronavirus

SARS-CoV-2, severe acute respiratory syndrome coronavirus 2

SKI-1, subtilisin kexin isozyme-1

SSU72, SSU72 homolog, RNA polymerase II CTD phosphatase

STAT, signal transducer and activator of transcription

TCRV, Tacaribe virus

TNF, tumor necrosis factor

TFIIB, transcription factor II B

TJ, tight junction

TLR, toll-like receptor

TMPRSS2, transmembrane protease, serine 2

TNF, tumor necrosis factor

TRIM2, tripartite motif containing 2

VGCC, voltage gated calcium channel

VHF, viral hemorrhagic fever

VSV, vesicular stomatitis virus

WT, wild type

SUMMARY

Arenaviruses are single-stranded RNA viruses that can infect and cause disease in humans. It is important to study arenavirus biology as hemorrhagic fever-causing arenavirus infection is associated with a high mortality rate and can lead to neurological complications in infected humans. A siRNA screen previously performed in our lab identified novel antiviral host factors that may play a role in viral entry. Importantly, one factor identified in the RNAi screen, TRIM2 (Tripartite Motif Containing 2), was shown to be antiviral against New World Arenaviruses (NWAs) when tested with murine leukemia virus (MLV) pseudotyped with glycoproteins from NWAs and with the replication-competent Junín virus vaccine strain Candid 1 (JUNV C1). *In vitro* and *in vivo* studies carried out in our lab using *Trim2*-knockout mice showed that TRIM2, at minimum, requires its Filamin domain for its antiviral activity.

Trim2-knockout mice carried an ataxic phenotype and the underlying neurological conditions were investigated in this mouse model which can be used to study Charcot-Marie-Tooth (CMT) disease (a degenerative nerve disease that may be related to TRIM2).

TRIM2 was investigated by using several different strains of arenaviruses. It was found that it plays a role in restricting NWAs but does not affect the Old World Arenaviruses (OWAs). The results found by using replication-competent viruses overlapped with the results found by using the pseudotyped viruses. The other two factors found in the screen were CLDN2 (Claudin-2) and SSU72 (SSU72 Homolog, RNA Polymerase II CTD Phosphatase). In this study, CLDN2 and SSU72 were further analyzed for their effects on NWA and OWA infections. They have the potential for being broad antiviral factors. The findings in this study might shed light upon a broader discovery for future studies with a greater range of emerging viruses.

I. INTRODUCTION

1.1 Arenaviruses

Arenaviruses are enveloped, negative-sense single-stranded RNA viruses. The genus of mammal-infecting viruses, known as *Mammarenaviruses*, are capable of infecting rodents and in some instances humans. Mammarenaviruses are divided into two different clades: New World Arenaviruses (NWAs) and Old World Arenaviruses (OWAs). Within these clades, the viruses are usually associated with a particular rodent species. These viruses have the potential to zoonose into humans. NWAs Junín, Machupo, Guanarito, Sabia and Chapare and the OWAs Lassa, Lujo, and lymphocytic choriomeningitis virus (LCMV) are the viruses that can cause various illnesses including hemorrhagic fever as well as other pathologies, such as neurological disease, in humans. These viruses are transmitted from rodents to humans via aerosolization (Hallam et al., 2018).

1.1.1 Taxonomy, Epidemiology, Structure, Replication and Cellular Entry

The family *Arenaviridae* is comprised of three genera: *Hartmanivirus*, *Reptarenavirus* and *Mammarenavirus*. The genus *Mammarenavirus* is subdivided into the New World Arenaviruses (NWAs) and Old World Arenaviruses (OWAs) (Hallam et al., 2018; Radoshitzky et al., 2015). The genus *Mammarenavirus* encompasses a wide range of viral species found in a variety of animal hosts. Arenaviruses are asymptomatic within the host -mostly rodents- and research suggests that there is a coevolution between the host and the virus (Gonzalez et al., 2007). Some species of *Mammarenaviruses* are capable of infecting humans and are transmitted to humans from rodents via aerosolized excreta. These hemorrhagic fever-causing species of arenaviruses require biosafety level 4 (BSL-4) for handling and are identified as bioterrorism threats due to their potential for airborne transmission (Iwasaki & de la Torre, 2018).

NWAs and OWAs which are capable of infecting humans, demonstrate a great diversity in their geographical areas of emergence. OWA-related hemorrhagic fever incidents are mainly present in Africa while NWAs are endemic to the Americas. Despite these geographic locales, OWA and NWA species have been observed to be present in the United States with the LCMV strain of OWA being the primary instigator of human infection within US borders (Milazzo et al., 2011).

Arenaviruses' typical electron microscopy shows grainy ribosomal particles giving the virion a sandy ("*arena*" in Latin) look which is how their family obtained their name (Hallam et al., 2018).

The genome of arenaviruses is composed of two RNA segments; the large (L) segment encodes for the RNA polymerase (L) and Z protein, while the short (S) one encodes for the viral glycoprotein precursor (GPC) and nucleoprotein (NP) (Figure 1) (Rojek et al., 2008). Replication of the viral genome for *Mammarenaviruses* starts with the transcription of NP and L from the (S) and (L) segments (Rojek et al., 2008). Following this, the viral polymerase makes the antigenome of the small and large segments which then translates the GPC and Z protein respectively (Rojek et al., 2008). The GPC is then cleaved by the cellular proprotein convertase (PC) subtilisin kexin isozyme-1 (SKI-1)/site-1 protease (S1P) for the generation of GP1 and GP2 subunits of the viral glycoprotein (Rojek et al., 2008). Prior to spreading to another cell, virus progeny egress by budding from the plasma membrane (Hallam et al., 2018). The strategy these viruses use to infect cells and the cellular immune responses and restriction strategies the host uses to block infection warrant additional investigation as it may assist in preventing post-infection complications.

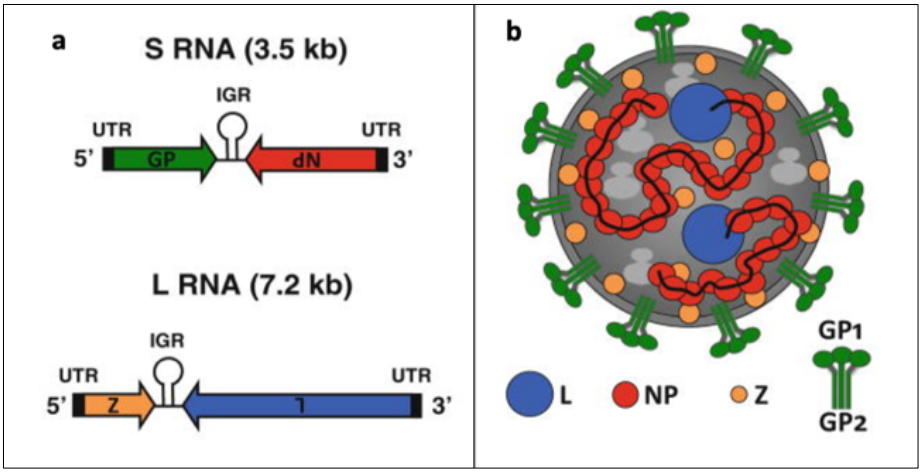

Figure 1: The structure of arenaviruses (Reprinted from Rojek et al., 2008)

(a) Arenaviral genome organization, (b) virion structure

There is a divergence in the receptors used by NWAs and OWAs (Vela, 2012). α-dystroglycan is the cellular receptor for OWAs while transferrin receptor 1 (TfR1) is the main cellular receptor for NWAs (Abraham et al., 2009; Hickerson, Westover, et al., 2020; Radoshitzky et al., 2007). Other than NWAs, mouse mammary tumor virus (MMTV) is a *Betaretrovirus* that uses TfR1 as an entry receptor (Ross et al., 2002). It has been suggested by our lab that voltage-gated calcium channels (VGCCs) are also used by NWAs and CACNA1S haploinsufficiency conferred resistance

to NWA infection (Lavanya et al., 2013; Nicolás Sarute & Ross, 2020). Mice given Gabapentin – a drug specific to the α2δ2 subunit of VGCC CACNA2D2- were infected by JUNV C1 with a smaller likelihood than untreated mice (Lavanya et al., 2013). This study showed that, VGCC is important in NWA Junin virus entry, evident by the decreased infection when these channels were blocked by gabapentin (Lavanya et al., 2013). Besides the difference in receptor preference, NWAs and OWAs were suggested to use different strategies during internalization (Martinez et al., 2007). The viral fusion of arenaviruses, upon internalization was described to be pH-dependent for both NWAs and OWAs while the delivery of the arenaviral content into the host cells requires acidic endosomes for a productive arenavirus infection and replication (Martinez et al., 2007, 2009). Following entry, the fusion of the virus from the late acidic endosome delivers the genome of the virus into the cytoplasm of the host cell (Martinez et al., 2007, 2009). There are other viruses where a low pH is necessary for the viral fusion such as for certain retroviruses and coronaviruses (White & Whittaker, 2016). Most influenzas are dependent on low pH (~pH 5) to fuse and they fuse from late endosomes, whereas viruses depend on relatively higher pH (~pH 6) for fusion such as vesicular stomatitis virus (VSV) generally fuse from early endosomes (White & Whittaker, 2016).

1.1.2 Viral Targets, Immune Response and Treatment

When transmitted to humans by means of inhaling the aerosolized infected sample or transmission by infected blood, *Mammarenaviruses* are known to cause fever, malaise, fatigue, confusion, convulsion, coma, seizure, encephalitis and edema which can lead to mucosal bleeding, hemorrhaging/petechia or in some cases, to death (Forni et al., 2018; Giusti et al., 2011; Gonzalez et al., 2007; Lascano et al., 1992; McKee et al., 1988). The initial targets of these viruses are thought to be the sentinel cells of the immune system (Geisbert & Jahrling, 2004). Alveolar macrophages and dendritic cells are hypothesized to be cell types that get infected first as a result of arenavirus exposure (Aronson et al., 1994; Fedeli et al., 2018). Infected macrophages can spread the arenavirus into various cells such as hepatocytes, vascular endothelial cells, and the epithelia of the kidney (Fedeli et al., 2018). The levels of interferon (IFN) are higher in some of the infected cell types (Huang et al., 2012; Levis et al., 1984). Although wild type (WT) laboratory mice do not get sick by arenaviral infections, the depletion of critical innate immune genes, such as IFN receptors or Signal transducer and activator of transcription 1 (STAT1) can result in infection

without the symptoms observed in humans (Hickerson, Sefing, et al., 2020; Koma et al., 2013). So, these mice and the cells obtained from them may be used as models to study and characterize arenavirus infection. Tacaribe virus (TCRV) is a nonpathogenic member of NWAs which cannot cause disease in animals due to its inability antagonize the host interferon (IFN) response (Gowen et al., 2010; Hickerson, Sefing, et al., 2020). Newborn mice or IFN knockout models, must be used to study TCRV. Other species that are used to study arenavirus pathogenesis include Syrian golden hamsters, non-human primates and outbred guinea pigs (Carballal et al., 1983; Hickerson, Westover, et al., 2020; Molinas et al., 1983; Xiao et al., 2001).

Studies done with rhesus macaque models and infected patient serum showed that the samples had elevated levels of cytokines IL-6, IL-8 and IL-10 and tumor necrosis factor (TNF)-α after infection (Koma et al., 2013). Similarly, proinflammatory cytokine levels, such as IL-6, TNF-α and IFN-γ, were found to be heightened in MACV (Machupo virus, a NWA)-infected STAT-1 KO mice (Hickerson, Westover, et al., 2020; Koma et al., 2013). Studies done using mouse macrophages showed that JUNV glycoproteins (GPs) are capable of elevating IFN-β and TNF-α levels (Cuevas & Ross, 2011). These viruses are recognized by toll-like receptor 2 (TLR2)-, but not TLR4 (Cuevas & Ross, 2014). It was observed that CD8+ T cells played a critical role in NWA clearance via TLR2-mediated immune response (Cuevas & Ross, 2014).

The treatments for arenavirus infection are limited. As preventative care, the elimination of rodents can be effective in fighting against these viruses, as was the case in Bolivia against MACV (Kuns, 1965). However, this strategy is impractical in some instances. The virus is capable of spreading through the air, so the development of therapeutic strategies is essential to cure the Viral Hemorrhagic Fever (VHF) cases that arise from NWA infection. While palliative care is provided for all VHFs, there are a small number of specific therapies available for only a few viruses (Vela, 2012). Ribavirin (a nucleoside analog antiviral medication) was used in the past as a treatment against diseases caused by arenaviruses, Lassa fever and, Argentine and Bolivian hemorrhagic fevers (Enria & Maiztegui, 1994; Kilgore et al., 1997; McCormick et al., 1986). Using ribavirin as part of the treatment, there is a chance of a decrease in the mortality rate if given at early onset of the disease (Gowen et al., 2008). However, this drug raises concerns regarding lack of specificity and toxicity with potential to cause severe side effects such as anemia and common

complications such as nausea, muscle pain and mood changes (Gowen et al., 2008; McCormick et al., 1986; McKee et al., 1988). There are not any licensed vaccines currently available for VHF agents except for Junín virus. The live attenuated vaccine derived from Argentinian hemorrhagic fever (AHF) causing JUNV called Candid-1, also known as JUNV C1, is effective, yet only licensed in Argentina and not in other countries (Martínez-Sobrido et al., 2016). The plasma from recovered patients was highly effective when used in people infected by JUNV (Maiztegui et al., 1979). Early treatment with serum can bring the mortality rate from about 30% to 1% (Enria et al., 2008) Yet, it was seen that the therapeutic treatment with convalescent plasma of patients recovered from AHF yielded significant late-onset neurological complications of unknown etiology (Geisbert & Jahrling, 2004; Maiztegui et al., 1979). Similar concerns have recently been raised with COVID-19 treatment, as a number of potential therapeutics such as convalescent serum are being explored with for infected patients (Jarrahi et al., 2020). It is suspected that COVID-19 therapies may cause adverse neurological effects on top the neurological manifestations of the virus itself (Bridwell et al., 2020). In some instances, neurological complaints reported by SARS-CoV-2 infected patients, such as seizures, were attributed to adverse effects from the use of antiviral medication such as ribavirin (Niazkar et al., 2020). Further research and a better understanding of viral infection is critical and can enable the development of effective and safe therapeutics as well as new vaccine development strategies.

