

Investigating the effects of protein disulfide isomerase A4 on influenza virus replication in mice
and rabbit hemorrhagic disease virus 3C-like protease in cell death

by

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Abstract

Influenza A virus (IAV) infection is one of the important viral infections with a wide host range including mammals and birds. Protein disulfide isomerases (PDIs) are a diverse family of proteins that act mainly as thiol-disulfide oxidoreductases and redox chaperones facilitating proper folding and rearrangement of proteins. We have previously reported that the knockdown of PDI1, PDIA3, or PDIA4 using siRNAs resulted in a substantial decrease in the replication of both influenza A and B viruses in cells. Therefore, we investigated the effect of PDIA4 on the replication of IAV in constitutive PDIA4-knockout (KO) and wild-type (WT) C57BL/6J mice and PDIA4 KO and WT A549 cells. We observed comparable survival rates and lung viral loads between KO and WT mice and lower virus titers in A549 KO cells compared to WT cells, which indicate that constitutive knockout of PDIA4 did not reduce viral replication in mice, which may be due to compensatory host pathways.

Rabbit Hemorrhagic Disease Virus (RHDV) can cause a highly contagious and often fatal disease that mainly affects domestic and wild European rabbits (*Oryctolagus cuniculus*). RHDV 3C-like protease (3CLpro) processes most cleavage sites on the viral polyproteins to produce functional non-structural proteins and is crucial in viral replication. The 3CLpro and 3C proteases of some viruses have also been reported to play a role in host immunity and inducing apoptosis. Therefore, we investigated the effect of RHDV2 3CLpro in cytotoxicity and its mechanism, and the roles of the catalytic activity of 3CLpro in mediating cell death and cytokine production. Our results showed that the expression of active RHDV2 3CLpro in HEK293T cells led to a significant reduction in cell viability and increased cell death, compared to those of the inactive 3CLpro or the empty vector, via activation of caspase 3/7 activity. The active RHDV2 3CLpro increased IFN-

β and the gamma-activated sequence (GAS) elements promoter activity compared to the inactive 3CLpro or the empty vector. However, a significant increase in IFN- β mRNA expression levels was not observed. These results indicate that RHDV2 3CLpro induces apoptosis by activating caspase 3/7 via the catalytic activity of 3CLpro. Additionally, it suggests that RHDV2 3CLpro may mitigate IFN- β mRNA expression induced by cell death.

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Dedication

I dedicate this dissertation to my advisor Dr.Yunjeong Kim, my family, my love Bingun Perera and my best friend Shashika for their unwavering support, guidance, and encouragement throughout my academic journey .

Chapter 1 - Review of Literature

Classification of influenza viruses

Influenza viruses are single-stranded negative-sense RNA viruses that belong to the family *Orthomyxoviridae*. The name, *Orthomyxoviridae*, was derived from two Greek words, ‘*Orthos*’ referring to ‘correct’, ‘standard’, and ‘*myxso*’ referring to ‘mucus’ (Kibenge & Kibenge, 2016; Kuchipudi & Nissly, 2018). According to the most recent taxonomy release from the International Committee on Taxonomy of Viruses (ICTV), the family *Orthomyxoviridae* is comprised of seven genera and 21 species (*ICTV Taxonomy Release*, 2022). The seven genera are *Alphainfluenzavirus* consisting of Influenza A viruses (IAV), *Betainfluenzavirus* comprised of Influenza B viruses (IBV), *Gammainfluenzavirus* encompass Influenza C viruses (ICV), *Deltainfluenzavirus* consisting of Influenza D viruses (IDV), *Isavirus*, *Mykissvirus*, *Quaranjavirus*, *Sardinovirus* and *Thogotovirus* (*ICTV Taxonomy Release*, 2022; Kuchipudi & Nissly, 2018) (Figure 1.1). Classification of influenza viruses into types, A, B, C, and D is based on the antigenic difference in nucleoprotein (NP) and matrix protein 1 (M1) (Kuchipudi & Nissly, 2018; Shantanam & Muller, 2018).

IAVs have a wide range of host species including humans, birds, swine, horses, seals, mink, anteaters, and toothed whales (Landolt & Olsen, 2007). Wild aquatic birds serve as the natural reservoir for all the subtypes of IAV, and phylogenetic evidence also indicates that all mammalian IAV strains are originated from avian IAV pools (Landolt & Olsen, 2007; Yoon et al., 2014). IBVs and ICVs are predominantly found in humans, while IBVs have been reported in seals and ICVs in pigs and dogs (Wright PF, Neumann G, 2007). IDV was initially isolated in a diseased pig in

Oklahoma in USA in 2011 (Hause et al., 2013), but later cattle were found to be the primary reservoir for IDV (Hause et al., 2014). Even though antibodies against IDV were found in people who were exposed to cattle, whether it can cause infections in humans is unclear (Hause et al., 2014). IAV and IBV cause seasonal influenza epidemics in humans, and IAV is the only influenza virus that has caused flu pandemics (Taubenberger & Morens, 2008). ICV infection in humans is usually associated with mild respiratory diseases, but children younger than 2 years of age can develop severe lower respiratory infections (Sederdahl & Williams, 2020).

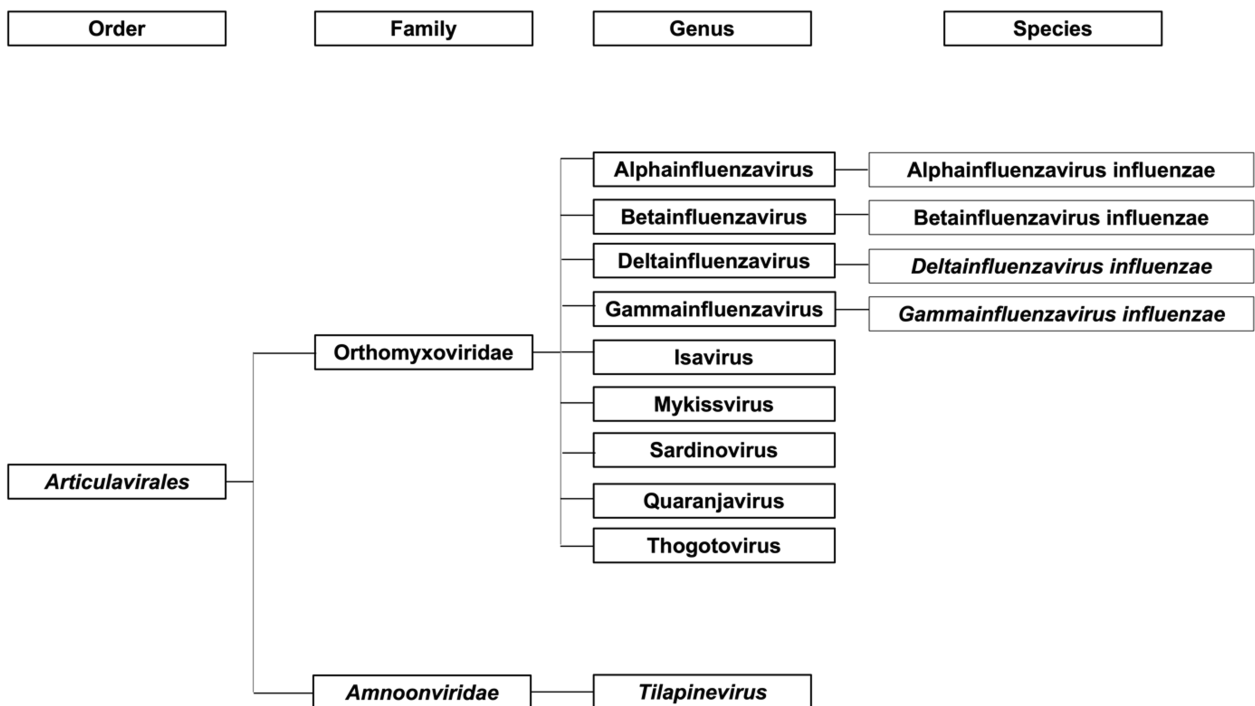


Figure 1.1. Classification of the family *Orthomyxoviridae* based on the International Committee on Taxonomy of Viruses (ICTV) (ICTV Taxonomy Release, 2022).

Structure and genome organization of influenza viruses

Influenza viruses are pleomorphic, spherical, or elliptical and around 80-120 nm in diameter (Abdelwhab & Mettenleiter, 2023; Kuchipudi & Nissly, 2018). Some strains of IAV, IBV, ICV, and IDV can appear as filamentous shapes of varying lengths (Abdelwhab & Mettenleiter, 2023; Wolff & Veit, 2020). Influenza viruses are enveloped viruses with segmented genomes. IAV and IBV have eight single-stranded, negative-sense RNA segments that encode 10-11 proteins. Each segment encodes at least one structural protein, and some segments encode more than one protein (Griffin & Tompkins, 2023; Jeffery & John, 2010; Wolff & Veit, 2020). IAV proteins include polymerase basic 2 (PB2), polymerase basic 1 (PB1), polymerase acidic (PA), hemagglutinin (HA), neuraminidase (NA), nuclear protein (NP), matrix protein 1 (M1), matrix protein 2 (M2), nonstructural protein 1 (NS1) and nuclear export protein (NEP, previously known as NS2) (Neumann G, 2007) (Figure 1.2).

The viral envelope is composed of HA, NA, and M2. HA is the most abundant glycoprotein in the envelope followed by NA and M2 (Hutchinson et al., 2014). Based on the properties of HA and NA, IAV is further divided into 18 HA subtypes (H1-H18) and 11 NA subtypes (N1-N11). The HA binds to sialic acid on the host cell receptor and facilitates cell entry and the NA cleaves sialic acids on the surface of infected host cells and viral particles facilitating the release and spread of newly formed viral particles from the host cell. The M2 protein is an ion channel protein and helps the virus uncoating in the endosome. Three polymerase proteins, PB1, PB2, and PA, are packaged with NP and vRNA to form the viral ribonucleoprotein (vRNP) complex. Some IAV has an accessory protein PB1-F2, which serves as a virulence factor contributing to viral pathogenicity

(Palese, 2011). IBV has four envelope proteins, HA, NA, NB, and M2 protein of influenza B (BM2), where NB and BM2 are unique to IBV and correspond to the M1 protein of IAV (Hatta & Kawaoka, 2003; Neumann, 2007). ICV and IDV have only 7 genome segments (Wolff & Veit, 2020).