2.1 Host Factors screened for the assessment of Viral Entry

A high-throughput RNAi screen done by our lab resulted in the identification of new antiviral host factors involved in virus entry. CLDN2 was found to be antiviral against murine leukemia virus (MLV) pseudotyped with glycoproteins from NWAs (Junín), retrovirus mouse mammary tumor virus (MMTV), and the rhabdovirus vesicular stomatitis virus (VSV). Another factor identified in the RNAi screen SSU72 was antiviral against pseudoviruses bearing glycoproteins from Junín and MMTV but was not antiviral against VSV pseudovirus. TRIM2 was antiviral only against NWA Junin virus (Figure 2). (Lavanya et al., 2013)

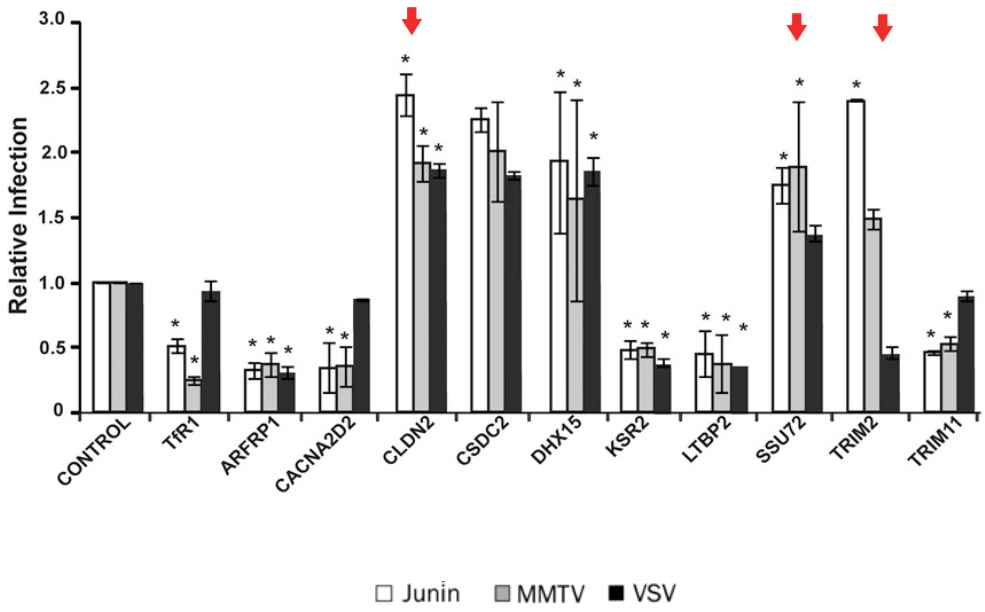

Figure 2: Gene knockdown effects on pseudovirus infection (Reprinted from Lavanya et al., 2013)

Infection levels were measured by luciferase assays; of U2OS (human cell line) stably expressing mouse TfR1 transfected with indicated siRNAs(x-axis) and infected with MLV pseudo-typed with the Junín (white bars), MMTV (gray bars), or VSV (black bars) GPs (48 hpi) (Lavanya et al., 2013) The factors investigated in this thesis were identified by red arrows.

2.1.1 CLDN2 (Claudin-2)

Claudins are transmembrane proteins that provide intercellular barrier function and membrane integrity and also play a role in paracellular ion passage. Tight junctions (TJ) on the other hand are cell-cell contacts in polarized cell tissues that appear to be fused as two cells when viewed under a microscope. TJ strands contain different proteins such as occludin (OCLN) and some claudins. The molecular mass of claudin proteins (mammals have ~ 24) is between 20 to 27 kDa. The proteins of the claudin family and occludin share the same topology as they feature tetraspan transmembrane domains with two extracellular loops (ECL-1 and ECL-2) (Krause et al., 2008).

The distribution and pore functions of claudins on the cell membrane are greatly dynamic. Although they are quite conserved, different claudin proteins have different strand formations and they were identified in different functions. In the studies done by Furuse et al., it was revealed that claudin-1 (CLDN1) showed continuous strands while claudin-2 had discontinuous strands (Furuse et al., 1998). Moreover, ECL-1 of CLDN-2 is non-conserved and it has been suggested to be important for cation pore formations at TJ which leads to a decrease in transepithelial resistance

(Amasheh et al., 2002). Evidence suggest that Claudin-1 (CLDN1) serves as a co-receptor in Hepatitis C virus (HCV) infection and is required for its late step entry (Evans et al., 2007). The internalization of HCV occurs via co-endocytosis of virus and its receptor (Farquhar et al., 2012). The extracellular loop of CLDN1 ECL-1 is where HCV binds to CLDN1 (Evans et al., 2007; Farquhar et al., 2012; Harris et al., 2010). Studies done using animal cells, showed that OCLN is critical in mediating HCV entry as well (Ploss et al., 2009; Tong et al., 2011). On the other hand, tight junction proteins CLDN1 and OCLN not only control HCV entry but they also get downregulated and hinder superinfection (Liu et al., 2009). Studies performed by using humanized mice suggested that CLDN1 monoclonal antibodies may be used in an effort to control HCV infection (Mailly et al., 2015). CLDN6 and CLDN9 are coreceptors for HCV in endothelial cells while CLDN1 is more efficient in hepatoma cells (Zheng et al., 2007).

The functions determined for Claudin-2 (CLDN2) include that it enhances paracellular permeability (Amasheh et al., 2002). Many studies observed that CLDN2 enhances the permeability of the cell and it has a specific selectivity on the conductance of cations such as Na+ as well as paracellular water transport (Furuse et al., 2001; Hou et al., 2006; Krug et al., 2012). CLDN2 is also a key regulator in calcium excretion, so it was proposed as a potential target in mitigating kidney stones (Curry et al., 2020). Other studies propose CLDN2 has a role in the absorption of vitamin D in a Ca^{2+} dependent manner and found that CLDN2 gets downregulated by vitamin-D itself upon STAT-6 phosphorylation (Domazetovic et al., 2020; Fujita et al., 2008).

Additionally, it was found that CLDN2 gets upregulated in response to the inflammatory cytokines under some pathological conditions such as active Crohn's disease and necrotizing enterocolitis; the upregulation plays a role in increasing epithelial permeability due to a disruption in TJ barriers (Ares et al., 2019; Zeissig et al., 2007). Studies report that CLDN2 transcription is induced by IL-6, IL-13 and TNF - via phosphatidylinositol-3-kinase signaling-, (Al-Sadi et al., 2014; Mankertz et al., 2009; Weber et al., 2010). It was also observed that CLDN2 expression was higher in HIV-1 patients, which is thought to be due to increased proinflammatory cytokines (Epple et al., 2009). The increase in TNFα and IL-6 observed in HIV-1 infected cells further supports the notion that the mucosal barrier reduction in these patients was associated with the TJ protein expression (Epple et al., 2009; Nazli et al., 2010). The upregulation of CLDN2 expression can be promoted by

Symplekin (SYMPK) (a protein is known to be directly interacting with SSU72)(Buchert et al., 2010).

The expression of CLDN2 in humans is primarily found in the liver, gallbladder, stomach, duodenum, small intestine, seminal vesicle and kidney, mostly at the tight junction of leaky epithelial cells of renal tubules (Kiuchi-Saishin et al., 2002). CLDN2 deficient mice are viable yet are defective in the renal proximal tubules for their leaky and cation-selective paracellular permeability. The administration of 2% NaCl may help these mice to compensate the loss of ions, due to disruptions in their tight junction. Thus, they provide a good model for investigations involving CLDN2. (Muto et al., 2010)

2.1.2 SSU72 (SSU72 Homolog, RNA Polymerase II CTD Phosphatase)

SSU72 is a phosphatase which originally was identified in a screen performed for finding the suppressors of *sua7-1* - a cold-sensitive mutant of TFIIB in yeast - and acquired its name as suppressor of *sua7-1* clone 2 (Sun & Hampsey, 1996). Mammals carry a single SSU72 gene and the protein is localized partly in the nucleus and mostly in the cytoplasm (St-Pierre et al., 2005). Studies done in COS-7 cells, an African green monkey kidney fibroblast-like cell line, suggests that suppression of its expression by using siRNAs does not reduce survival nor increase proliferation (St-Pierre et al., 2005).

Biochemical studies show that SSU72 interacts with TFIIB, yet this interaction was not found to inhibit the phosphatase activity of human SSU72 (St-Pierre et al., 2005). It is implied that SSU72 can play a role in RNA processing and it can recruit factors that are important for transcription termination and cleavage of pre-mRNA (Krishnamurthy et al., 2004; Steinmetz & Brow, 2003). Another study using *in vitro* transcription assays with HeLa nuclear extracts, suggests that SSU72 acts in the early elongation stage or can affect transcription prior to initiation and its activity is dependent on the nascent RNA (Spector et al., 2019).

It has been suggested that SSU72 has a role in regulating the immunometabolism. One type of various post-translational modifications in eukaryotic cellular signaling pathways is reversible protein phosphorylation. Studies done using a conditional knock-out mice specific to adipose tissue indicates that SSU72 has a role in thermoregulation as well and its depletion results in a decrease in macrophage populations (S.-Y. Lee et al., 2018). Another function described for

this protein is the fine-tuning of TCR signaling (ko et al., 2020). SSU72 has been identified to have a role in suppressing STAT-3 activation, the suppression might cause inflammatory responses as a result. The same study showed that the overexpression of SSU72 had a therapeutic effect on the arthritic mouse model indicating a potential for the development of treatment against autoimmune arthritis (S. H. Lee et al., 2017). Additionally, a patent application that was created for treating autoimmune disease discloses a composition in which SSU72 is an active ingredient (Cho et al., 2018). Another signaling pathway regulated by SSU72 is GM-CSF receptor signaling (Woo et al., 2020). The research done by using conditional SSU72 KO used as allergic asthma mouse models presents a role for SSU72 in regulating allergic airway inflammation and alveolar macrophage development (Woo et al., 2020). Amongst the findings, a decrease of IL-4 and IL-5 production in mature alveolar macrophages in KO mice was noted (Woo et al., 2020). Another study adds one more to list of many functions of this protein. Based on this study, the reduction of the SSU72 in the liver leads to liver problems in KO mice (Kim et al., 2016). SSU72 is a factor that can also preserve the chromosome homeostasis (Kim et al., 2016). A gene specific role described for SSU72 is that Tat protein of HIV-1 alters the SSU72 function in the infected cell to boost the viral transcription (Chen et al., 2014). Based on the findings, Tat can exploit SSU72 machinery to promote viral gene expression (Chen et al., 2014).

2.1.3 TRIM2 (Tripartite Motif-containing Protein 2)

The TRIM family proteins are known ubiquitin ligases, yet the specific function of a majority of the proteins of this family are unknown (Williams et al., 2019). This protein family has been shown to function in some hereditary diseases as well as defending against viral infections and its family members may have many more undiscovered functions (Williams et al., 2019). Amongst 70 members of the TRIM protein family, multiple TRIM proteins such as TRIM8 and TRIM21, can be upregulated by IFN and have regulatory activities in IFN stimulation (Ozato et al., 2008). TRIM5α restricts retroviruses (Kaiser et al., 2007). TRIM2 is known as a E3 ubiquitin ligase and is largely localized to the cytoplasm (Williams et al., 2019). It is composed of the following domains: RING, B-box (BB2), Coiled-coil region (RBCC), followed by Filamin (FIL) domain and NHL repeats at the C-terminus (Figure 3) (Sarute et al., 2019). The RING domain contains the ubiquitin ligase activity while the BBox and Coiled Coil (CC) domains are thought to provide a binding site for ubiquitinated

substrates and promote the formation of protein complexes along with the FIL domain (Tocchini & Ciosk, 2015). FIL is also proposed to be important in mRNA regulation, while the NHL repeats bind to RNA and form protein/RNA complexes (Tocchini & Ciosk, 2015). It is critical to investigate TRIM2 protein's function and clarify the cellular events in which it modifies viral infection.

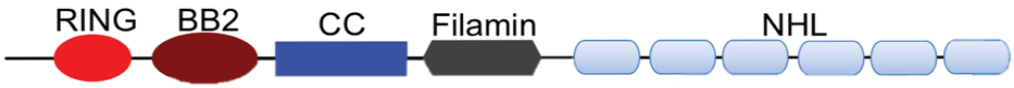

Figure 3: The domains of TRIM2 protein (Reprinted from Sarute et al., 2019)

TRIM2 protein is composed of RING, B-box2 (BB2), Coiled-Coil (CC), Filamin and NHL repeats (Sarute et al., 2019)

The TRIM2 interactome includes the proteins SIRPA, NEFL, BIM and MYO5A. These proteins were tested to investigate whether any of these factors could act together with TRIM2 against NWA infection (N. Sarute et al., 2019). The JUNV C1 infection increased only when SIRPα (also known as SIRPA and SHPS-1) was depleted, which suggests that SIRPA is antiviral against NWAs similar to TRIM2 (Figure 4) (N. Sarute et al., 2019).

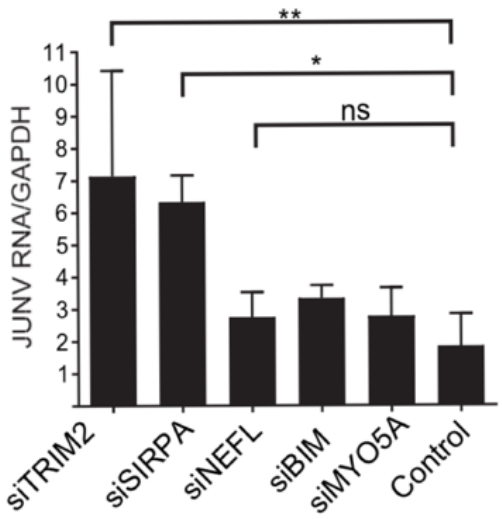

Figure 4: The effect of TRIM2-interacting factors on JUNV C1 infection levels (Reprinted from Sarute et al., 2019)

RNA samples collected from U2OS cells transfected with the indicated siRNAs were analyzed for the viral RNA levels by RT-qPCR 24 hours post infection (hpi). (Nicolas Sarute et al., 2019). Experiment was performed by N. Sarute.

Neurofilament light (NF-L) chains are a component of the intermediate filament in axons (Balastik et al., 2008). TRIM2 was held responsible for ubiquitination of NF-L chains in the brain which is thought to play a critical role in preventing neurodegeneration (Balastik et al., 2008). Mice deficient in TRIM2 were found to have NF-L chain filled axonal swellings in the cerebellum, cerebral cortex, retina and the spinal cord (Balastik et al., 2008). Recent findings by our lab using different TRIM2 knockout models did not point to a relationship between the increase in neurofilaments and the loss of TRIM2 (Li et al., 2020). The observed ataxic phenotype was not dependent on the RING domain, which is known to be the E3 ubiquitin ligase responsible domain of TRIM2 (Li et al., 2020). These findings suggested that the ataxia phenotype is regulated by another mechanism independent of TRIM2 activity (Li et al., 2020).

Hereditary Charcot–Marie–Tooth (CMT) disease is a peripheral nerve disorder that typically appears with the symptoms of loss of muscle control, deformities in muscle bulk in legs and feet, difficulty in walking and weakness in the arms, legs and feet. This disease manifests itself with signs ranging from multisystem disorders to mild peripheral neuropathies (Ylikallio et al., 2013). It is one of the most common inherited neuromuscular disorders which leads to a progressive damage in the motor and sensory nerves. When nerve conduction velocity is decreased, a demyelinating type of CMT (CMT1) occurs and the CMT2 is classified as an axonal type, however an overlapping phenotypes exists between CMT1 and CMT2. Although there are over 40 genes described in causing CMT to date, it is thought that the genes responsible for most of the genetic disorders that cause CMT have not been identified yet (Patzkó & Shy, 2011). One of the genes described as responsible from CMT disease was TRIM2. A patient with heterozygous mutations in TRIM2 displayed low weight and small muscle mass and was found to have childhood-onset axonal neuropathy (Ylikallio et al., 2013). Again, in Ylikallio et al. (2013), it was discussed that the loss-of-function mutations in TRIM2 were responsible for the observed axonopathy and neurodegeneration with juvenile-onset tremor and ataxia (Ylikallio et al., 2013). The phenotype observed in the mutants is suggested to have developed as a result of a decreased ubiquitination of NF-Ls by TRIM2 (Ylikallio et al., 2013). Yet the studies carried out using the mice from our lab did not yield the NF-L augmentation in the mutants (Li et al., 2020). Aside from the patient described in Ylikallio et al. (2013), there are 3 other patients whose disease is related to

functional and genetic abnormalities in TRIM2. One of the patients carrying a homozygous missense mutation had vocal cord paralysis together with peripheral neuropathy (Pehlivan et al., 2015). In addition, Magri et al. (2020) corroborated two novel TRIM2 mutations related to CMT by genetic and neurophysiological analyses of the patients presenting progressive axonal neuropathy. These patients had vocal cord paralysis as well along with other impairments in cranial nerves (Magri et al., 2020). Given the expansion in the genotypic and phenotypic spectrum of TRIM2 deficiency in relation to the patients described above the mice generated by our lab presented a promising disease model for the ongoing study of CMT. Understanding the function of TRIM2 given the abundance of it in the brain, may assist in developing neuroprotective strategies.

II. MATERIALS AND METHODS

Under the materials and methods section, section II, a part of previously published work, was presented. (Previously published as Sarute, N., Ibrahim, N., Fagla, B. M., Lavanya, M., Cuevas, C., Stavrou, S., ... & Ross, S. R. (2019). TRIM2, a novel member of the antiviral family, limits New World arenavirus entry. PLoS biology, 17(2), e3000137).

Under the materials and methods sections 2.12 and 2.13 previously published work was presented. (Previously published as Li, J. J., Sarute, N., Lancaster, E., Otkiran-Clare, G., Fagla, B. M., Ross, S. R., & Scherer, S. S. (2020). A recessive Trim2 mutation causes an axonal neuropathy in mice. Neurobiology of Disease, 104845.)

2.1 Ethics Statement

Mice used for the studies carried out for this thesis were housed according to the policies of the Institutional Animal Care and Use Committee of the University of Pennsylvania and of the Animal Care Committee of the University of Illinois at Chicago. They were performed according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Designed experiments were approved by the University of Pennsylvania IACUC (protocol #803700) and University of Illinois at Chicago ACC (protocol #18–168).

2.2 Cell Lines and Viruses

Cells lines and viruses were grown as described in Sarute et al., 2019. Briefly, Vero, U2OS, and 293T cells were cultivated in Dulbecco's modified Eagle Medium (DMEM; Gibco) supplemented with glutamine (2 mM), 10% fetal bovine serum (FBS; Invitrogen), and penicillin (100 U/ml)-streptomycin (100 µg/ml) (Invitrogen). NR-9456 cells were cultivated in Dulbecco's RPMI medium supplemented with glutamine (2 mM), 10% fetal bovine serum (FBS; Invitrogen), penicillin (100 U/ml)-streptomycin (100 µg/ml) and 0.1 % sodium pyruvate (Invitrogen). Candid 1 was propagated in Vero cells, whereas LCMV and Tacaribe virus (TRVL-11573; BEI Resources) were propagated in BHK-21 cells. Cells monolayers were infected when they reached 70%–80% confluency with a multiplicity of infection (MOI) of 0.01–0.03. Viruses were diluted in infection medium containing 2% FBS instead of 10% FBS. 24 hr post infection (hpi), media were removed and the cells were fed with fresh media supplemented with 2% FBS. Media containing viruses were harvested at 3, 4, and 5 dpi to obtain LCMV and at 7, 8, 9, and 10 dpi for JUNV C1 and Tacaribe virus. Virions in collected media were partially purified through a 30% sucrose cushion by ultracentrifugation. Supernatant was decanted and the pellet was resuspended in 1:10 of original volume of DMEM supplemented with 2% FBS. Virions were stored at −80°C until use. Pseudoviruses bearing the different viral GPs and MLV core and the reporter encoding the luciferase gene were created as previously described in Cuevas et al., 2011.

2.3 Virus Titration

JUNV C1 was titrated by infectious center assays (ICAs) and LCMV titers were determined by plaque assay as described in Sarute et al., 2019. Tacaribe virus and pseudovirus titers were determined by $TCID_{50}$ as described in Pedras-Vasconcelos et al., 2006 and Cuevas et al., 2011 respectively. Pseudoviruses low in titer were concentrated by using Amicon® Ultra Centrifugal Filters (Millipore Sigma).

2.4 Knockout Mice

2.4.1 *Trim2*-knockout mice

CRISPR/Cas9 technology was used to generate *Trim2*-knockout mice. Two sgRNAs targeted exon 3 and exon 9 were designed and synthesized by N. Ibrahim. The sgRNAs and

CRISPR RNAs were microinjected into zygotes obtained from C57BL/6N mice (Charles River) by the University of Pennsylvania Transgenic and Chimeric Mouse Facility. By sequencing genomic DNA (all strains) and cDNA (strains B and C) generated from total cellular RNA, the exact deletions found in each strain were determined (Nicolas Sarute et al., 2019). For genotyping, the PCR primers were designed as described in Sarute et al., 2019 and the products were analyzed on 2% agarose gels.

2.4.2 Ssu72-knockout mice

The *Ssu72*-knockout mice strain ($Ssu72^{em1(IMPC)J}$) obtained from The Jackson Laboratory was generated by the Knockout Mouse Phenotyping Program. The obtained strain was generated by using CRISPR technology by electroporating Cas9 protein and 2 guide sequences GCCCTAACATGGGAAGCACA and ATACAGGTCAGAGGATGTGG. The knock-out mutant of the *Ssu72* gene was generated as a result of the deletion of 439 bp in exon 3 and 299 bp of flanking intronic sequence, including the splice acceptor and donor, and this deletion is predicted to cause a change of amino acid sequence after residue 75 and early truncation 23 amino acids later.

2.5 RNA Interference

In order to deplete the selected targets in cell lines, siRNAs from Qiagen were used for TRIM2 (SI04165602), CLDN2 (SI00347893 and SI00952595), OCLN (SI05054385), SSU72 (SI03060533 and SI04923562), SYMPK (SI01439018) and control (1022076); from Ambion for SIRPA (109944); and from Dharmacon for TfR1 (L-003941). Knockdown experiments were performed as described in Sarute et al., 2019. Using Lipofectamine RNAi Max (Invitrogen), siRNAs were transfected using the forward transfection method. 48 hr after siRNA transfection, cells were infected with viruses. Cells infected with replication competent viruses were incubated for another 24 hr, cells infected with pseudoviruses were incubated for another 48 hr.

2.6 Generation of TRIM2, SSSU72 and CLDN2 Constructs

The c-myc-tagged human TRIM2 and TRIM5α constructs and RFP construct were generated as described in Lavanya et al., 2013. The full-length coding sequence of human SSU72 and CLDN2 were amplified by PCR using cDNA reverse transcribed from U2OS cells RNA with

primers 5′-TAA TGT GGA TCC GCA ATG CCG TCG TCC CCG CTG-3′ and 5′-TTG TTG TCT AGA CTT GTC GTC ATC GTC TTT GTA GTC GTA GAA GCA GAC GGT GTG CAG-3′; 5′-TAA TGG GGA TCC GCA ATG GCC TCT CTT GGC CTC-3′ and 5'-TTG TTG TCT AGA CTT GTC GTC ATC GTC TTT GTA GTC CAC ATA CCC TGT CAG-3′ respectively. A FLAG-tag was included in the reverse primer. The purified DNA from each construct was cloned into a pcDNA3.1 (+) *myc*-His vector (Thermo-Fisher); the myc and His tags were in frame with the coding regions of the constructs.

2.7 Procurement and Transfection of Plasmid Constructs for Infection Assays

Full-length SIRPA (Addgene), TRIM2, SSU72, and CLDN2 plasmids were transfected into U2OS cells using Lipofectamine 3000 (ThermoScientific) for 24 hr according to the manufacturers' instructions. Then, the cells were infected with viruses and kept at 37°C for 24 hr. Cells were collected and the expression of target genes was confirmed by western blot while their RNA was used to determine the infection levels using RT-qPCR as described below.

2.9 Infection of Cell Lines

Cells were infected with viruses (MOI of 1), after for 1 hr of adsorption at 37°C, shaking the plate at every 15 mins during this 1 hr. Cells were washed with PBS, supplied with fresh media, and were incubated at 37°C for 48 hr. 48 hpi, total RNA was isolated and the samples were analyzed by RT-qPCR.

2.10 RNA Isolation and RT-qPCR

RNeasy kit (Qiagen) was used to isolate RNA from cells infected with replication competent viruses. The isolated RNA was used as a template for cDNA synthesis using the SuperScript III First-Strand Synthesis System (Invitrogen) and random hexamer primers following the manufacturer's specifications. To determine the expression, specific primer pairs (listed in Table 1, see appendix A) were used to perform RT-qPCRs. Obtained cDNAs were tested by using Power SYBR green PCR kit (Applied Biosystems) in 384-well plates. The reaction plate was run at QuantStudio 5 Real-Time PCR System (Applied Biosystems). RNA quantifications were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The amplification

conditions were 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s, and 60°C for 1 min. To determine the amplification efficiency, a standard curve with 10-fold serial dilutions of a known concentration of DNA was generated for each primer. The slope values of the standard curves for the primer pair amplicons ranged from 3.5 to 3.2, indicating 90%–100% efficiency. For each primer pair, a no-template control was included, and each sample was run in triplicate.

2.11 Western Blot Analysis

Equal amounts of protein extracts (50 μg) were resolved by 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Detection of JUNV NP was done using a monoclonal antibody NA05-AG12 (BEI Resources). Myc-tagged TRIM2 proteins were detected with an anti-Myc antibody (Cell Signaling Technologies [CST]), and FLAG-tagged SIRPA, SSU72 and CLDN2 were detected with an anti-FLAG M2 antibody (Sigma). Endogenous and transfected TRIM2 was detected by rabbit anti-TRIM2 antibodies (Sigma SAB4200206).

2.12 Quantification of Cerebellar Ataxia

Ataxia disease severity in mouse models of cerebellar ataxia was carried out by following the scoring system described in Guyenet et al., 2010 by an observer blinded to the genotype. Scores from the ledge test, hindlimb clasping, gait and kyphosis were averaged to give a total score per mouse.