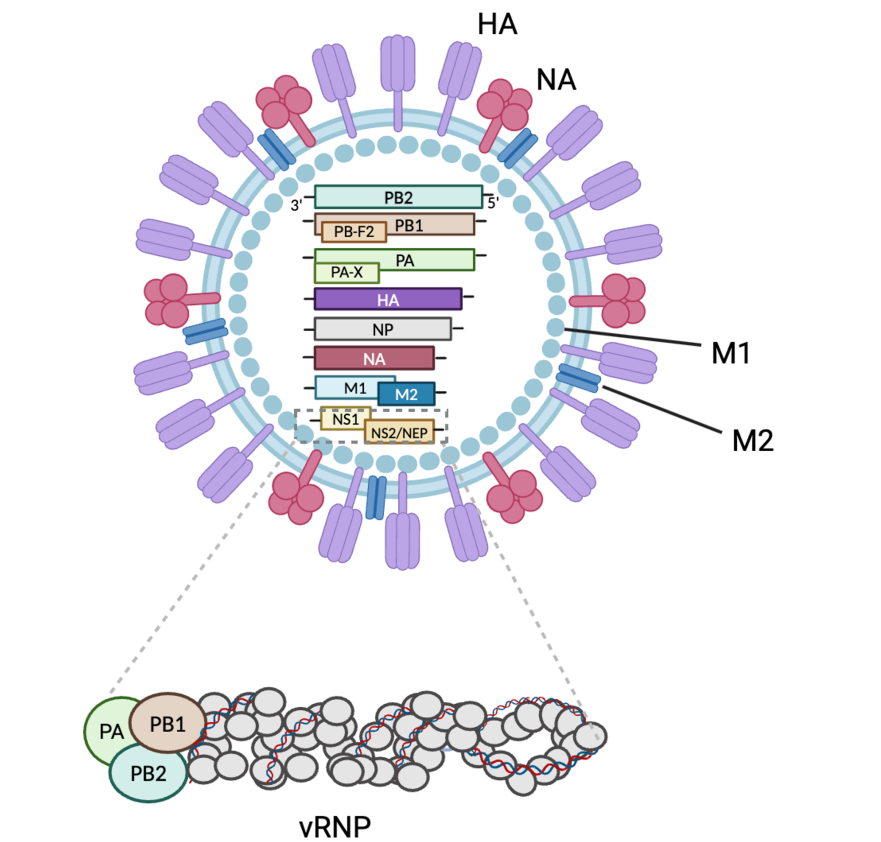


Figure 1.2. Structure and genome organization of IAV. The genome **consists** of 8 segments of negative-sense, single-stranded RNA segments encapsidated by viral ribonucleoprotein (vRNP). The whole genome encodes 10-11 proteins with each segment encoding at least one structural protein. Each vRNP is associated with three polymerase proteins, basic protein 1 (PB1), basic protein 2 (PB2), and the acidic protein (PA), which forms the RNA-dependent RNA polymerase (RdRP) complex. (Image created with BioRender.com)

Replication of influenza viruses

Influenza virus primarily replicates in the epithelial cells of the respiratory tract in humans and other mammals, and the epithelial cells lining the intestinal tract in avian species (Krammer et al., 2018). The virus enters the host cells following the binding of HA and sialic acid receptors on the host cells. Sialic acid present in the terminal of glycans on the host cell surface glycoproteins and glycolipids are linked to the oligosaccharides with different sialic linkages (Baigent & McCauley, 2003; Van Riel et al., 2006). Most human influenza viruses prefer α 2,6-linked sialic acids as the receptor, which are abundantly present in the upper respiratory tract of humans, while avian influenza viruses bind to α 2,3-linked sialic acids, which are abundantly present in the avian gastrointestinal tract (Baigent & McCauley, 2003). Swine acts as a mixing vessel of influenza viruses because they possess both avian and mammalian sialic acid linkages, which allows for co-infection of different viral strains leading to genetic reassortment of influenza viruses (Griffin & Tompkins, 2023).

After binding to the host cell receptor, virus enters the cells via receptor-mediated endocytosis. Acidic pH inside the endosome leads to a conformational change in HA, exposing its fusion peptide, which triggers the fusion of viral and endosomal membranes (Taubenberger & Kash, 2010). Ion channel M2 protein further acidifies the virus interior allowing vRNP release to cytoplasm in a process of virus uncoating (Chauhan & Gordon, 2022). Subsequently, viral genome replication and transcription occur in the nucleus. The nuclear import of newly released vRNPs in the cytoplasm largely depends on several host proteins (Dou et al., 2018; Gabriel et al., 2011; Staller & Barclay, 2021). Viral RNA dependent RNA Polymerase (RdRP) initiates replication by first generating complementary RNA (cRNA) or positive-sense RNA that acts as the template for

the generation of viral RNA (vRNA) (Robb et al., 2016). Viral mRNA transcribed from vRNA lacks a 5' methylated cap, and the viral mRNA obtains the caps from cellular mRNAs by a mechanism called 'cap snatching' to generate mature mRNA (Plotch et al., 1981). Unlike cellular mRNAs, newly generated viral mRNA lacks a polyadenylation signal (AAUAAA) that initiates polyadenylation (Samji, 2009) and undergoes a stuttering mechanism where viral RdRp adds a stretch of U residues by moving back and forth leading to the formation of poly-A tail on the 3' end of viral mRNA and subsequent termination of transcription (Poon et al., 1999). The protein synthesis of IAV completely depends on the translation machinery of the host cell. The newly generated IAV mRNAs undergo translation through two distinct pathways. Cytosolic ribosomes are responsible for translating PA, PB1, PB2, NP, NS1, NS2, and M1 transcripts (AbuBakar et al., 2023), and the nascent proteins are transported into the nucleus. Translation of HA, NA, and M2 transcripts occur in the Endoplasmic reticulum (ER)-associated ribosomes and nascent proteins travel through the Golgi apparatus to reach the plasma membrane (AbuBakar et al., 2023).

HA is initially synthesized as a precursor protein, HA₀, which becomes N-glycosylated and trimerized in the ER. The proteolytic cleavage of precursor HA₀ into disulfide bonded HA1 and HA2 is needed to generate infectious virus particles. This cleavage can be initiated either in the Golgi network or in the plasma membrane by host proteases (Carter & Iqbal, 2024). HA, which contains a polybasic cleavage site is cleaved by furin, a ubiquitous endoprotease, in the trans-Golgi network. HA with a monobasic cleavage site is cleaved by cellular trypsin-like serine proteases, which are found in the respiratory or intestinal tract. HA cleavage can occur when HA is present on the plasma membrane or in the trans-Golgi network, during assembly and budding or extracellularly after the virus is released from the cell (Eva Böttcher-Friebertshäuser et al., 2014).

HA cleavability may affect tissue tropism, pathogenicity, and virulence of influenza infection in the host (Bruin et al., 2022; Steinhauer, 1999). Once vRNA copies are synthesized in the nucleus, NP binds to them, initiating the assembly of PA, PB1, and PB2 proteins and ultimately forming the vRNP complex (Dou et al., 2018). Subsequently, the vRNPs are exported from the nucleus with the help of M1, NP, and NS2/NEP (Brunotte et al., 2014). The vRNPs are then directed to the plasma membrane where HA, NA, and M2 are located. When all the newly synthesized viral components are assembled at the membrane, budding of viruses occurs which is facilitated by NA cleaving the sialic acid residues on the newly formed virion and host receptors (AbuBakar et al., 2023; Dou et al., 2018; Nayak et al., 2009)

Influenza A viruses in animals

IAV infects a broad range of hosts including avian and mammalian species (Landolt & Olsen, 2007; Munster et al., 2007). The primary reservoir for the antigenically diverse IAV is believed to be wild aquatic birds (Taubenberger & Kash, 2010). IAV mainly infects the gastrointestinal tract of aquatic birds and can be transmitted among wild birds via the fecal-oral route (Caliendo et al., 2020). Unlike DNA polymerase, vRdRp of the influenza virus lacks a proofreading function, leading to higher mutation rates. Antigenic drift allows immune escape of IAVs and IBVs and may lead to yearly seasonal influenza epidemics (Moghadami, 2017; van der Sandt et al., 2012). Genetic reassortment can occur when two or more influenza viruses co-infect a host and undergo genetic exchange by swapping gene segments (Balish et al., 2009; van der Sandt et al., 2012). If this leads to novel reassortant viruses which can be efficiently transmitted among humans it may lead to epidemics or pandemics (De Jong et al., 2000).

Avian influenza

The major reservoirs for IAV are aquatic birds and shorebirds in the orders *Anseriformes* and *Charadriiformes* (Bodewes & Kuiken, 2018; Munster et al., 2007; Yoon et al., 2014). Wild birds are mostly asymptomatic following IAV infection (Bodewes & Kuiken, 2018) and often spill the viruses to domestic poultry and sometimes to mammals. Avian influenza in poultry can be divided into low pathogenic avian influenza (LPAI) and high pathogenic avian influenza (HPAI) (Horimoto & Kawaoka, 2005). This classification is mainly attributed to the presence of monobasic or multibasic cleavage sites in the HA protein, which affects the virus's ability to spread systemically within the host.

LPAI virus (LPAIV) has a monobasic cleavage site in HA, which is cleaved by cellular trypsin-like serine proteases, which are found in the respiratory or intestinal tracks in birds (Horimoto & Kawaoka, 2005). Some HA subtypes of avian IAV, such as HA5 and HA7, have a polybasic sequence R-X-R/K-R at the cleavage site in HA (Taubenberger & Kash, 2010; Krammer et al., 2018), which is associated with severe disease outcomes (Steinhauer, 1999). The polybasic cleavage site in the HA of HPAI viruses increases the cleavage of HA by ubiquitous cellular proteases such as furin, allowing systemic replication of the virus (Compans & Oldstone, 2014). In recent years, transmission of HPAIV strains, including H5N1, H5N8, and H7N9, has posed notable threats to public health (Sutton, 2018). Among them, H5N1 is of particular importance for its pathogenicity, demonstrating high mortality rates in both chickens and humans (Kim et al., 2023; Oduoye et al., 2023).

More recently, H5N1 infection has been reported in a wide range of mammals, including ferrets, opossums, pigs, raccoons, wild otters, sea lions, black bears, badgers, fisher cats, leopards, foxes, bobcats, and cattle (Abdelwhab & Mettenleiter, 2023; Marangon, et al., 2023; Melidou, et al., 2023; Lindh et al., 2023). Most of these infections did not lead to mammals to mammal transmission (Gilbertson & Subbarao, 2023), but outbreaks of H5N1 in seals (Callan et al., 1995; Hinshaw et al., 1984; Puryear et al., 2023), mink farms (Agüero et al., 2023), and the most recent outbreak of H5N1 in dairy cows in 2024 have shown more efficient mammal-to-mammal transmission of the disease. According to the Centers for Disease Control (CDC), more than 890 human infection cases with H5N1 IAV have been reported since 1997 in 22 countries with approximately 50% case fatality rate. In 2024, several dairy workers, who had been exposed to infected dairy cows, were infected with H5N1 with no reported human deaths among these cases. (Bird Flu, CDC, 2024). There is no evidence of human-to-human transmission in the current outbreak and reported cases have been linked to direct contact with infected dairy cows.

AIV infection can spill over to humans and other mammals mainly in two ways. IAV can transmit directly from birds to mammals and undergo natural selection over time to better adapt to mammals (M. Chen et al., 2023; Meng et al., 2022). Another way has been the coinfection of pigs with an avian influenza virus and a swine influenza virus, resulting in reassortant viruses that are capable of infecting mammals (M. Chen et al., 2023; Lipsitch et al., 2016). H5N1 viruses isolated from mammals exhibit mammalian adaptive mutations in the PB2 gene that increase virus binding to α 2,6-linked receptors over α 2,3-linked receptors (Alkie et al., 2023; Bussey et al., 2010; Du et al., 2020). These outbreaks of H5N1 with mammal-to-mammal transmission and sporadic human infections raise a concern of creating future avian influenza epidemics and pandemics.

Swine influenza

Swine influenza can cause an important swine disease in pigs and swine influenza has become endemic in many regions of the world with a high morbidity of up to 100% and mortality of around 10-15% (Ma, 2020). At present, the major swine influenza subtypes circulating in swine herds globally are H1N1, H3N2, and H1N2 (Lewis et al., 2016). The history of swine influenza dates back to 1930 when the first swine influenza A was isolated in the USA (Shope, 1931). This swine influenza isolate was similar to the 1918 H1N1 human influenza A pandemic strain and was named ‘classical swine influenza A H1N1’ (Schultz-Cherry et al., 2011). Animal exportation from the USA to Europe led to the emergence of an antigenically distinct strain of classical swine influenza A H1N1 strain in Europe in 1979 (Zell et al., 2012). This new strain is of avian origin and was known as ‘Eurasian avian-like H1N1’ (Anderson et al., 2016), and it has rapidly surpassed the classical swine influenza A H1N1 by spreading rapidly across Europe and Asia (Brown et al., 1998).

Until 1990, these two swine influenza virus lineages were the main ones circulating in swine, and later multiple reassortment viruses from pigs led to the diverse swine influenza viruses in different parts of the world (Mancera Gracia et al., 2020). Triple reassortant swine influenza H3N2 with genes derived from avian (PB2 and PA avian genes from North America), human (PB1, HA, and NA genes from human seasonal H3N2), and swine (NP, M, and NS from classical swine H1N1) were isolated from pigs in the USA in 1998 (Webby et al., 2000; Zhou et al., 1999). The triple reassortant H3N2 viruses were further reassorted with existing swine virus lineages to generate novel triple reassortants such as H1N1 or H1N2 (Newman et al., 2008; Shinde et al., 2009; Vincent et al., 2008). The emergence of pandemic swine influenza H1N1 in 2009 (H1N1pdm2009) was

the result of reassortment between the triple reassortant H1N1 subtype from the USA with Eurasian avian-like H1N1 viruses in Europe (Mena et al., 2016; Vincent et al., 2009). H1N1pdm2009 has genes PB2, PB1, and PA, HA, NS, and NP derived from triple reassortant H1N1 subtype and other two genes NA and MP derived from Eurasian avian-like H1N1 (Brockwell-Staats et al., 2009; Shinde et al., 2009). Similar to the H1N1 virus from the 1918 pandemic, the H1N1pdm2009 virus has repeatedly undergone transmission from humans to swine (reverse zoonosis) (Nelson & Vincent, 2015), which resulted in the generation of multiple swine influenza reassortants affecting both humans and pigs (Watson et al., 2015).

Protein disulfide isomerases

Protein disulfide isomerases (PDIs) are members of the thioredoxin superfamily (Jacquot et al., 2002), which are involved in oxidation, reduction, and isomerization of disulfide bonds (Wilkinson & Gilbert, 2004) and act as molecular chaperones in ER (Hillson et al., 1984; Y. Zhang et al., 2022). PDI family is comprised of 21 members, their expression levels and patterns can vary significantly depending on the cell type, tissue, developmental stage, and physiological or pathological conditions. This differential expression contributes to the specialized functions of PDIs in protein folding, quality control, and cellular homeostasis in different contexts within the human body. Some PDI family members, such as PDIA1 are widely expressed in almost all cell types and tissues. (Galligan & Petersen, 2012). All the members of PDIs have at least one thioredoxin (TRX)-like a structural domain with or without catalytic cysteine (Cys) motif (Cys-x-x-Cys) (Ellgaard & Ruddock, 2005; Galligan & Petersen, 2012; Kozlov et al., 2010). Most PDIs have four thioredoxin-like domains identified as a, a', b, b' (Ellgaard & Ruddock, 2005). The a and a' domains have a catalytic motif and functionally resemble thioredoxin. The b and b' domains

lack catalytically active Cys and act as spacers, which are often involved in bringing the protein substrate closer to the catalytic domains for disulfide bond rearrangement (Kozlov et al., 2010). N- and C-terminus of mammalian PDIs are important in calcium binding and facilitating protein interactions with other ER chaperones (Kozlov et al., 2010; Pollock et al., 2004).

Functions of Protein disulfide isomerases

PDIs are mainly confined to the ER, but they are also found in other cellular compartments such as mitochondria, cytosol, extracellular matrix, plasma membranes, and nuclear envelope (Turano et al., 2002). PDIs also play a role in the pathophysiology of diseases such as cancer (S. Xu et al., 2014), cardiovascular disease (Khan & Mutus, 2014), neurodegenerative conditions (Andreu et al., 2012), diabetes (Grek & Townsend, 2014) and alcoholic and non-alcoholic liver diseases. Upregulation of PDIs has been reported in some cancers including lung, brain, ovarian, prostate, kidney, melanoma, and male germ tumors (S. Xu et al., 2014), which suggests their potential use as a diagnostic marker (Goplen et al., 2006; Thongwatchara et al., 2011). In addition to their involvement in diverse diseases, PDIs have been reported to play a role in the infections of some parasites, viruses, and bacteria (Abromaitis & Stephens, 2009; Auwerx et al., 2009; Stolf et al., 2011).

Protein disulfide isomerase A3 (PDIA3), which is also known as ERp57, is a 57 kDa protein with four TRX-like domains: a, b, b', and a' where a and a' domains have the tetrapeptide catalytic sites Cys-Gly-His-Cys for redox activity. PDIA3 is involved in the proper folding of glycoproteins in the ER by interacting with ER chaperones calnexin (CNX) and calreticulin (CRT) (Oliver et al., 1999). The terminal glucose residues of the nascent N-glycosylated glycoproteins in the ER are

sequentially removed by Glucosidase I and II before they enter the CNX-CRT cycle. PDIA3 interacts with CNX and CRT to facilitate the proper folding of the glycoproteins by disulfide bond formation. PDIA3 also plays a major role in the presentation of antigens by major histocompatibility complex I (Mahmood et al., 2021).

Protein disulfide isomerase A4 (PDIA4), also known as ERp72, is comprised of 645 amino acids (Wang et al., 2020) and has five TRX-like domains including three 'a' domain that have three Lys-Glu-Glu-Leu active sites (Sato et al., 2005). PDIA4 is a crucial component of the multiprotein chaperone complex comprised of proteins such as Ig binding protein (BiP), PDI, ERp29, and Glucose-regulated protein 94 (Grp94). This complex is integral in regulating the redox state of proteins and influencing cellular secretion (Meunier et al., 2002). PDIA4 interacts indirectly with CRT through PDIA5 (PDIR) and helps the proper formation of disulfide-bonded glycoproteins (Patel et al., 2020; Solda et al., 2006). PDIA4 shares a 40% amino acid sequence identity with PDIA3 (Powell & Foster, 2021).

PDIA3 and PDIA4 have been studied as a potential marker and a therapeutic target for some cancers such as renal carcinoma, lung cancer, breast cancer, gliomas, and hepatocellular carcinomas (Kang et al., 2023; Wang et al., 2020; Yang et al., 2022). The involvement of PDIA3 has also been studied in various neurological diseases, including Alzheimer's disease and amyotrophic lateral sclerosis (Bargsted et al., 2016; Montibeller & de Belleruche, 2018). PDIA4 and PDIA3 are also associated with an increased risk of metabolic syndrome related to insulin resistance, obesity, glucose intolerance, hypertension, and dyslipidemia (Antoniotti et al., 2022; Chien et al., 2017; Clement et al., 2022).