2.13 Quantification of Purkinje Cells

Cerebellum sections from WT and Trim2-knockout mice were cut and stained with a rabbit antiserum against calbindin-D-28K (1:2000, Millipore Sigma #C2724), and visualized with goat-anti-rabbit Alexa-594 (1:250, Thermo A-11012) by the RRC core facility at UIC. Images were made of these stained sections and I quantifed the number of Purkinje cells in a blinded experiment (without knowing the genotype of the mouse). Purkinje cells were counted by using ImageJ in an average of 3 sections from 8 to 12 weeks old Trim2A/A and WT mice (3 mice of each genotype). The average number of Purkinje cells in the 3 sections for each mouse were plotted.

2.14 Statistical Analysis and Data

Each experiment was done with 3 technical replicates/experiment. Data shown in the results are the average of at least 3 independent experiments, or as indicated in the figure legends. For *in vivo* experiments, the number of mice used in each experiment is shown in the graphs. Statistical analysis was performed using the GraphPad/PRISM software.

III. RESULTS

Under the results section, 3.1 assesment of antiviral host factors, a part of previously published work, was presented. (Previously published as Sarute, N., Ibrahim, N., Fagla, B. M., Lavanya, M., Cuevas, C., Stavrou, S., ... & Ross, S. R. (2019). TRIM2, a novel member of the antiviral family, limits New World arenavirus entry. PLoS biology, 17(2), e3000137).

Under the results section, 3.2 neurological function of TRIM2, previously published work was presented. (Previously published as Li, J. J., Sarute, N., Lancaster, E., Otkiran-Clare, G., Fagla, B. M., Ross, S. R., & Scherer, S. S. (2020). A recessive Trim2 mutation causes an axonal neuropathy in mice. Neurobiology of Disease, 104845.)

3.1 Assesment of Antiviral Host Factors

3.1.1 TRIM2 is Antiviral against New World Arenaviruses

TRIM2 was found to be antiviral against NWAs when tested with pseudotyped viruses carrying the glycoprotein from a NWA Junin virus, VSV and MMTV, as mentioned in Lavanya et al., 2013 (Lavanya et al., 2013). Furthermore, when infected with the replication competent attenuated version of the Junín virus JUNV C1, TRIM2 and SIRPA (a factor TRIM2 interacts with) were both found to be antiviral against JUNV1 C1 (Nicolas Sarute et al., 2019).

Here in this study, I wanted to test to see whether TRIM2 and SIRPA have antiviral activity against another NWA; Tacaribe virus (TCRV). The knockdown and infection experiments confirm that TCRV can replicate better in TRIM2 depleted cells than in control cells (Figure 5A). SIRPA exemplifies a similar interaction with TRIM2 against Tacaribe virus. When the level of SIRPA is comparatively less than the constitutive level, the detected TCRV levels are higher than the control

(Figure 5A). On the contrary, when TRIM2 and SIRPA levels were increased the detected TCRV levels were lower than the control cells (Figure 5B).

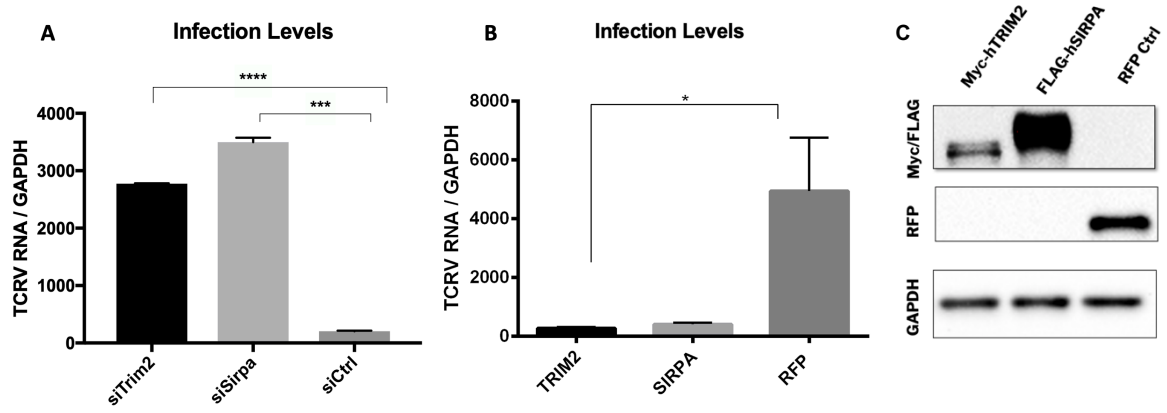

Figure 5. Relative TCRV infection levels when TRIM2 and SIRPA levels were changed

(A) The relative TCRV RNA levels of U2OS cells transfected with the indicated siRNAs, analyzed by RT-qPCR 24 hpi
(B) The relative TCRV RNA levels of U2OS cells transfected with the the plasmids expressing the indicated tagged proteins, analyzed by RT-qPCR 24 hpi
(C) The confirmation of overexpressed TRIM2 and SIRPA when U2OS cells were transfected with the expression plasmids. The lysate from transfected cells were run and transferred onto the PVDF membrane. Then, the membrane was blotted with the antibodies against the tags. RFP expressing plasmid was used as a control. Its expression was confirmed with the antibody against the RFP. GAPDH levels in lysates were checked by using anti-GAPDH antibody.
Values seen in both panel A and panel B represent the average of 3 independent experiments with triplicate experimental replicates ± SD. Statistical significance was calculated by one-way ANOVA. *P ≤ 0.01; **P ≤ 0.002; ****P ≤ 0.001; ****P ≤ 0.0001.
TCRV, Tacaribe virus; RT-qPCR, real-time quantitative PCR; siRNA, short interfering RNA; hpi, hours post infection; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; h-, human; RFP, red fluorescent protein.

TRIM2 was evaluated to observe whether it is antiviral against OWAs as well. For this purpose, three different pseudoviruses bearing different virus glycoproteins and containing the luciferase gene, were used. These glycoproteins were of Junin (a NWA), LCMV and Lassa (two different species of OWAs) (Figure 6) (Nicolas Sarute et al., 2019). When infected with a NWA, Junin pseudovirus, cells that have lower TRIM2 levels than the endogenous levels got infected at a significantly higher rate while the cells expressing higher levels of TRIM2 were infected at lower levels in comparison to the control. However, the infection levels were not affected by the OWA pseudoviruses bearing glycoproteins from LCMV or Lassa (Figure 6).

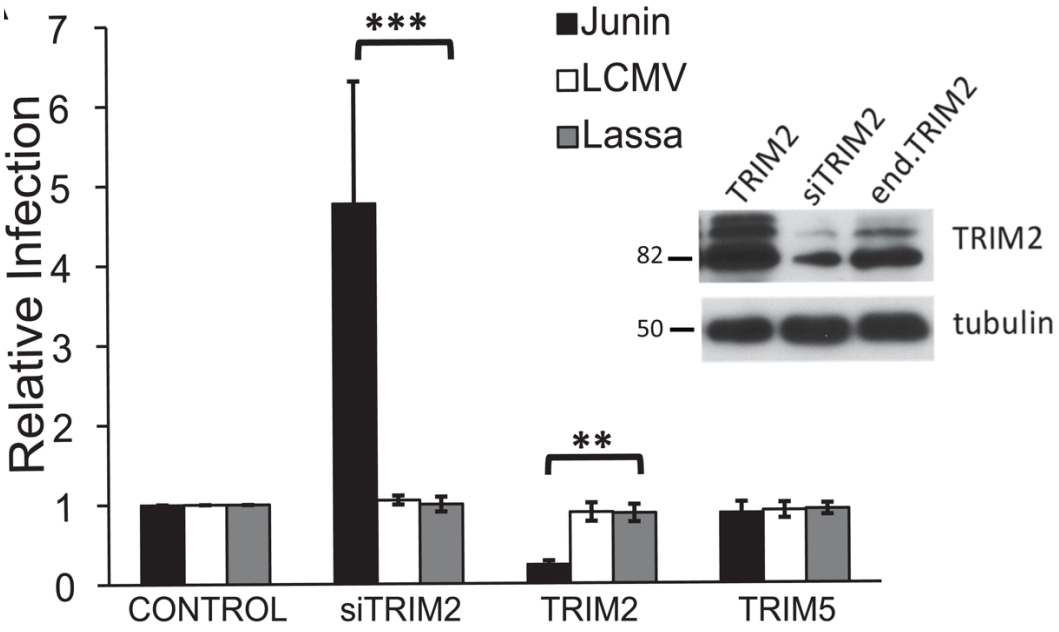

Figure 6. TRIM2 subsides Junín virus but not LCMV and Lassa virus infections (Reprinted from Sarute et al., 2019). All experiments in this figure were performed by M. Lavanya.

U2OS cells were transfected with siTRIM2, TRIM2 or TRIM5α expression vectors and 24 hr later were infected with Junín virus, Lassa virus, or LCMV GP-pseudoviruses containing the luciferase gene. Control refers to cells treated with a control siRNA. The data shown are the average and SD of 3 independent experiments. Western blot is from U2OS cells and those transfected with the TRIM2 expression vector or siRNA. Blots were probed with anti-TRIM2 and anti-β-tubulin antisera.

One-way ANOVA was used to determine significance. **P ≤ 0.005; ***P ≤ 0.0005.

LCMV, lymphocytic choriomeningitis virus; OWA, Old World arenavirus; RT-qPCR, real-time quantitative PCR; siRNA, short interfering RNA (N. Sarute et al., 2019).

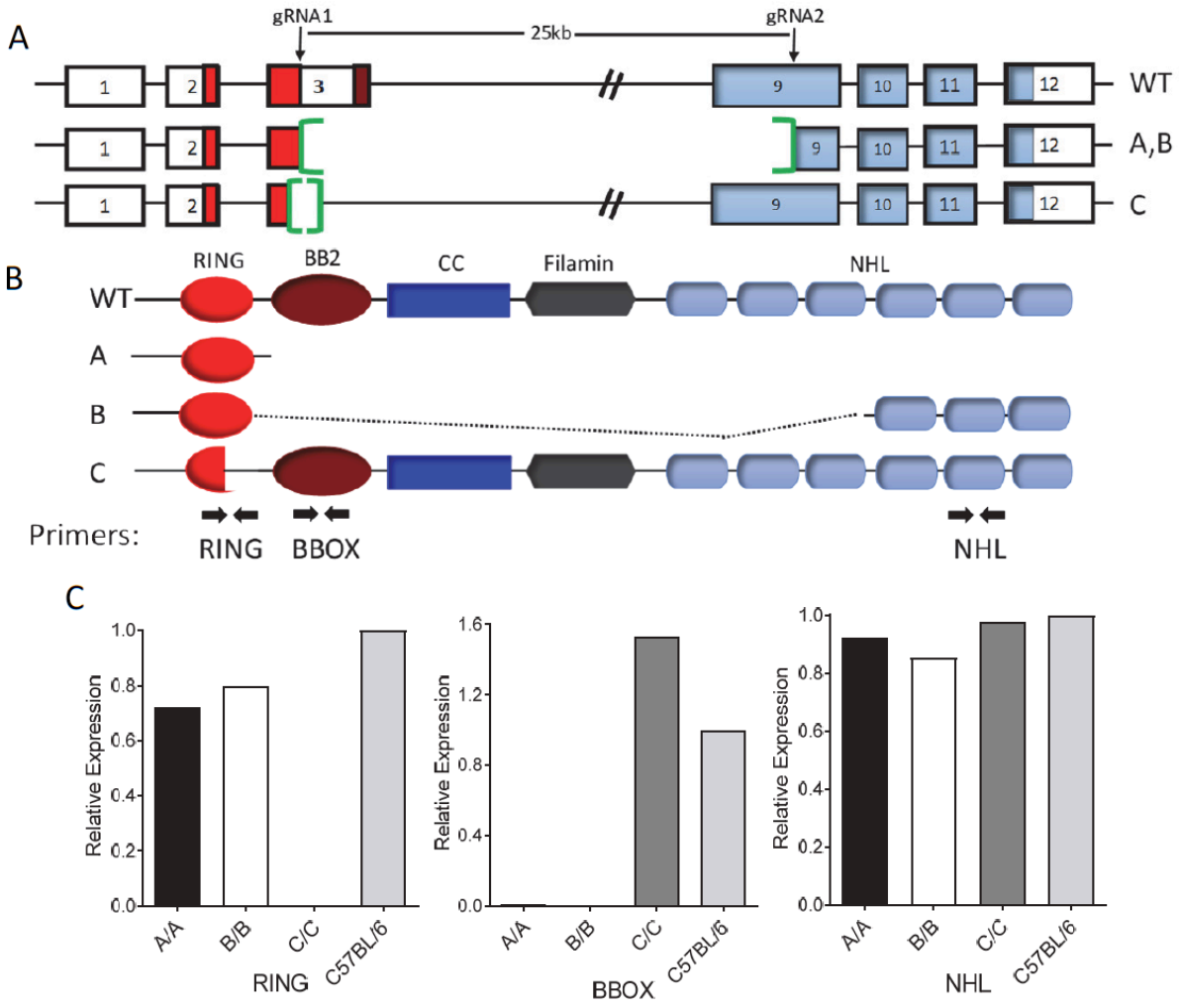

Figure 7. The targeted regions and domains of TRIM2 in different *Trim2*-knockout strains (Reprinted from Sarute et al., 2019). Experiments were performed by N. Sarute.

(A) Diagram showing the position of the guide RNAs used to generate the TRIM2 KO mice. Shown are the genomic deletions found in strains A, B, and C.

(B) Diagram of the domains of TRIM2 present in the mutant mice. Shown are the primers used to analyze TRIM2 expression.

(C) RT-qPCR analysis of RNA isolated from the brains of strains A, B, and C, using the indicated primers (N. Sarute et al., 2019)

We also tested the effect of TRIM2 on NWA infection *in vivo* by testing JUNV C1 and TCRV on different strains of TRIM2 knockout mice (see Figure 7 for the targeting regions of guide RNAs (7A) and the strains obtained (7B) as well as the expression profile of each domains in each strain

(7C)). Strain C is different than the WT strain with only a 30 amino acids difference in the RING domain (Figure 7B). While strain B consists of the RING domain and partial domain of NHL repeats, strain A consists of the RING domain only (Figure 7B). When a TRIM2 antisera to an epitope in the CC domain (see the red arrow in Figure 8A) was used to detect the TRIM2 protein in brain samples from each strain, TRIM2 was found in heterozygous mice in both the A and the B strains but not in the knockout mice (Figure 8B). We confirmed that the TRIM2 CC domain is present both in heterozygous and knockout forms of the strain C (Figure 8B).

Bone marrow derived macrophages (BMDMS) of three different strains and WT (C57BL/6) were infected with JUNV C1, TCRV and LCMV *ex vivo* (Figure 8C, 8D and 8E) and the brains of these strains were infected with JUNV C1 and TCRV *in vivo* (Figure 8F and Figure 8G). Then, the samples were analyzed by RT-qPCR as described in the previous section. JUNV C1 and TCRV viruses showed higher infectibility in the strains A and B than in the WT strain both *ex vivo* and *in vivo* (Figure 8C, 8D, 8F and 8G). Strain C, however, did not get infected with any significant difference from the WT strain (Figure 8C, 8D, 6F and 8G). None of the infection levels of the knock-out strains were different than the WT strain when infected with an OWA LCMV *ex vivo* (Figure 8E).

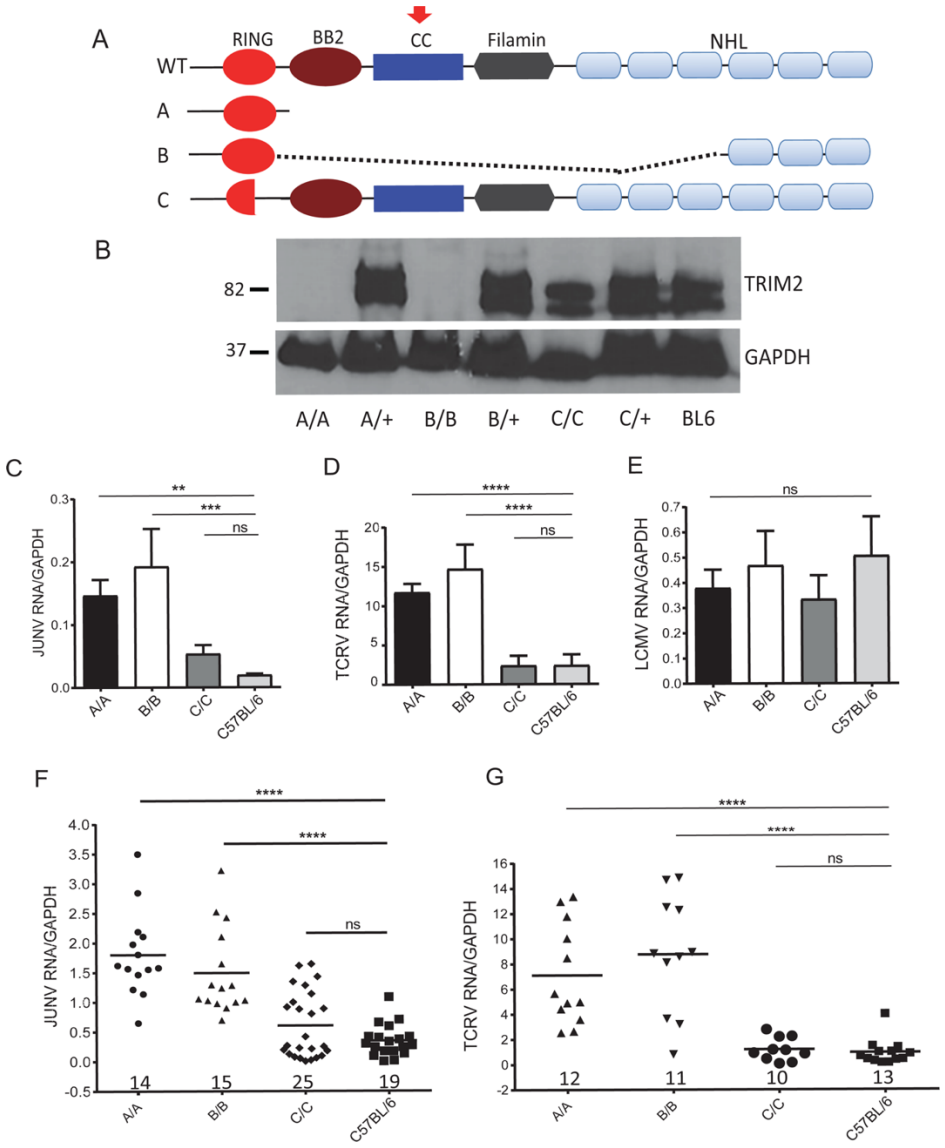

Figure 8. TRIM2 knockout mice are more susceptible to infection with new world arenaviruses (Reprinted from Sarute et al., 2019). All experiments in this figure were performed by N. Sarute.

(A) Diagram of the ***Trim2*** WT and deletion alleles in strains A, B, and C (See also Figure 7A). The red arrow indicates the epitope recognized by anti-TRIM2 antisera.
(B) Western blot analysis of brain extracts from the different homozygous and heterozygous mouse strains, using TRIM2 antisera to an epitope in the CC domain (red arrow in panel A).
(C, D, and E) Primary bone marrow–derived macrophages from the different knockout strains were infected with JUNV C1, TCRV, and LCMV, respectively, and analyzed by RT-qPCR for viral RNA levels at 24 hpi. Shown are the averages ± SD of 3 different experiments. One-way ANOVA was used to determine significance. **P ≤ 0.005; ***P ≤ 0.0006.
(F) Mice of the indicated genotype were infected by intracranial inoculation with 2 × 10^4 PFU of JUNV C1, and at 5 dpi RNA isolated from brains was analyzed for viral RNA. P values were determined by unpaired ***t*** tests; ****P ≤ 0.0001. Number of mice in each group is shown above the x-axis.
(G) Mice of the indicated genotype were infected intraperitoneally with 2 × 10^3 PFU of TCRV by intraperitoneal injection, and at 7 dpi, RNA isolated from spleen was analyzed for viral RNA. ***P*** values were determined by unpaired ***t*** tests; ****P ≤ 0.0001. Number of mice in each group is shown above the x-axis.
dpi, days post infection; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hpi, hours post infection; JUNV, Junín virus; LCMV, lymphocytic choriomeningitis virus; ns, not significant; PFU, plaque-forming units; RT-qPCR, real-time quantitative PCR; TCRV, Tacaribe virus; WT, wild type (N. Sarute et al., 2019)

3.1.2 The Assessment of the Effects of Selected Host Factors on Arenavirus Infection

The factors screened for Junín virus entry as described at Lavanya et al., 2013 included CLDN2 and SSU72 in addition to TRIM2. When tested with pseudotyped viruses carrying the glycoprotein from NWA, VSV and MLV; CLDN2 showed antiviral activity against these 3 viruses. SSU72 showed antiviral activity against Junin and MMTV pseudoviruses but not the VSV pseudovirus. Here these factors were further investigated by using replication competent viruses.

First, CLDN2 was investigated together with OCLN (a factor is known to be interacting with CLDN2 (Daugherty et al., 2007)). The purpose of this test was to investigate the relationship of the above factors with JUNV C1 and TCRV viruses. U2OS cells were transfected with siRNAs against the factors CLDN2, OCLN, SIRPA, TRIM2 and TfR1 (the receptor for JUNV virus) and infected with JUNV C1 (Figure 9A). U2OS cells were transfected with siRNAs against CLDN2, OCLN, SIRPA and A2D2 (a subunit of calcium channel which is known to be proviral for TCRV (Lavanya et al., 2013; N. Sarute et al., 2019)) and then they were infected with TCRV, another NWA (Figure 9B). In the cells where TRIM2 levels were lower, the JUNV C1 virus was detected to be significantly higher than in the control group (Figure 9A). Although CLDN2 was not significantly antiviral against JUNV C1, the depletion of CLDN2 in these cells resulted in higher JUNV C1 infection in comparison to the control (Figure 9A). The depletion of OCLN in cells, resulted in lower levels in JUNV C1 infection (Figure 9A). In the cells where A2D2 levels were lower, the virus was detected to be significantly lower than in the control group when tested with TCRV (Figure 9B). The lower levels of CLDN2 resulted in significantly higher TCRV levels while the lower levels of OCLN resulted in significantly lower TCRV levels in comparison to the control (Figure 9B).

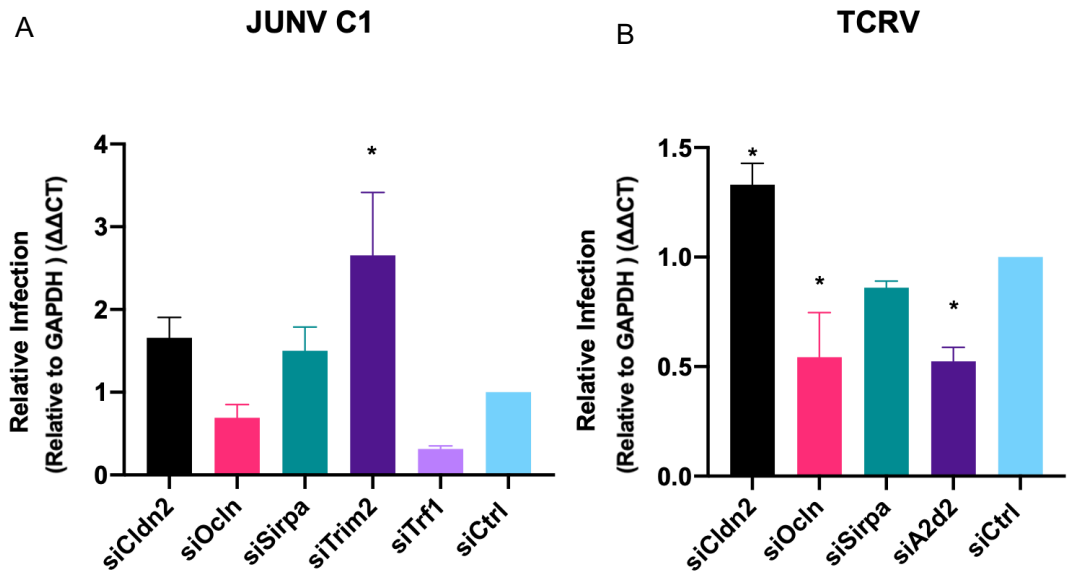

Figure 9. CLDN2 subsides while OCLN intensifies JUNV and TCRV infection.

(A) U2OS cells were transfected with the indicated siRNAs and infected with JUNV C1 (MOI: 0.1), then RNA was isolated 24 hpi and analyzed for viral RNA by RT-qPCR.

(B) U2OS cells were transfected with the indicated siRNAs and infected with TCRV (MOI:1), then RNA was isolated 24 hpi and analyzed for viral RNA by RT-qPCR.

Values in A and B represent the average of 3 ore more independent experiments with triplicate experimental replicates ± SD. Statistical significance was calculated by one-way ANOVA. *P ≤ 0.01.

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MOI, multiplicity of infection; hpi, hours post infection; JUNV C1, Candid-1; TCRV, Tacaribe virus; RT-qPCR, real-time quantitative PCR; siRNA, short interfering RNA; TfR1, transferrin receptor 1; A2D2, calcium channel subunit α2δ2.

Second, SSU72 was investigated together with SYMPK (a factor is known to be interacting with SSU72) to test the relationship of these factors with NWA. U2OS cells were transfected with siRNAs againt the factors SYMPK, SSU72, TRIM2, SIRPA and TfR1 (the receptor for JUNV virus) to test how the infectivity by JUNV C1 is altered (Figure 10A). Murine macrophage cell line NR-9456 cells were transfected with siRNAs against SSU72, SYMPK, TRIM2 and JUNV C1 infectivity was assesed by RT-qPCR in each group (Figure 10B). In U2OS cells where TRIM2 and SSU72 levels were lower, the JUNV C1 virus was detected to be significantly higher than in the control group (Figure 10A). Although SYMPK was not significantly antiviral against JUNV C1, the depletion of this factor in these cells resulted in higher JUNV C1 infection in comparison to the control (Figure

10A). When tested in NR-9456 cells, where TRIM2 levels were lower, the JUNV C1 virus was detected to be significantly higher than in the control group (Figure 10B).