PDIA3 has been reported to play key roles in some viral infections including dengue virus, zika virus, Newcastle disease virus, hepatitis B virus, and human immunodeficiency virus (HIV) (Auwerx et al., 2009; Jain et al., 2009; Wan et al., 2012). In the influenza virus, PDIA3 is reported to be directly involved in the folding of influenza virus HA and NA (Chamberlain et al., 2022). PDIA3 knockdown using siRNAs has been shown to inhibit the maturation of influenza virus HA and suppress influenza virus replication in cells (Solda et al., 2006). Moreover, suppression of IAV burden and inflammation has been also shown using mice treated with LOC14, a PDIA3 inhibitor, and transgenic mice where lung specific PDIA3 knockout is induced with doxycycline (Chamberlain et al., 2019, 2022). However, constitutive knockout of PDIA3 was observed to be embryonic lethal in mice (Coe et al., 2010). PDIA4 has also been reported to play crucial roles in several virus infections. It has been identified as a host factor that facilitates lymphocytic choriomeningitis virus (LCMV) infection in cells, and elevated PDIA4 levels in plasma samples from HIV patients correlate with inflammation, marked by significant expression of IL-6 and tumor necrosis factor- α (Wang et al., 2023; Xu et al., 2023). The PDIA4 levels in the plasma samples of HIV patients were positively associated with inflammation with significant expression of IL-6 and tumor necrosis factor- α (Wang et al., 2023). Additionally, PDIA4 involved in the genome uncoating process during the entry of human astrovirus into host cells (Aguilar-Hernández et al., 2021).

We have previously reported the effects of siRNA knockdown of PDIA1, PDIA3, or PDIA4 expression resulted in a substantial decrease in the replication of both influenza A and B viruses in cells with the most pronounced effect observed with PDIA4 suppression (Kim & Chang, 2018). However, the role of PDIA4 in IAV replication in transgenic mice with the constitutive knockout

of PDIA4 has not been reported to date. Unlike PDIA3 mice, PDIA4 knockout mice do not exhibit noticeable phenotypes except for abnormality in blood clotting due to reduced platelet aggregation (Almeida et al., 2011). Therefore, we conducted experiments in Chapter 2 to determine the role of PDIA4 in IAV replication and inflammation in transgenic mice with a constitutive knockout of the PDIA4 gene.

Classification of rabbit hemorrhagic disease viruses

Rabbit Hemorrhagic Disease (RHD) is caused by a non-enveloped, single-stranded positive-sense RNA virus that belongs to the genus *Lagovirus* in the family *Caliciviridae* (Ohlinger et al., 1990). According to the proposed unified classification system by Le Pendu et al. (2017), the genus *Lagovirus* has a single species of *Lagovirus europaeus* (Le Pendu et al., 2017). The species is subdivided into two genogroups, GI and GII, based on the VP60 capsid sequence. GI comprises four genotypes GI.1 to GI.4. GI.1 (classical RHDV) and GI.2 (RHDV2) are pathogenic viruses and GI.3 and GI.4 are non-pathogenic viruses. GI.1 is further subdivided into G1.1a– GI.1d. GII.1 are European brown hare syndrome virus (EBHSV) and GII.2 are non-pathogenic hare calicivirus (HaCV) (Figure 1.3) (Le Pendu et al., 2017). Recombinant RHDVs have been reported to occur within the same or between different genotypes (Mahar et al., 2021; Szillat et al., 2020). Recombination of RHDV mostly occurs at the junction between nonstructural gene RdRp and structural gene VP60 (Mahar et al., 2021). The recombinant RHDVs are designated as [RdRp genotype] P-[capsid genotype] (Le Pendu et al., 2017). Phylogenetic analysis has shown that all RHDV2 are recombinants containing orphan capsid (Abrantes, Droillard, et al., 2020).

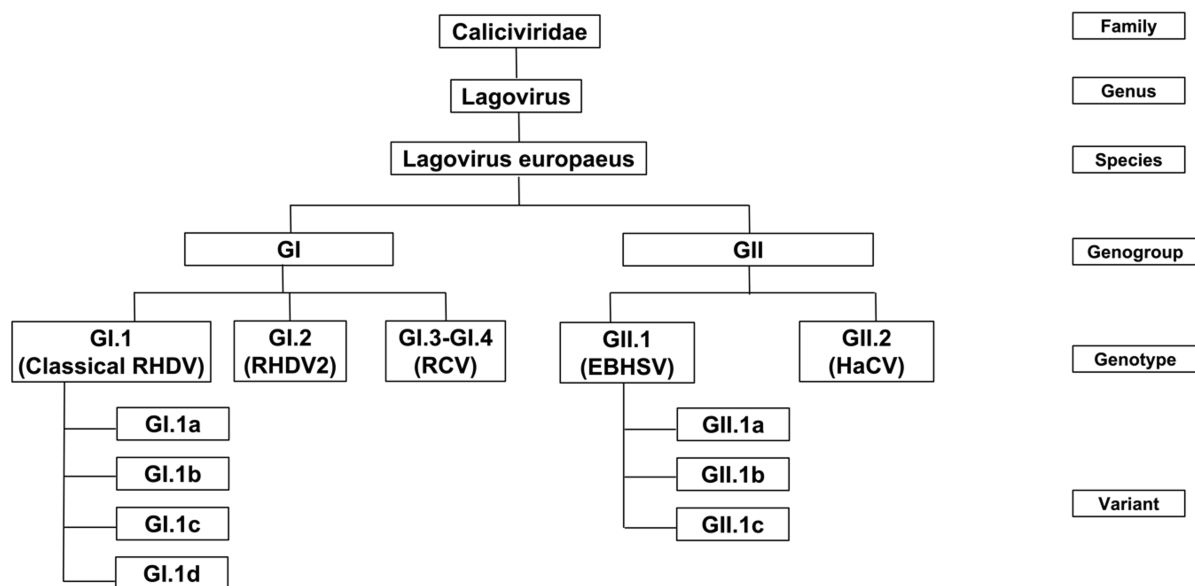


Figure 1.3. Classification of lagoviruses. The taxa organization of lagoviruses within the *Caliciviridae* family proposed by Le Pendu and colleagues (Le Pendu et al., 2017). The genus *Lagovirus* is classified into two genogroups, GI and GII, which are further divided into genotypes and variants.

Rabbit hemorrhagic disease viruses

Rabbit hemorrhagic disease virus (RHDV) is a highly contagious and pathogenic lagovirus that affects primarily wild and domestic European rabbits (*Oryctolagus cuniculus*) (Lavazza et al., 1996). Classical RHDV was first detected in 1984 in China among rabbits imported from Europe, and later it spread rapidly through Asia, Australia, and Europe to become endemic in many countries (Abrantes et al., 2012). Adult rabbits infected with classical RHDV exhibit high morbidity, reaching up to 100%, and high mortality rates ranging from 70% to 90%. Young rabbits aged 6 to 8 weeks are less prone to disease while kits younger than 30 days old remain unaffected by the virus (Abrantes et al., 2012).

In 2010, RHDV2, a genetically and antigenically distinct variant of classical RHDV, emerged in France (Le Gall-Reculé et al., 2011). Within a few years, it spread across Europe and other regions of the world (Abrantes, Droillard, et al., 2020; Asin et al., 2021; Le Gall-Reculé et al., 2017; Rouco et al., 2019). Before 2020, sporadic and isolated incidences of RHDV2 infection in European rabbits had been reported in the USA (Rouco et al., 2019). However, in 2020, RHDV2 was reported in native North American lagomorphs, including wild desert cottontail (*Sylvilagus auduboni*) and black-tailed jackrabbit (*Lepus californicus*) in the state of New Mexico, USA (United States Department of Agriculture factsheet, 2020). RHDV2 has a wider host range than the classical RHDV, and it can infect various lagomorphs, including European rabbits (*Oryctolagus cuniculus*), cottontail rabbits (*Sylvilagus spp.*) and some hare (*Lepus*) species (Dalton et al., 2014; Hall et al., 2017; Le Gall-Reculé et al., 2011).

RHDV2 can cause disease in young rabbits as early as 15-20 days of age and older rabbits with variable mortality rates ranging 5%-70% (Capucci et al., 2017). The incubation period for RHDV is 1-3 days. There are three forms of RHD. In the peracute form of RHD, rabbits may undergo sudden death without showing premonitory clinical signs (Bonvehí et al., 2019). In the acute form, infected rabbits show depression, anorexia, neurological abnormalities, and respiratory signs before death, and in the chronic form rabbits exhibit anorexia, generalized jaundice, and lethargy and may recover in several weeks (Abrantes et al., 2012). Rabbits with subclinical infection continue to shed the virus for months without obvious disease (Kerr & Donnelly, 2013). Major pathological features of RHD include massive hepatic necrosis, splenomegaly, hemorrhages, and congestion in several organs such as the trachea, heart, lungs, and kidneys, which are associated with disseminated intravascular coagulation (Abrantes et al., 2012; Lankton et al., 2021). Both

classical RHDV and RHDV2 can be transmitted directly through oral, nasal, or conjunctival mucosa, exposure to infected carcasses, urine, feces, hair, food, water or indirectly spread via fomites, clothing, cages, and equipment. Mechanical vectors including fleas, sandflies, blowflies, and scavenging foxes and crows are also involved in disease transmission. (Gleeson & Petritz, 2020). These caliciviruses exhibit remarkable environmental resilience where they can endure freezing conditions and survive in infected carcasses for extended periods (Abrantes et al., 2012).