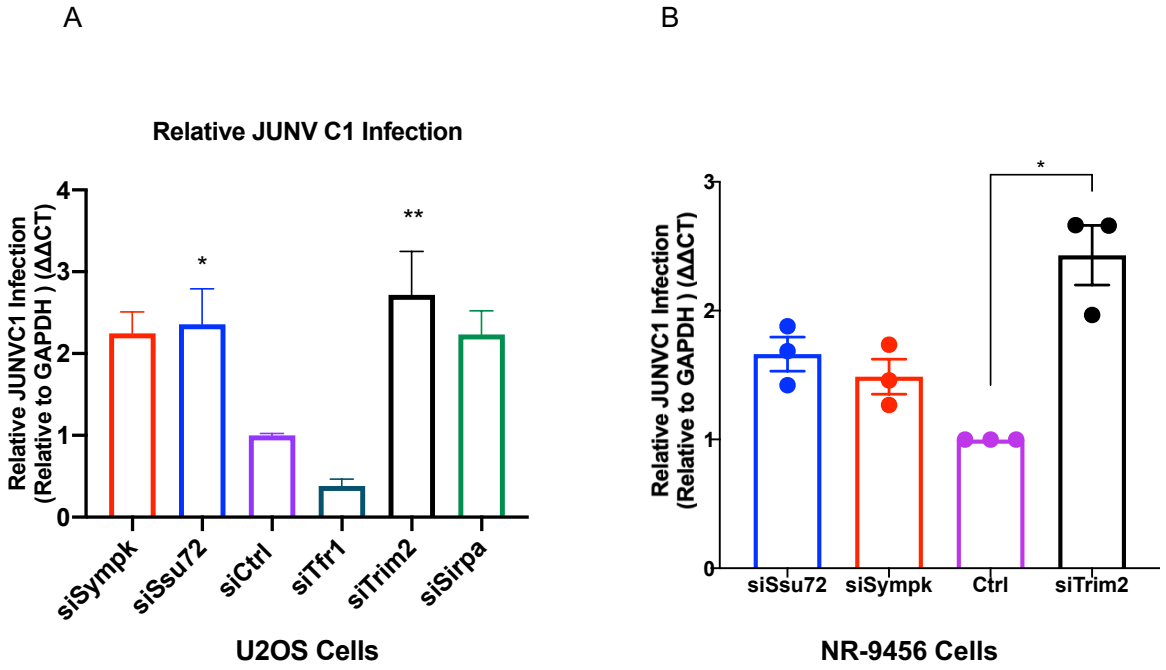

Figure 10. SSU72 subsides JUNV C1 infection in U2OS cells.

(A) U2OS cells were transfected with the indicated siRNAs and infected with JUNV C1 (MOI: 0.1), then RNA was isolated 24 hpi and analyzed for viral RNA by RT-qPCR.

(B) U2OS cells were transfected with the indicated siRNAs and infected with JUNV C1 (MOI: 0.1), then RNA was isolated 24 hpi and analyzed for viral RNA by RT-qPCR.

Values in A and B represent the average of 3 or more independent experiments with triplicate experimental replicates ± SD. Statistical significance was calculated by one-way ANOVA. *P ≤ 0.01; **P ≤ 0.005.

SSU72 and CLDN2 overexpresion plasmids were obtained by molecular cloning. U2OS cells were transfected with these plasmids as well as the plasmids expressing SIRPA, TRIM2 and RFP in order for validation (Figure 11).

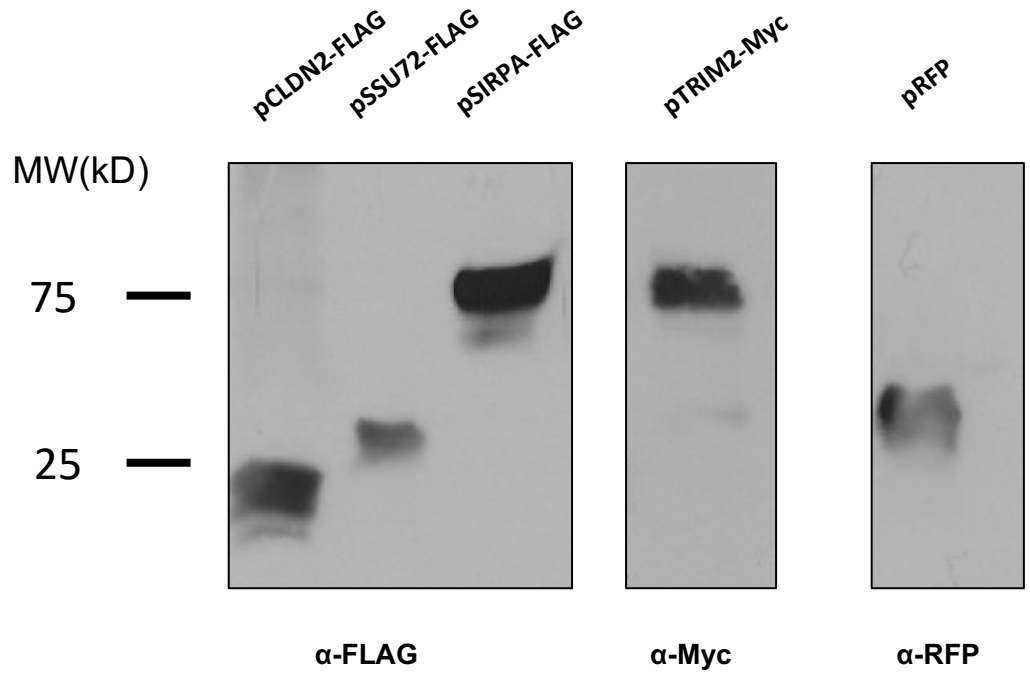

Figure 11. Confirmation of the cloned overexpression plasmids of CLDN2 and SSU72.

Western blot from the lysates of U2OS cells transfected with expression plasmids; CLDN2, SSU72, SIRPA, TRIM2 or RFP. The lysates were purified, run and resolved on acrylamide gel and then transferred onto PVDF membrane. CLDN2, SSU72 and SIRPA expressing vectors carried a FLAG tag, while TRIM2 plasmid carried a Myc tag. The membrane was blotted against FLAG, Myc and RFP.

MW, molecular weight; kD, kilodalton; p-, plasmid; RFP, red fluorescent protein

JUNVC1 (a species of NWA) and LCMV (a species of OWA) were tested on U2OS cells transfected with the plasmids mentioned above in Figure 11. U2OS cells transfected with CLDN2 and SSU72 overexpression plasmids reduced JUNV C1 levels, similar to the TRIM2 and SIRPA transfected cells (Figure 12A). TRIM2 did not alter LCMV levels, but all cells transfected with CLDN2, SSU72 and SIRPA plasmids resulted in having less infection than in control RFP (Figure 12B) .

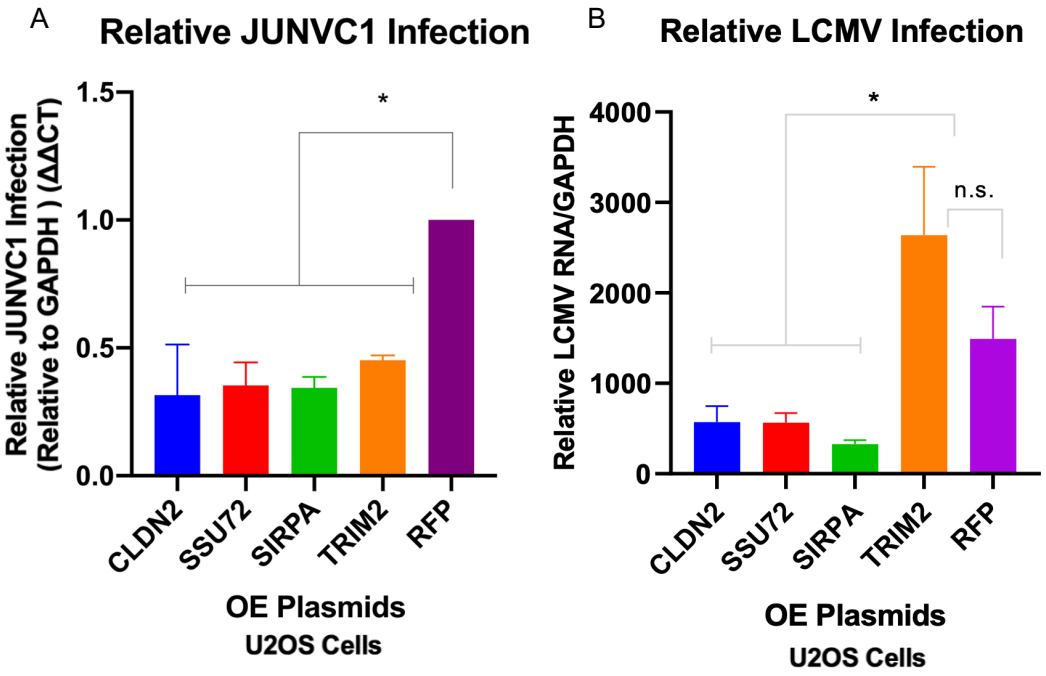

Fig 12. Overexpression of SSU72 or CLDN2 decreases NWA and OWA infection.

(A) U2OS cells were transfected with CLDN2, SSU72, SIRPA, TRIM2 or TRIM5α expression vectors and 24 hr later were infected with JUNV C1 (MOI: 0.1). RNA was isolated 24 hpi and analyzed for viral RNA by RT-qPCR.

(B) U2OS cells were transfected with CLDN2, SSU72, SIRPA, TRIM2 or TRIM5α expression vectors and 24 hr later were infected with LCMV (MOI: 1). RNA was isolated 24 hpi and analyzed for viral RNA by RT-qPCR.

The data shown in A and B represent the average of 3 or more independent experiments with triplicate experimental replicates ± SD. Statistical significance was calculated by one-way ANOVA. *P ≤ 0.01.

LCMV, lymphocytic choriomeningitis virus; MOI, multiplicity of infection; NW, New Word arenavirus; OWA, Old World arenavirus; RT-qPCR, real-time quantitative PCR; RFP, red fluorescent protein; OE, overexpression.

SSU72 knock-out embryonic cells were obtained from the Jackson Laboratory as mentioned in section II. The strain created by using CRISPR technology was predicted to cause a truncation with a 23 amino acid change in the sequence after the residue 75. Heterozygous mice were bred in the C57BL/6 background at BRL facility at the University of Illinois at Chicago. After many generations of crossing, we failed to obtain a viable homozygous *Ssu72*- knockout mouse. In order to test whether there is haploinsuffiency, fibroblasts were obtained from WT and heterozygous mice. They were analyzed for their SSU72 expression by using RT-qPCR. The levels of SSU72 were not significantly different in heterozygous mice than in WT mice (Figure 13A). When fibroblasts and macrophages of WT and heterozygous mice were infected with JUNV C1 there was no difference observed in their infectibility (Figure 13B and 13C).

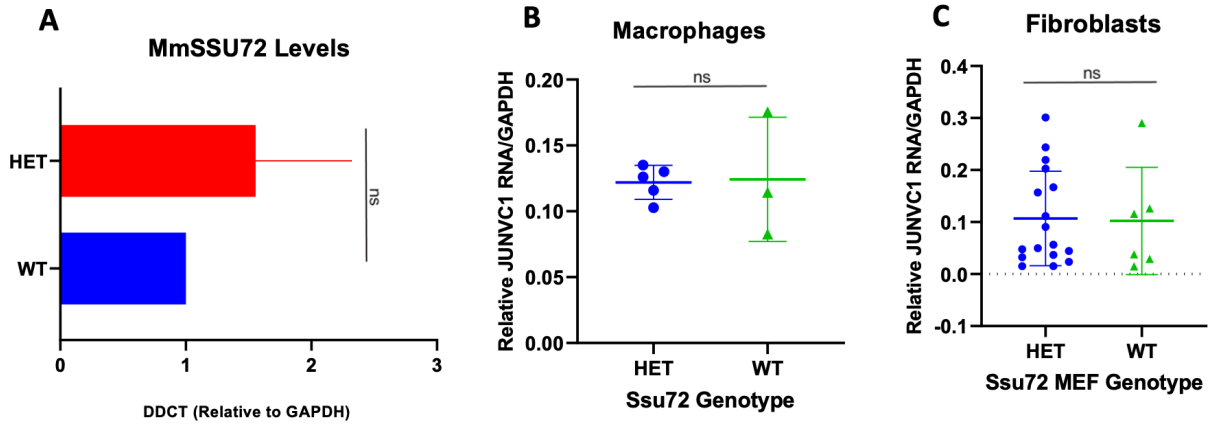

Fig 13. SSU72 does not show haploinsuffiency.

(A) Isolated RNAs were tested from fibroblasts obtained from three different WT and SSU72 HET mice with triplicate experimental replicates by using RT-qPCR for mouse specific SSU72 expression. One-way ANOVA was used to determine the statistical significance.

(B) Macrophages isolated from WT and SSu72 HET mice and infected with JUNV C1 (MOI: 0.1), then RNA was isolated 48 hpi and analyzed for viral RNA by RT-qPCR.

(C) Fibroblasts isolated from WT and SSU72 HET mice and infected with JUNV C1 (MOI: 0.1), then RNA was isolated 48 hpi and analyzed for viral RNA by RT-qPCR.

Values in B and C represent the average of 3 or more samples from different mice with triplicate experimental replicates ± SD. Statistical significance was calculated by one-way ANOVA.

WT, wild type; HET, heterozygous; Mm, *mus* musculus; dpi, days post infection; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hpi, hours post infection; ns, not significant; MOI, multiplicity of infection; RT-qPCR, real-time quantitative PCR.

3.2 Neurological Function of TRIM2

3.2.1 Behavioral Assessment of the Mutant and Wild-Type Mice

CRISPR/Cas9 technology was used to generate *Trim2*-knockout mice. Two sgRNAs targeted exon 3 and exon 9. The sgRNAs and CRISPR RNAs were microinjected into zygotes obtained from C57BL/6N mice (Charles River) by the University of Pennsylvania Transgenic and Chimeric Mouse Facility as previously described in section II. Here, we further investigated the *Trim2*-knockout mouse strain A for its phenotype. While crossing heterozygous *Trim2*-knockout mice and breeding knockout mice A/A, it was observed that the knockout mice were more prone to have tremor and shaking phenotype. Their uncoordinated movements suggested an ataxia phenotype and these mice were subjected to a test for scoring their ataxia levels. Ataxic behavior was tested by using ledge, clasp, gait and kyphosis levels of mice as described in Guyenet et al. (2010). WT and knockout mice were compared in the study and the evaluations suggested that *Trim2*$^{A/A}$ mice develop a progressive ataxia (Figure 14). It was observed that the ataxia starts as early as 2 months in these mice with a significant difference from their WT littermates and worsens overtime.

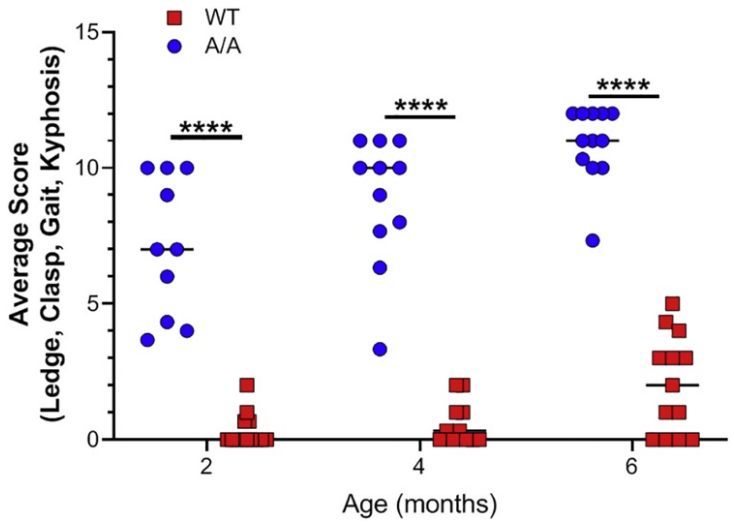

Figure 14. *Trim2*$^{A/A}$ mice develop a progressive ataxia (Reprinted from Li et al., 2020). The experiment was performed by N. Sarute and G. Otkiran Clare and analyzed by G. Otkiran Clare.

A cohort of *Trim2*$^{A/A}$ mice and their *Trim2*$^{+/+}$ (WT) littermates were scored for ataxia (Guyenet et al., 2010) at the indicated ages. The ataxia score worsens over time in *Trim2*$^{A/A}$ mice; two month-old Trim2 A/A mice were statistically different from 6 month old mice but not from 4 month old mice ($P < .0007$). *P* values were determined by two-way ANOVA. **** $P < .0001$ (Li et al., 2020)

3.2.2 The Effects of TRIM2 depletion on Purkinje Cell Density

Trim2-knockout mice were tested for their levels of Purkinje cells to see whether there is an underlying correlation between the ataxic phenotype and the presence of these cells. For this, the density of Purkinje cells found in *Trim2*$^{A/A}$ mice and their WT littermates was compared. Fluorescent images of the brain sections obtained from knockout and WT mice showed that the density of Purkinje cells is lower in *Trim2*-knockout mice than in WT mice (Figure 15A and 15B). Note the row of Purkinje cell bodies are diminished in the Trim2$^{A/A}$ cerebellum in comparison to the in the WT cerebellum. The average density of these cells is significantly less in *Trim2*-knockout mice than in WT mice, which outlines that *Trim2*$^{A/A}$ mice substantiate loss of Purkinje cells in the cerebellum (Figure 15B).

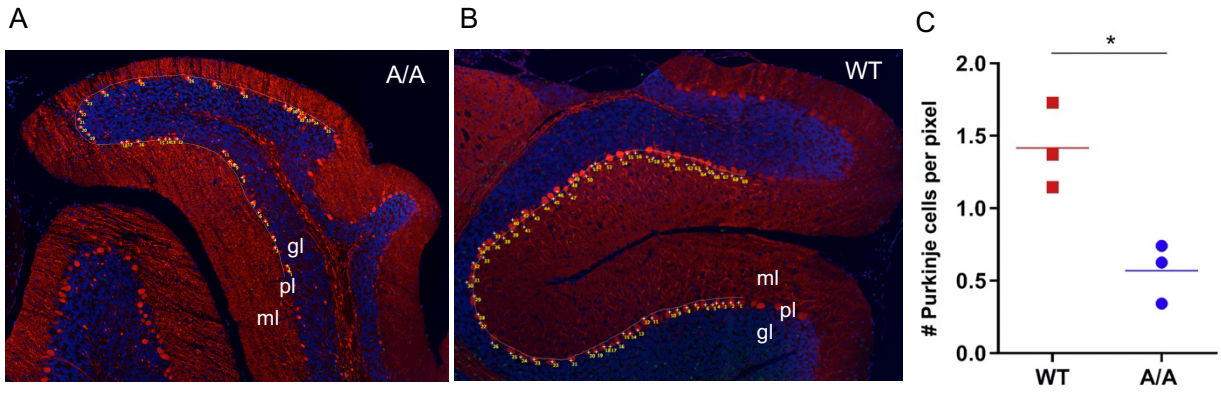

Figure 15. Loss of Purkinje cells in the cerebellum of *Trim2*$^{A/A}$ mice (Panel C only, reprinted from Li et al., 2020). Experiments were performed by G. Otkiran Clare.

(A) Sample images of semi-thin, sagittal sections of the cerebellar vermis from an 11-week-old *Trim2*$^{A/A}$ mouse (B) and its WT littermate, immunostained for calbindin (red) and counterstained with DAPI (blue) to label nuclei. Purkinje cells seen as red round dots lying on the Purkinje layer (pl) found in between the molecular (ml) and granular layer (gl), on a pre-set line traced on the images at same magnification were counted (yellow).

(C) Purkinje cell density found on the traced line were plotted for WT and knockout (A/A) strains. The graph shows that the number of Purkinje cells is significantly reduced in *Trim2*$^{A/A}$ mice (*N* = 3) compared to their WT littermates (N = 3). *P* value was determined by an unpaired *t*-test, **P* ≤ .02. Scale bars: 25 μm for A-D, H&I; 50 μm for E&F (Li et al., 2020)

IV. DISCUSSION

3.1 TRIM2 and the Peripheral Neuropathy

Several clinical reports identified cases of patients with many physiological symptoms such as muscle weakness due to *TRIM2* mutations they carried. One of the patients had a compound heterozygous *TRIM2* mutation (c.680A>T/p.Glu227Val and c.1966delA/p.Lly567fs, 1- bp deletion causing a frameshift which introduced a premature stop codon (Ylikallio et al., 2013)); the other patient had a homozygous c.2000A>C/p.Asp667Ala mutation (Pehlivan et al., 2015). Additionally, two recent cases expanded on these observations with four novel *TRIM2* variants. One patient was compound heterozygous with a missense mutation (c.1012 T > C [p.Ser338Pro]) in the paternal allele while in the maternal allele it caused a frame shift (c.642delT [p.Glu215LysfsTer6]) resulting in 1-bp deletion. The other was detected with a severe axonal motor-sensory neuropathy which carried a maternally inherited in-frame deletion (c.1859_1861delACA [Asn621del]) that eliminated asparagine 621 in the third NHL domain and a paternally-inherited nonsense mutation (c.2302C > T [p.Arg768Ter]) (Magri et al., 2020). Humans with *TRIM2* mutations do not have ataxia but demonstrate various sensory and motor disorders as reported in the above cases yet, the cases confirm that they express axonal neuropathy (Li et al., 2020). In chapter 3.2 we assessed the *Trim2*-knockout mice to address whether the ataxia phenotype observed in strain A/A could present these mice as animal models to study CMT disease since both human cases and our mouse model point to the presence of an axonal neuropathy. The results of scoring the ataxic phenotype confirmed that indeed, these mice had succumbed to ataxia and severity increased as they aged (Figure 14). When mutant (A/A) and WT mice were blindly scored and compared for ataxia, a significant difference in their ataxia phenotype scoring were detected between the mutant versus WT mice. The ataxia score heightened over time in A/A mice starting as early as the age of two months old (Figure 14). The score of two-month-old A/A mice was significantly different than the 6-month-old mice. The cases from the reported patients above with varying pathologies were observed over time as these patients got older. This overlap in the phenotype as well as the reported neuromuscular problems found both in the patients and A/A mice suggest the mice could be used as CMT disease model.

The pathological findings in the images of cerebellar sections from an 11-week-old A/A mouse (Figure 15A) and its WT litter mate (Figure 15B), evidenced loss of Purkinje cells in mutant mice. The row of Purkinje cell bodies observed with Calbindin staining (a Purkinje cell marker) between the gl and ml were diminished in A/A cerebellum in comparison to WT sections. Examination done through the use of more samples asserts a significant difference in the number of Purkinje cells from WT versus A/A mice. Purkinje cells are important in the process of learning and motor control; the loss of them can be one of the reasons why the patients experienced the loss of motor controls. Taken together the findings identify that TRIM2 has a significant role in the central nervous system (CNS) so we postulate that the pathologies seen in the CNS are related to the cell autonomous effects of TRIM2, which is highly expressed in human CNS (to a higher degree than in any other tissues). TRIM2 is an important factor in maintaining neurological health.

The differences observed above, between the A/A and WT mice, were not observed between the mice of the C/C strain and the WT (data not shown). There was not any loss of Purkinje cells in C/C mice, nor did they have any visible phenotype for ataxia. The C/C strain does not retain the entire RING domain, while the A/A strain retains the RING domain (the domain which was identified to be important for the ubiquitin activity of TRIM2) (Figure 7C). Our observations indicate that the neurological disease is not strictly associated with the ubiquitin activity of TRIM2. While C/C mice were the same with the WT mice, A/A mice developed an axonal neuropathy and Purkinje cell loss even though they still expressed the RING domain (Li et al., 2020). In the event the loss of ubiquitination activity was not the reason behind the acquired phenotype (as our data suggests), then another function of TRIM2 must be disrupted in A/A mice, but not in C/C mice for this phenotype to occur. The detailed functional role of TRIM2 is yet to be determined and not yet obvious. There are several other proteins aside from NF-L that interact with TRIM2 including myosin5 (which reciprocally interacts with NF-L) and SIRPA (that is expressed by macrophages and neurons and its binding to CD47 on the surface of target cells inhibits their engulfment by macrophages) (Li et al., 2020). In maintaining the protective function in CNS, other members of the TRIM2 interactome, such as SIRPA, might be involved, or they might play a role in the development of axonopathy in mice and humans in the absence of TRIM2.

Overall, the neurological phenotype in A/A mice was demonstrated in this study by showing the loss of Purkinje cells and the development of ataxia in the knockout mice. We were able to address the similarities in phenotypes between CMT patients (caused by the mutations in TRIM2) and the A/A mice. Thus, our A/A mice represent a substantial model of CMT and with further studies, will enable potential therapies to be developed.

3.2 Anti-Arenaviral Entry Genes

It is quite critical to understand the biology of viruses and to reveal the mechanisms behind virus-host interactions to seek new ways to develop treatment against viruses that cause diseases in humans. SARS-CoV-2, which recently caused the unforeseen and abrupt global COVID-19 pandemic, belongs to the family *Coronaviridae*, a large family of single-stranded RNA viruses (Machitani et al., 2020). It shares common features with previously described SARS-CoV and MERS which needs ACE-2 and TMPRSS2 for the viral entry (Hoffmann et al., 2020; Kleine-Weber et al., 2018). Sequence analyses examining the evolutionary changes on these viruses suggest a significant divergence between these viruses has occurred (Tang et al., 2020). The ability of SARS-CoV-2 to spread quickly via airborne methods has proven one of the most difficult aspects in limiting increases in infections globally. Arenaviruses, as members of an RNA-based virus family can also be transmitted by air and, they have the potential to infect many people in a short time, similar to SARS-CoV-2. Arenaviruses are a potential bioterror agent as they too, can easily be transmitted by air (Gowen & Bray, 2011). As mentioned in Chapter 1, the arenaviral pool is growing with the emerging possibility of zoonosis. The use of some side-effective and non-specific drugs such as ribavirin in the treatment of infected patients, the absence of a vaccines against NWAs other than JUNVC1, and even the fact that this vaccine is not approved in many countries, adds importance to the investigations against arenaviruses.

We searched for the host factors that could be important in arenavirus infection by performing an siRNA screen. Ten genes scored well in the screen and were further tested using MLV pseudotyped viruses bearing glycoproteins from a NWA, a retrovirus, and a rhabdovirus (Figure 2). The first of these factors tested was TRIM2 which appeared to be antiviral only against NWAs when tested with pseudoviruses. This factor was chosen for further pursuit as it raised a possibility of

contributing to the development of specific anti-arenaviral treatments. We investigated this factor with another NWA, TCRV, and OWAs to further test its specificity (Figure 5 and 6). In the *in vitro* study conducted with TRIM2 and SIRPA (a factor known to interact with TRIM2) performed in Figures 5, it was observed that the amount of TCRV (a replication competent virus) infection increased in cells where TRIM2 and SIRPA levels were lower than the control (Figure 5A). On the other hand, the amount of virus infection was under control in cells where the levels of these factors were increased by using overexpression plasmids (Figure 5B, 5C and 12A). However, changing the TRIM2 levels did not affect pseudotyped LCMV nor Lassa virus infection levels -OWAs- but instead, only affected Junin virus infection -a NWA- (Figure 6). Parallel results were obtained when replication competent JUNV C1 and LCMV were used upon overexpression of TRIM2 (Figure 12). The results show that SIRPA is also an anti-arenaviral factor like TRIM2. This is because the absence of it not only made the U2OS cells more prone to the infection, but the supplement of SIRPA could protect the cells from NWA infection (as evidenced by the increase in TCRV infection when SIRPA expression was suppressed by siRNAs) (Figure 5A). This was also made evident by the decrease in TCRV and JUNV C1 infection when SIRPA was overexpressed (Figure 5B and Figure 12A). Unlike TRIM2, SIRPA was antiviral against OWAs as well, evident by the decreased LCMV infection levels when overexpressed (Figure 12B). This means that TRIM2 is a specific molecule against NWAs while SIRPA is an antiviral with a broader capacity. The fact that SIRPA behaves in the same way against JUNV C1 indicates that this factor may work with TRIM2 in NWA restriction. Future studies will shed light on the way to resolve the specificity of the anti-arenaviral mechanisms of TRIM2 and the action of SIRPA in working together with TRIM2.