Structure and genome organization of Rabbit hemorrhagic disease viruses

RHDV is a non-enveloped, icosahedral virus of 30-40 nm in diameter (Abrantes et al., 2012). It contains a polyadenylated positive-sense, single-stranded RNA genome of ~7.4 kb (Wu et al., 2020). The genome consists of two open reading frames (ORF) and is covalently linked to the viral protein VPg via a Tyr-21 residue at the 5' untranslated region (5'UTR) (Meyers et al., 2000). ORF1 encodes a polyprotein that contains seven non-structural proteins of p16, p23, helicase, p29, VPg, 3CLpro, RdRp, and a major structural capsid protein (VP60). ORF2 encodes the minor structural protein VP10 (Droillard et al., 2021). During replication, the viral polyprotein undergoes post-translation cleavage by virus-encoded 3CLpro into mature nonstructural proteins, which are required for virus replication (Figure 1.4). The subgenomic polyadenylated RNA (sgRNA) of about 2.2 kb also contains these two overlapping ORFs, however, the first ORF lacks the coding sequence for the non-structural proteins (Abrantes, Lopes, et al., 2020). sgRNA is packaged with the virion along with genomic RNA. VP10, together with VP60, forms the capsid and it has also been reported that VP10 plays an important role in regulating virus replication (Liu et al., 2008). The capsid protein VP60 is crucial for host range, antigenicity, and pathogenicity (Mahar et al., 2021). VP60 comprises three domains, the N-terminal arm (NTA), the shell (S), and the protrusion

(P). P domain is further divided into two subdomains called P1 and P2. The P2 subdomain is mainly associated with antigenic diversity where most of the variations between different strains of RHDV occur (Debbink et al., 2013).

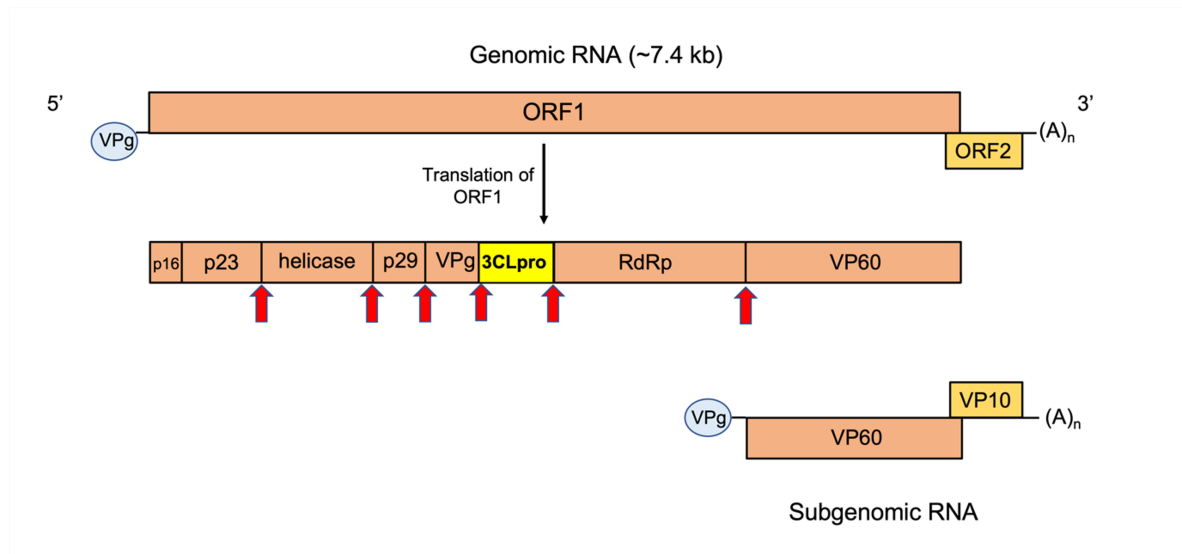


Figure 1.4. Genomic organization of RHDV. RHDV genome consists of two ORFs, ORF1 and ORF2 which are overlapping. ORF1 encodes a polyprotein which is cleaved by virus-encoded 3CLpro into mature nonstructural proteins; p16, p23, helicase, p29, VPg, and RdRp and capsid protein VP60. ORF2 encodes for the minor structural protein VP10. The 3CLpro cleavage sites processed by 3CLpro in the polyprotein are indicated with red arrows. A subgenomic RNA can be found within the virus that encodes for structural proteins VP60 and VP10. Both genomic and subgenomic RNAs are polyadenylated at the 3' end and covalently bound to the virus-encoded protein VPg at the 5' end.

Structure and function of RHDV 3CLpro

The virus families of *Picornaviridae*, *Caliciviridae*, and *Coronaviridae* encode a trypsin-like cysteine protease 3C (*Picornaviridae*) or 3CL protease (*Caliciviridae* and *Coronaviridae*) (Sun et al., 2016), which are responsible for most of the cleavage of RHDV polyprotein. The common characteristics of the 3Cpro or 3CLpros of these virus families include having a typical chymotrypsin-like fold, Cys residue serving as the neutrophile in the active site of catalytic triad (Cys, His, Glu/Asp), or dyad (Cys, His), and a preference for Glu/small apolar residues such as Ala or Gly residues at the cleavage site (Kim et al., 2012). RHDV 3CLpro is comprised of 143 amino acids and is notably smaller than picornavirus 3C or coronavirus 3CLpro, which is generally larger than 180 amino acids (Wirblich et al., 1995). RHDV 3CLpro has a catalytic triad of Cys104, His27, and Asp44 (Figure 1.5) (Boniotti et al., 1994).

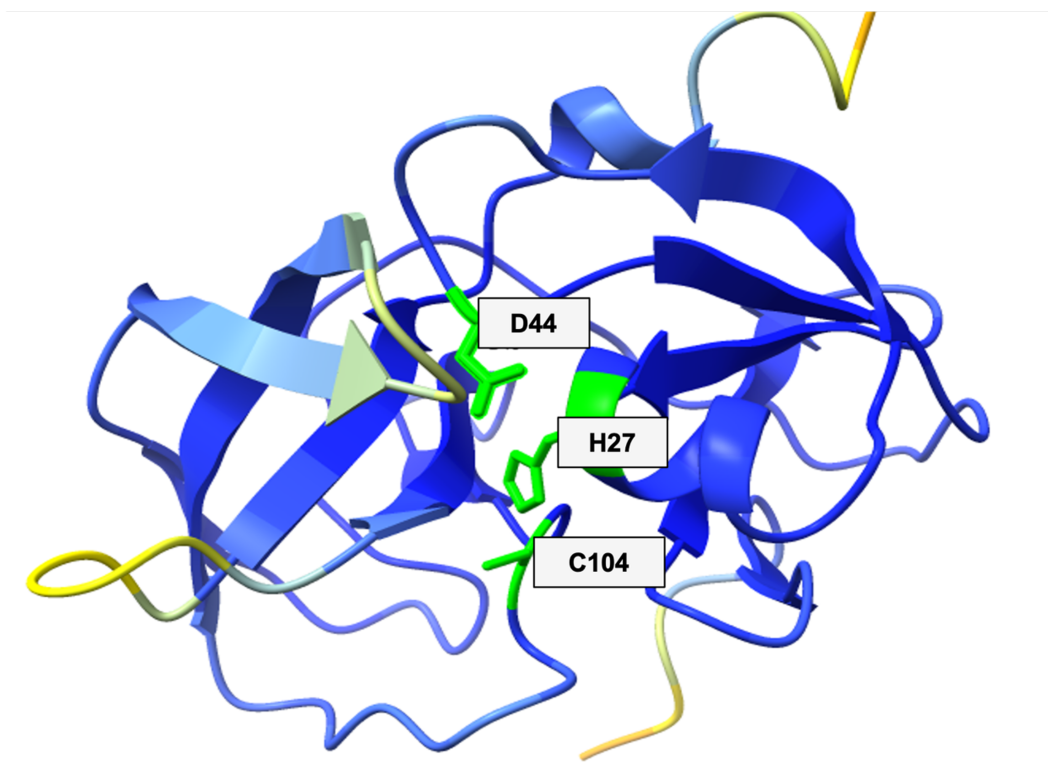


Figure 1.5 The homology model of RHDV2 3CLpro. RHDV2 protein structure (GenBank accession number: QDF46325.1) was generated using AlphaFold 2 and Google CoLab. The amino acids in the catalytic triad of the protease C104, H27, and D44 are marked in green. The 3CLpro is colored in blue (best) to red (worst) according to the confidence level values from the predicted local distance difference test (pLDDT) by AlphaFold 2 software (Jumper et al., 2021).

Role of 3CLpro in apoptosis and modulating host immunity

Previous publications by others reported that 3Cpros and 3CLpros of several viruses induce apoptosis. Picornaviral 3Cpros, including rhinovirus and poliovirus, are involved in the cleavage of transcription and translation initiation factors that are important in cell survival and death pathways, which can lead to apoptosis (Barco et al., 2000; Li et al., 2002; Shubin et al., 2015). Poliovirus 3Cpro and Seneca virus 3Cpro induced apoptosis by degrading cellular DNA by cleavage of poly (ADP-ribose) polymerase (PARP), an enzyme involved in DNA repair (Barco et al., 2000; Fernandes et al., 2019). Enterovirus 71 3Cpro is linked to apoptosis in human neural cells, which can be blocked by caspase inhibitors, suggesting the involvement of caspase activation in the process (Li et al., 2002). SARS-CoV 3CLpro has been shown to induce caspase-dependent apoptosis, although conflicting results have been reported on SARS-CoV-2 3CLpro's ability to induce apoptosis (Komissarov et al., 2022; Lin et al., 2006).

The roles of some viral 3Cpro/3CLpros in modulating host immunity has been reported. The 3Cpro or 3CLpros of hepatitis A virus, Foot and mouth disease virus (FMDV), enterovirus (EV), SARS-CoV-2, and Porcine epidemic diarrhea virus (PEDV) have been reported to inhibit type I interferon (IFN) responses and the nuclear transcription factor kappa B (NF- κ B) signaling pathways by cleaving the key proteins in these pathways (Santos et al., 2007; D. Wang et al., 2016; H. Zhang et al., 2022). Picornavirus 3Cpro and calicivirus 3CLpro are shown to cleave host poly(A)-binding protein which is important for cellular mRNA translation (Joachims et al., 1999; Kerekatte et al., 1999; Kuyumcu-Martinez et al., 2004).

It has been reported that overexpression of classical RHDV 3CLpro mediates caspase-dependent apoptosis through both death receptor-mediated and mitochondrial apoptotic pathways in cells (Chen et al., 2019). In chapter 3 of this study, we investigated the cytotoxic effects and mechanism of cell death of RHDV2 3CLpro in HEK293T cells, and the roles of the catalytic domain of 3CLpro in mediating cell death and cytokine production.

Chapter 2 - Effects of protein disulfide isomerase A4 on influenza virus replication in mice

Influenza viruses are enveloped, negative sense, single-stranded viruses with a segmented genome and belong to the family *Orthomyxoviridae* (Bouvier & Palese, 2008.). Based on the antigenic differences in nucleoprotein (NP), they are classified into four types, A, B, C, and D (Bouvier & Palese, 2008.). Among these, influenza A viruses (IAV) are the most diverse and able to infect a wide range of hosts, including humans, birds, pigs, and other mammals (Landolt & Olsen, 2007). IAVs can be further categorized into subtypes based on the differences in the two major glycoproteins, hemagglutinin (HA) and neuraminidase (NA). HA and NA play a main role in virus entry and release, respectively (Abdelwhab & Mettenleiter, 2023; Hutchinson et al., 2014). Ability of IAVs to undergo genetic drift and genetic shift may lead to the emergence of novel strains with altered virulence and transmissibility (Moghadami, 2017; van der Sandt et al., 2012).

Swine influenza A virus (SIAV) subtypes H1N1, H1N2, and H3N2 are primarily endemic in pig populations globally (Lewis et al., 2016). SIAV infection may cause high morbidity of up to 100% and low mortality in infected pigs, while the mortality of naïve pigs can reach up to 10-15% (Ma, 2020). When Pigs are co-infected with other pathogens including *Mycoplasma hyopneumoniae* , Porcine reproductive and respiratory syndrome virus, and Porcine circovirus 2, which are the agents of the Porcine Respiratory Disease Complex, can lead to higher rates of mortality (Ellis et al., 2004; Thacker et al., 2001) Currently, the economic loss from SIAV alone is estimated to be \$1-5 per pig each year, which is a significant economic burden in the pork industry (Chen et al., 2023). Avian influenza viruses (AIVs) preferentially bind to α -2,3-linked sialic acid, which is

predominantly found in the respiratory and intestinal tracts of birds. In contrast, human-adapted influenza viruses and SIAVs prefer α -2,6-linked sialic acid, a receptor type that is more common in the upper respiratory tract of humans. (Suzuki et al., 2000). Pigs are susceptible to avian, human, and swine-adapted influenza virus infections due to the presence of both α -2,3-linked sialic acid receptors and α -2,6-linked sialic acid receptors in their respiratory tract (Mancera Gracia et al., 2020b). This makes pigs "mixing vessels" for avian and mammalian influenza viruses allowing swine to be a major host reservoir for novel IAVs. Therefore, the availability of effective control and prevention strategies against influenza infections in swine is critical.

There are currently four FDA-approved antiviral drugs for influenza A and B viruses. Zanamivir, Oseltamivir, and Peramivir are NA inhibitors, while Baloxavir marboxil is an endonuclease inhibitor (Jones et al., 2023). Amantadine and Rimantadine are M2 ion channel inhibitors but are no longer used due to increased resistance of seasonal IAVs to these drugs (Ziegler et al., 1999). oseltamivir resistance was rarely seen prior to 2007, but later increasing numbers of oseltamivir resistant IAVs were identified due to the occurrence of NA mutations in influenza variants (Collins et al., 2008; Lampejo, 2020). Furthermore, antiviral susceptibility of Zanamivir and Peramivir has also been reported (Hussain et al., 2017; Leang et al., 2014). In order to avoid the emergence of antiviral resistance, there are efforts to develop combinational drug therapy using two or more antiviral drugs with different mechanisms of action, (Nguyen et al., 2009, 2010)

A number of host factors are associated with influenza virus replication (Husain, 2020). Protein disulfide isomerases (PDIs) are a diverse family of proteins that act mainly as thiol-disulfide oxidoreductases and redox chaperones facilitating proper folding and rearrangement of proteins (Wilkinson & Gilbert, 2004). All the members of the PDI family have at least one thioredoxin-like

structural domain with or without catalytic Cys-x-x-Cys motif (Galligan & Petersen, 2012). PDIs are mainly confined to the ER, but they have also been detected in different cellular compartments including mitochondria, cytosol, extracellular matrix, plasma membranes, or nuclear envelope (Turano et al., 2002). Several studies have reported the roles of PDIs in diverse diseases and host-pathogen interactions of parasites, viruses, and bacteria (Abell & Brown, 1993; Conant & Stephens, 2007; Gilbert et al., 2006). Additionally, PDIs are involved in the pathophysiology of diseases, such as cancer (S. Xu et al., 2014), cardiovascular disease (Khan & Mutus, 2014), neurodegenerative conditions (Andreu et al., 2012) and diabetes.

PDIA3 and PDIA4 are members of the PDI family involved in the proper folding and rearrangement of disulfide bonds. PDIA3 is mainly involved in the folding of newly synthesized glycoproteins in association with calnexin and calreticulin in the ER (Frasconi et al., 2012). PDIA4 is in the ER network with other chaperone proteins such as Hsp70 (BiP), Grp94, and ERp29 to act on unfolded protein substrates (Z. Wang et al., 2020). PDIA4 is also involved in the glycoprotein folding process by indirectly interacting with calreticulin via PDIA5 (PDIR) (Patel et al., 2020). PDIA3 plays roles in mediating various immune responses, including its key roles as a scaffold protein in the peptide loading complex for major histocompatibility complex (MHC) class I molecules (Mahmood et al., 2021). This complex is integral in regulating the redox state of proteins and influencing cellular secretion. PDIA4 and PDIA3 are implicated in different types of cancers (Li et al., 2021; Padariya et al., 2020; Z. Wang et al., 2020), and PDIA3 in neurodegenerative diseases (Andreu et al., 2012; Bargsted et al., 2016). PDIA4 and PDIA3 are also associated with increased risk of metabolic syndrome such as insulin resistance, obesity, glucose intolerance, hypertension, and dyslipidemia (Chien et al., 2017; Clement et al., 2022).

The roles of PDIA3 and PDIA4 in viral infections have also been reported. PDIA3 plays key roles in many viral infections including dengue, Zika, Newcastle disease virus, hepatitis B virus, and human immunodeficiency virus (HIV) (Auwerx et al., 2009; Jain et al., 2009; Wan et al., 2012). The role of PDIA3 in influenza virus replication has been reported in several studies. PDIA3 knockdown using siRNAs has been shown to inhibit the maturation of influenza virus HA and influenza virus replication (Roberson et al., 2012; Solda et al., 2006). Furthermore, the involvement of PDIA3 in the oxidative folding, activity, and stability of influenza A virus HA and NA glycoproteins has been demonstrated (Chamberlain et al., 2022; Wu et al., 2020). Reduction in IAV replication and lung inflammation was observed in PDIA3-ablated lung epithelial cells, mice treated with LOC14, a PDIA3 inhibitor, and transgenic mice with lung epithelial-specific deletion of PDIA3 (Wu et al., 2020).

PDIA4 has been identified as a host factor that facilitates the replication of Lymphocytic choriomeningitis virus infection (Xu et al., 2023). In HIV patients, elevated PDIA4 was associated with inflammation (Wang et al., 2023), and PDIA4 is implicated in the uncoating of human astrovirus upon entry into host cells (Aguilar-Hernández et al., 2021). Interestingly, PDIA3 knockout mice are reported to be embryonic lethal (Coe et al., 2010), while the absence of PDIA4 does not result in any observable altered phenotype in knockout mice, indicating that PDIA4 is not essential for the host (Wu et al., 2020). We have previously shown that the knockdown of PDIA1, PDIA3, or PDIA4 expression using siRNAs resulted in a substantial decrease in the replication of both influenza A and B viruses in cells with the most pronounced effect observed with PDIA4 suppression (Kim & Chang, 2018). In this study, we investigated the effect of PDIA4 on the

replication of influenza A viruses in PDIA4-knockout and wild-type A549 cells and PDIA4 knockout and wild-type C57BL/6J mice.

Materials and Methods

Cells and viruses

Madin-Darby canine kidney (MDCK) cells were cultured in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics penicillin (250 U/ml) and streptomycin (250 µg/ml) and 2 mM L-glutamine. A549 PDIA4 knockout (A549 KO) cells were generated by UBIGENE, Inc (Guangdong, China) by deleting the exon 2 of the PDIA4 gene in chromosome 6 and maintained in Ham's F-12K (Kaighn's) medium supplemented with 10% FBS and antibiotics (penicillin and streptomycin). Cells were cultured in a humidified incubator at 37°C with 5% CO₂. Human influenza A virus A/Porto Rico/8/1934 (H1N1) (PR8) was purchased from American Type Culture Collection (ATCC) (Manassas, VA)

Preparation of virus inoculum

PR8 viruses were grown in MDCK cells in MEM, supplemented with 1:1000 of 1 µg/mL of tosyl phenylalanyl chloromethyl ketone (TPCK)–trypsin (Sigma), and the supernatant was collected and centrifuged at 3000 rpm for 10 mins to remove cellular debris. The viruses were concentrated using sucrose gradient (30%) ultracentrifugation at 25,000 rpm for 2 hrs. at 4°C. Following ultracentrifugation, the pellet was resuspended in DMEM to make a 100-fold concentrated virus stock, which was divided into aliquots and stored at -80°C until use.

Virus infection

WT and KO A549 cells in a 48-well plate at 80-85% confluency was washed twice with PBS to remove any residual FBS and were infected with PR8. After 24 hrs of incubation, the plate was subject to repeated freeze-and-thaw cycles and the cell lysates were collected for qRT-PCR or virus titration using the TCID₅₀ method.