Studies using different strains of TRIM2 knockout mice shown in Figures 7 and 8, were tested to see whether TRIM2 is functional against arenaviruses *in vivo.* KO mice A/A and B/B were efficient in restricting arenavirus infection but not OWA as these mice, who lacked TRIM2, were worse in controlling NWA infection, evident by JUNV C1 and TCRV infections (Figure 8C and 8D) compared to their WT littermates (Figure 8C and 8D): the OWA infection was controlled at the same level with the WT mice, evident by LCMV infection (Figure 8E). The resulting findings from these experiments confirmed the importance of the activity of this protein in restricting NWA but not OWA infection *in vivo*. Moreover, the results of this investigation suggest a role for TRIM2 in its antiviral mechanism

that is different than of the E3 ubiquitin ligase role commonly described for TRIM2. This is because the animals strain A/A and B/B (in which the NWA infection is controlled better than in their WT littermates) were the animals that did not lose their RING domains unlike C/C. However, the C/C strain did not only operate the same upon OWA infection with the WT but also upon NWA infection although its RING domain is impaired (Figure 8C, 8D and 8E).

Specific studies pursued with these factors will not only reveal what kind of mechanism TRIM2 inhibits the virus, but also potential theurapeutic pathways or targets may be revealed. One of the possible factors could be acting in this mechanism is a factor called paxillin. Paxillin is a protein expressed at the focal cell membrane and known for its importance in vesicle transport (Nogalski et al., 2011). During cell adhesion and movement, paxillin is known to have a critical role in regulating the actin rearrangement (Nogalski et al., 2011). It was shown to be important for Human Cytomegalovirus (HCMV) entry while the infection leads to a rapid induction in phosphatidylinositol-3-kinase activity (Nogalski et al., 2011). Phosphatidylinositol binding clathrin assembly protein (PICALM) recruits clathrin at sites of clathrin-vesicle assembly and thus enables TfR1 recycling via clathrin-mediated-endocytosis (Kaksonen & Roux, 2018). Previous reports by Tsai & Discher have discussed that CD47-SIRPA affects paxillin and myosin 197 phosphorylation as well as F-actin recruitment (Tsai & Discher, 2008). This might be the mechanism TRIM2-SIRPA use when they act upon NWA infection. Upon NWA infection an activate cytoskeletal assembly with rapid accumulation of phosphopaxillin may be playing a part in the act of TRIM2 and SIRPA on controlling infection. The putative crosstalk between TRIM2-SIRPA-paxillin should be examined as it might reveal which molecules undergo a couple of modifications such as phosphorylation before, during and after arenavirus internalization.

CLDN2, another factor examined, was found to be antiviral against all pseudoviruses carrying glycoproteins from VSV, MMTV and NWAs (Figure 2). Evident by the results in Figure 9, where the studies performed with replication competent JUNV C1 and TCRV NWAs, proved CLDN2 is not a significant antiarenaviral protein against JUNV C1 (Figure 9A) unlike what was expected based on the presented results in Figure 2. This can be due to the use of pseudoviruses where CLDN2 was revealed as an antiviral factor when tested with a replication competent yet attenuated virus strain, JUNV C1. This finding might be suggesting that the virus could not increase a cell autonomous

immune response high enough to restrict the viral entry; or perhaps due to the fact that the experiments performed with JUNV C1 used less virus per cell than TCRV virus (MOI:0.1 versus MOI:1). Nevertheless, the infection with the TCRV virus shows that CLDN2 is significantly antiviral to this virus (Figure 9B). Comparing the two tables, although not significantly, it can be seen that JUNV C1 actually causes an increase in infection in cells where CLDN2 is depleted by using siRNAs, in comparison to the control cells (Figure 9A) along with TCRV (Figure 9B). Occludin is defined as a type of TJ that could interact with CLDN2. The increase in this protein caused a decrease in viral RNA level contrary to CLDN2. This decrease is seen when tested with both JUNV C1 and TCRV, although not significantly in JUNV C1 infection (Figure 9A and 9B). JUNV C1 is an attenuated virus with the differences in its sequence and the amino acid structure from the disease causing WT virus (Goñi et al., 2006), and this might be the reason behind why it does not activate some factors as it does in wild type virus. Ultimately, OCLN can be a factor that works with CLDN2 either directly or indirectly in response to NWA infection. There might be overlapping signaling cascades that serve CLDN2 to act as an antiviral, while OCLN being a factor the virus exploits. Based on a study, OCLN and CLDN1 can be used by HCV-1 (Evans, 2007). My data suggests that occludin is proviral for NWAs as evidenced through the downregulation of occludin which caused an increase in JUNV C1 and TCRV infection (Figure 9). If this is the case, occludin might be targeted for a potential NWA therapy. Baicalin, a drug identified for targeting occludin, may be effective against NWAs (Shen et al., 2020). If occludin is proviral against NWA (Figure 9), CLDN1 might also be proviral for NWA because both are the factors that can facilitate HCV1. Both CLDN1 and occludin increase the epithelial barrier while sharing similarities in topology (Farquhar et al., 2012; Krause et al., 2008; Tong et al., 2011). If analyses confirm CLDN1 is proviral for NWAs, a curative monoclonal antibody therapy may be considered for NWA therapy similar to what was proposed in Mailly et al. (2015) against HCV. The findings might be effective against the other lethal viruses as well, expanding the scope of our investigations well beyond NWA therapies. When overexpressed, CLDN2 could cause a decrease in both JUNV C1 and LCMV infection (Figure 12). In restricting the arenavirus infection, proinflammatory cytokines might also be playing a role. This may be tested by measuring TNFα, IL-6 and IL-13 levels in infected cells knocked-down for CLDN2. It should also be considered that arenaviruses might indirectly cause an increase in CLDN2 levels,

causing a decrease in the progress of the infection. Future studies should analyze the effects of the infection on CLDN2 expression. CLDN2 is known to be able to affect the membrane dynamics, so the stimulation of CLDN2 in response to viral infection might be leading to a rearrangement as a result of a crosstalk between the other molecules and CLDN2. In this case, examining the localization of the CLDN2 levels, the receptor rearrangement and the position of the virus by imaging analyses might be helpful. A suitable model must be selected to perform such studies. A549 which is a human adenocarcinoma cell line which highly expresses CLDN2 protein or Caco-2 cells, a polarized cell line, can be used to observe the effect of loss of this factor in cells more significantly.

SSU72 was found to be antiviral against MMTV and NWAs but it did not have a significant effect on VSV infection (Figure 2). In the experiment performed in Figure 2, using glycoproteins from the three different virus families (Figure 2) was insightful. The findings can be informative of a potential overlapping mechanism if the observed effect on one factor is the same with another virus from a different family. For example, TfR1 can be critical in the action SSU72 plays against NWAs and MMTVs since both viruses usurp the receptor TfR1. The investigation of the relationship between SSU72 and TfR1 receptor may be incorporated into the future SSU72-NWA studies (Figure 2). VSV fuses through early endosomes while NWAs fuse through the late endosomes. Evident by the results in Figure 10, studies suggest that SSU72 is a significant anti-arenaviral protein against JUNV C1 (Figure 10A) further confirming the presented results in Figure 2. However, when tested in NR-9456 cells, the loss of SSU72 did not significantly affect the JUNV C1 levels. Again, this can be due to the use of an attenuated strain of JUNV that could not trigger a response as strong as the WT glycoprotein of the virus does. Symplekin (SYMPK) is a protein suggested to interact with SSU72 (Xiang et al., 2010). SYMPK did not have an effect on arenavirus infection, evident by the results obtained in Figure 10. The loss of SYMPK did not result in a significant increase in JUNV C1 infection in either NR-9456 or in U2OS cells. However, when overexpressed, SSU72 was able to significantly reduce both JUNV C1 and LCMV infection (Figure 12). Further studies performed using SSU72 may strongly and further validate it as an anti-arenaviral factor and might reveal that it can be effective against other viruses as well. If the suggested SSU72 drug described in Cho et al. (2018) is proven to be effective as a treatment

method, then it might be considered for use against arenaviruses in human trials (Cho et al., 2018). To this end, SSU72 may be useful against NWAs and perhaps other viruses.

The SSU72 KO embryonic mice obtained from the Jackson Laboratory did not yield any viable offspring capable of being used for *in vivo* infections. I obtained the samples from WT and HET mice to see whether there was any difference in the expression and whether they could be used as a model for *in vivo* infection. Based on RT-qPCR analyses, the expression levels of SSU72 in between WT and HET mice are not significantly different (Figure 13). There is no meaningful difference in JUNV C1 levels between WT and HET samples when infected *ex vivo* (Figure 13). Based on studies done in yeast, SSU72 is essential for cell viability and null mutation is lethal (Sun & Hampsey, 1996). So, for *in vivo* studies a conditional knockout mouse such as SSU72 KO specific to macrophages must be considered.

TRIM2 is specific in restricting the infection of NWA and must be considered for further studies. SIRPA was found to be antiviral against both JUNV C1 and LCMV. Overexpression and infection studies suggest SSU72, CLDN2 and OCLN may have a broad effect on the viruses from different families. These factors may be tested against SARS-CoV-2 virus or pseudotyped viruses with influenza or SARS-CoV-2 glycoproteins to see whether they would have any effect on infection levels. Research observed that SARS-CoV-2 disrupts the barrier integrity as early as 2 days post-infection (Pellegrini et al., 2020). Perhaps, the claudins or occludin could be studied as a target for a potential therapy in human trials. Unraveling the host-virus relations may lead to enabling the enhancement of new medical interventions.

APPENDICES

Appendix A

Sequence Name	Sequence of the Primers Used in RT-qPCR
JUNV-SF	GGGGCAGTTCATTAGCTTCATGC
JUNV-SR	CAAAGGTAGGTCATGTGGATTGTTGG
LCMV-SF	AGA ATC CAG GTG GTT ATT GCC
LCMV-SR	GTT GTA GTC AAT TAG TCG CAG C
SIRPA-F	TTC CAG TGC CTT CCA GCC CT
SIRPA-R	GGT GAT GTT ACC GAT GCG GAT G
SSU72-F	CCG ACA AGC CCA ATG TTT AT
SSU72-R	GCA GGT CTC CTG TTC TCT GG
CLDN2-F	GGG CTA CAT CCT AGG CCT TC
CLDN2-R	GAT GTC ACA CTG GGT GAT GC
TCRV-GPF	TCG GTC ACA GAT GGG ACC AGG
TCRV-GPR	CAG GGT TCT TCA CGT CCT CTG
mTRIM2-F	TGT CTG CAC ACT TTC TGC GAG AG
mTRIM2-R	GTT TGG GCA CGA AAG AGG CTT TC
MmSSU72-F	CAC TCT GGG AGC ATT CCT TAT C
MmSSU72-R	ACT CTT CTC CTC GAA CTC CT
mSIRPA-F	CAT CCA GCC AGC CAA TCC TGT
mSIRPA-R	TCC AGT TCG CCC TCT GGT TCT
mSYMPK-F	CAA GGA GGA GAA GGT GGT AAA G
mSYMPK-R	TGA GAG CCT ACC ACA GAG AT
OCLN-F	TGC ATG TTC GAC CAA TGC
OCLN-R	AAG CCA CTT CCT CCA TAA GG
GAPDH-F	CCCCTTCATTGAC CTCAACTACA
GAPDH-R	CGCTCCTGGAGGATGGTGAT

Appendix B

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

Office of Animal Care and
Institutional Biosafety Committees (MC 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

January 25, 2019

Susan Ross
Microbiology & Immunology
M/C 790

Dear Dr. Ross:

The protocol indicated below was reviewed at a convened ACC meeting in accordance with the Animal Care Policies of the University of Illinois at Chicago on **10-16-2018**. *The protocol was not initiated until final clarifications were reviewed and approved on* **12-12-2018**. *The protocol is approved for a period of* ***3*** years with annual continuation.

Title of Application: Genetics of Resistance and Susceptibility to Virus Infection and Cancer

ACC Number: 18-168

Initial Approval Period: 12-12-2018 to 10-16-2019

Current Funding: ***Portions of this protocol are supported by the funding sources indicated in the table below.***
Number of funding sources: 3

Funding Agency	**Funding Title**			**Portion of Proposal Matched**
NIH	*Role of ABOBEC3 in in viitro restriction of retrovirus infection (Institutional # 00017010)*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
RO1 AI085015	*Funded*	*201602743*	*UIC*	*Susan Ross*
Funding Agency	**Funding Title**			**Portion of Proposal Matched**
NIH	*Role of NA sensors in host-anti-retroviral defense (Institutional # 00021905)*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
RO1 AI121275	*Funded*		*UIC*	
Funding Agency	**Funding Title**			**Portion of Proposal Matched**

Phone (312) 996-1972 • Fax (312) 996-9088 • www.research.uic.edu

College	*Start up funds for APOBEC3-mediated damage of host geneomic DNA in mice and Novel genes involved in arenavirus*			*Other* *Start up funds*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
N/A	*Funded*		*UIC*	*Susan Ross*

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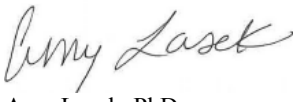

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