Influenza virus titration using the 50% Tissue Culture Infectious Dose (TCID₅₀) method

MDCK cells in a 96-well plate were washed twice with PBS to remove any residual FBS. Medium containing viruses were serially diluted by a tenfold in media supplemented with 1:3000 of 1 µg/ml TPCK–trypsin and added to each well in a 96-well plate. After 3-4 days of incubation of the plate at 37 °C, the plate was washed twice with PBS, and the cells were fixed with chilled 100% methanol. After washing the cells three times with PBS, cells were incubated with a 1:500 dilution of anti-Influenza A FITC-conjugated antibody (MAB8257F, Sigma-Aldrich) for one hr. Then, cells were washed three times with PBS and examined using a fluorescence microscope, and the TCID₅₀/ml was determined (Reed and Muench, 1938).

Quantitative Real-Time-PCR (qRT-PCR).

qRT-PCR was used for determining the levels of influenza A virus M mRNA, PDIA4 mRNA in A549 cells and mice, and lung cytokine levels of WT and KO mice. Viral RNAs were extracted using the RNeasy kit according to the manufacturer's instructions (Qiagen, Valencia, CA). Primers used for qRT-PCR and the conditions are listed in Table 1. A standard curve was generated by plotting the Ct values from 10-fold dilutions of the PR8 virus against the log₁₀ TCID₅₀/ml virus

titers. The $\Delta\Delta C_t$ method was used to calculate the relative expression levels of cytokine genes, where the C_t values of target cytokines were normalized to those of beta-actin.

Table 2.1: Primers and qRT-PCR profile

Primer sequence (5'-3')	qRT-PCR profile
Influenza A virus M gene Forward-5' CATGGAATGGCTAAAGACAAGACC 3' Reverse- 5' AAGTGCACCAGCAGAATAACTGAG 3' Probe- FAM/CT GCA GCG T/Zen/A GAC GCT TTG TCC AAA ATG /3IABkFQ/	Reverse transcription (RT) at 45°C for 30 min, activation of DNA polymerase at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 1 min, and elongation at 72°C for 30 s.
PDIA4 in WT/KO A549 cells Forward- 5' ATGCCATTGAGGATGAAGAGG 3' Reverse- 5' CATCATTTAGGACCAAGACTCCA 3'	Activation of DNA polymerase at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 1 min, and elongation at 72°C for 30 s.
PDIA4 in WT /KO mice Forward – 5' CTCCACCTGAAGTCACTCTTTC 3' Reverse – 5' GGGCATAGAACTCCACCAAA 3'	
β -actin Forward- 5' CATTGCTGACAGGATGCAGAAGG 3' Reverse- 5' TGCTGGAAGGTGGACAGTGAGG 3'	Activation of DNA polymerase at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 65°C for 1 min, and elongation at 72°C for 30 s.
IL-1 α Forward- 5' ACGGCTGAGTTTCAGTGAGACC 3' Reverse- 5' CACTCTGGTAGGTGTAAGGTGC 3'	
IL-1 β Forward- 5' TGGACCTTCCAGGATGAGGACA 3' Reverse- 5' GTTCATCTCGGAGCCTGTAGTG 3'	
IL-6 Forward- 5' TACCACTTCACAAGTCGGAGGC 3' Reverse- 5' CTGCAAGTGCATCATCGTTGTTC 3'	
IL-10 Forward- 5' CGGGAAGACAATAACTGCACCC 3' Reverse- 5' CGGTTAGCAGTATGTTGTCCAGC 3'	
TNF- α Forward- 5' GGTGCCTATGTCTCAGCCTCTT 3' Reverse- 5' GCCATAGAACTGATGAGAGGGAG 3'	
IFN- γ Forward- 5' CAGCAACAGCAAGGCGAAAAAGG 3' Reverse- 5' TTTCCGCTTCCTGAGGCTGGAT 3'	
TGF- β Forward- 5' TGATACGCCTGAGTGGCTGTCT 3' Reverse- 5' CACAAGAGCAGTGAGCGCTGAA 3'	
GM-CSF Forward- 5' AACCTCCTGGATGACATGCCTG Reverse- 5' AAATTGCCCGTAGACCCTGCT	

Generation of KO A549

The A549 PDIA4 KO cells were generated by UBIGENE, Inc (Guangdong, China) by deleting the exon 2 of the PDIA4 gene located on chromosome 6 using the CRISPR/Cas9 method. Cells are maintained using F12K medium supplemented with 10% FBS and penicillin (250 U/ml) and streptomycin (250 µg/ml) and 2 mM L-glutamine. Cells were maintained in a humidified incubator at 37°C with 5% CO₂.

WT and KO A549 genotyping

DNA was extracted from WT and KO A549 cells using the phenol/chloroform extraction method and amplified using PCR. The primers used for amplification were forward primer -5' CTGCTCCTGCTGCTCTTG 3' and reverse primer- 5'CTGACCTTGGCAACAATTTCTTC 3', which targets the upstream and downstream of deleted exon 2 of the PDIA4 gene respectively. The PCR products were then run on a 1% agarose gel electrophoresis system to visualize the band sizes. the expected band sizes are 473 bps for WT cells (including exon 2) and 290 bps for KO cells (excluding exon 2).

Generation of KO mice

The C57BL/6J PDIA4 knockout mice were generated by CYAGEN, Inc (Santa Clara, CA) using the CRISPR/Cas9 method to knock out the exons 2 to 7 of the PDIA4 gene (out of 10 exons) located on chromosome 6. The WT and KO breeding colonies were established at the animal facility at the comparative animal medicine at Kansas State University.

WT and KO mice genotyping

DNA was extracted from the lungs of the WT and KO C57BL/6J mice using the phenol/chloroform extraction method and amplified using PCR. The primers used for mouse genotyping were F1, F2, and R1; F1: 5'-CAATAACACTCTCTTGCATTCTGGC-3' targeting the upstream of the deleted exon 2 of the PDIA4 gene, F2: 5'-GTCAGTTCCTGGGTATTCTACGTG-3' targeting the deleted region between exons 2-7 of the PDIA4 gene and R1: 5'-TGAAGCTTAAGAGTGGGGTGAGAA-3' targeting downstream of the deleted region of exon 2 of the PDIA4 gene. Following the PCR, gel electrophoresis was carried out to visualize distinct bands for homozygous knockout mice for PDIA4 (one band at 657 bp), heterozygous mice (two bands at 657 bp and 445 bp), and wildtype mice (one band at 445 bp).

Mouse study

C57BL/6J Mice were housed and handled in accordance with the guidelines set by the Association for the Assessment and Accreditation of Laboratory Animal Care, and the study protocol was approved by the Institutional Animal Care and Use Committee. Mice experiment was conducted three times with 22-29 mice per experiment (**Experiment No.1:** Total of 22 mice -10 KO, 11 WT, **Experiment No.2:** Total of 29 mice -13 KO, 18 WT, **Experiment No.3:** Total of 23 mice -11 KO, 13 WT). Both female and male mice were included in each experiment and were assigned randomly into two groups. The mice were anesthetized with isoflurane and intranasally administered with 20 µl of PR8 (2.74×10^6 TCID₅₀/ml) or DMEM. Body weight was monitored daily for 10 days after infection. Mice were euthanized using CO₂ if the weight loss was 20% or more at the end of the study.

Tissue sample collection and preparation

The lung tissues were collected from mice with 20% or more weight loss and control mice with no infection on the 3rd day post-infection for experiments 1 and 2. The left lung lobes were homogenized in PBS using s 2.8mm ceramic beads at a speed of 5.65m/s, two cycles of 45 seconds with 30 sec pause using omni bead ruptor (Omni International, Inc, Kennesaw Georgia). The homogenates were centrifuged at 12,000 rpm for 5-10 mins to remove debris. The supernatant was collected and stored in a -80 °C freezer until qRT-PCR was performed to determine virus titers, cytokine levels, and PDIA4 levels.

Statistical Analysis

Data were analyzed using the GraphPad Prism 10.0 software version 10.2.2 (GraphPad, San Diego, CA) using unpaired t-test or one-way ANOVA.

Results

Genotyping of WT and KO A549 cells

To confirm the deletion of the PDIA4 gene in KO A549 cells, genomic DNA from WT and KO A549 cells were amplified using the primers targeting upstream and downstream of the deleted exon 2 region of the PDIA4 gene. On the gel, the PCR product from WT A549 cells yielded a single band of approximately 473 bp, which is the expected size for the wild-type PDIA4. In contrast, the PCR product from A549 KO cells yielded a band at 293 bp, which corresponds to PDIA4 KO (Figure 2.1).

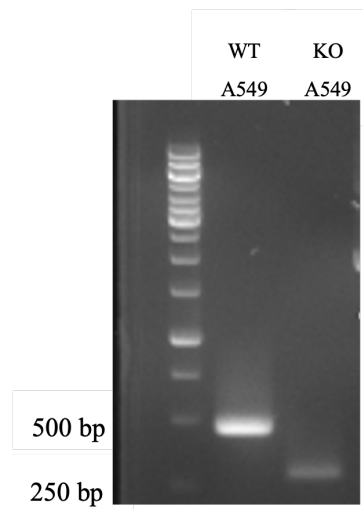


Figure 2.1. Gel Electrophoresis of the PCR Products for PDIA4 genotyping in A549 Cells. The band sizes for the WT and KO PDIA4 are approximately 473 bp and 293 bp, respectively.

PDIA4 mRNA levels in WT and PDIA4 KO A549 cells

PDIA4 mRNA levels were assessed in WT and KO A549 cells using qRT-PCR. The primers used for qRT-PCR targeted exon 2 of the PDIA4 gene, which was deleted in the generation of KO A549 cells. PDIA4 mRNA was not detectable for PDIA4 KO A549 cells, but PDIA4 mRNA expression was observed in WT cells. The high level of PDIA4 mRNA in WT A549 cells confirms the expression of PDIA4, which is absent in the KO A549 cells due to the specific deletion of exon 2 (data not shown).

Influenza virus infection of WT and KO A549 cells

Replication of PR8 in WT and KO A549 cells was determined using qRT-PCR. Influenza virus M mRNA levels in the infected and no infected WT and KO A549 cells that were determined using qRT-PCR were converted to TCID₅₀/ml values using a standard curve. Our findings revealed a significant decrease in viral titers of KO A549 cells compared to WT A549 cells, indicating lower virus replication in KO A549 cells compared to WT A549 cells (Figure 2).

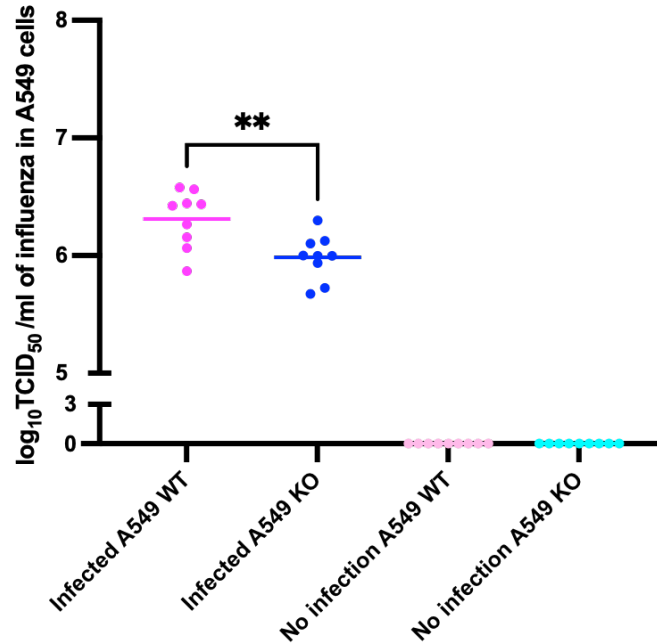


Figure 2.2. Virus titers in WT and KO A549 cells infected with PR8 or no-infection control. Influenza-M mRNA levels in PR8 infected or no infected WT and KO A549 cells were measured using qRT-PCR. The Ct values obtained from the qRT-PCR were converted to log₁₀ TCID₅₀/ml values using a standard curve. * $P < 0.05$, ** $P < 0.01$.

Genotyping of WT and PDIA4 KO mice

To confirm the deletion of PDIA4 in C57BL/6J KO mice, genomic DNA was extracted from the lung tissues of WT and KO mice and amplified by PCR. On a gel, amplification using the primers F1 and R1 yielded a single band of 657 bp for KO mice, but no band was observed for WT mice, which indicates the deletion of PDIA4 gene in KO mice (Figure 2.3B). When DNA was amplified using F2 and R1 primers, a single band of 445 bp was seen for WT mice, but no band was observed for KO mice on the gel (Figure 2.3A). These results confirm the successful knockout of the PDIA4 gene in the KO mice.

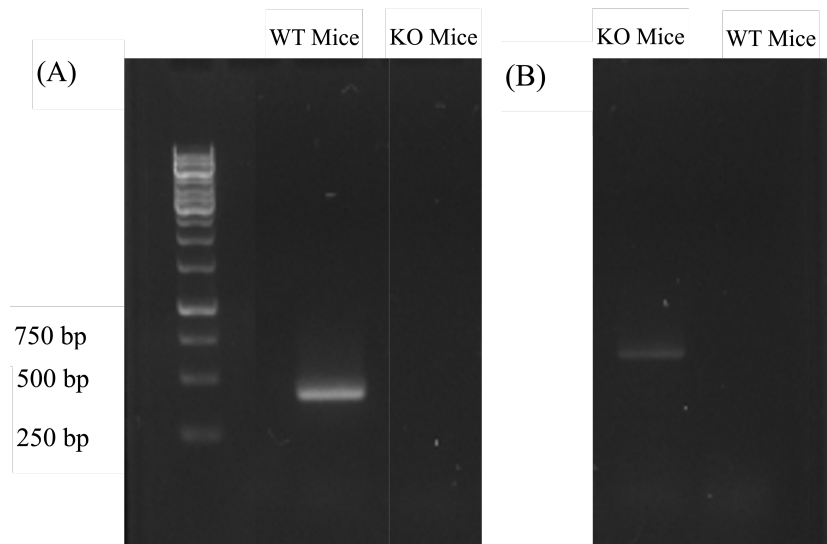


Figure 2.3. Validation of PDIA4 gene knockout in PDIA4 KO mice. DNA extracted from WT and PDIA4 KO mice was PCR amplified using two sets of primers to determine the presence of PDIA4 gene. (A) DNA of WT and KO mice amplified using F2 and R1 primers yielded a band of 445 bp while no band was seen for WT mice. (B) A single band of 657 bp was observed on the gel when the DNA of KO mice was amplified using F1 and R1 primers, whereas no band was seen for WT mice DNA amplified using the same primes.

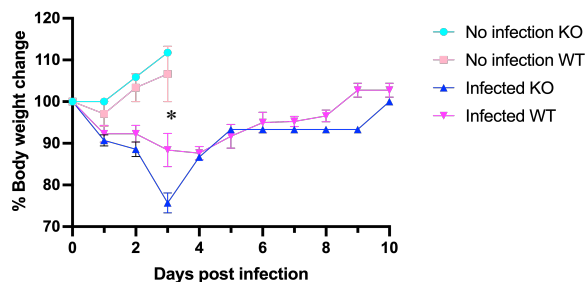
PDIA4 mRNA levels in WT and PDIA4 KO mice

PDIA4 mRNA expression levels in the lungs of WT and KO mice were determined using qRT-PCR. The Primers targeted the deleted exons 2-7 of the PDIA4 gene. As expected, KO mice exhibited very low mRNA expression levels of PDIA4, indicative of the successful knockout of the PDIA4 gene, whereas WT mice showed higher expression levels of PDIA4 mRNA (data not shown)

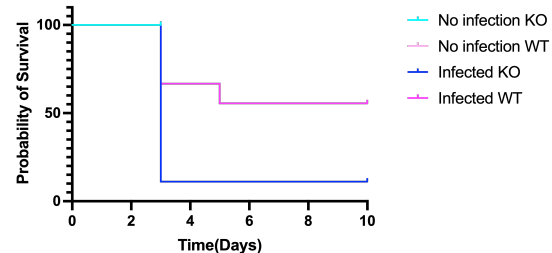
Body weight changes and survival rates in WT and KO mice infected with PR8

In all experiments, most mice lost 20% or more body weight on 2nd or 3rd day post-infection and were euthanized. For the duration of the study in all experiments, the infected KO mice and WT mice did not show significant differences in body weight loss (Figure 2.4A, C, and E), except for the KO mice compared to the WT mice on day 3 after infection in experiment 1 (Figure 2.4A). All surviving mice recovered their body weight by the end of the study. Significant differences in the survival of WT and KO mice were not observed in experiments 2 and 3 (Figure 2.4D and F), but in experiment 1, survival of the KO mice significantly decreased compared to that of the WT mice (figure 2.4B).

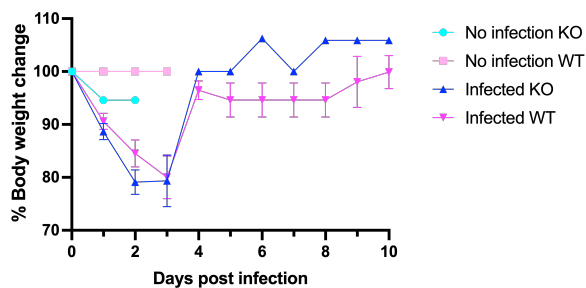
(A)



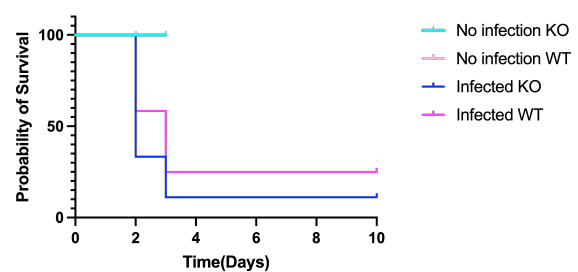
(B)



(C)



(D)



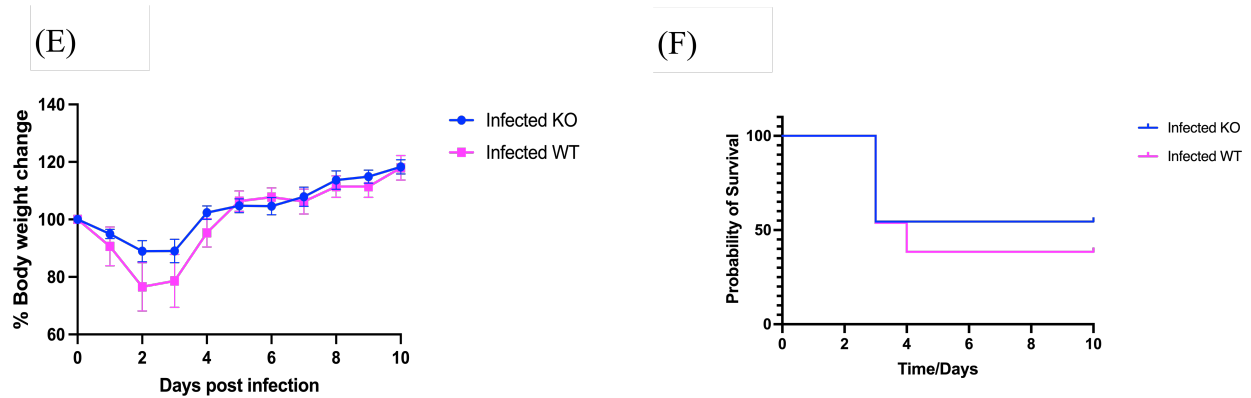
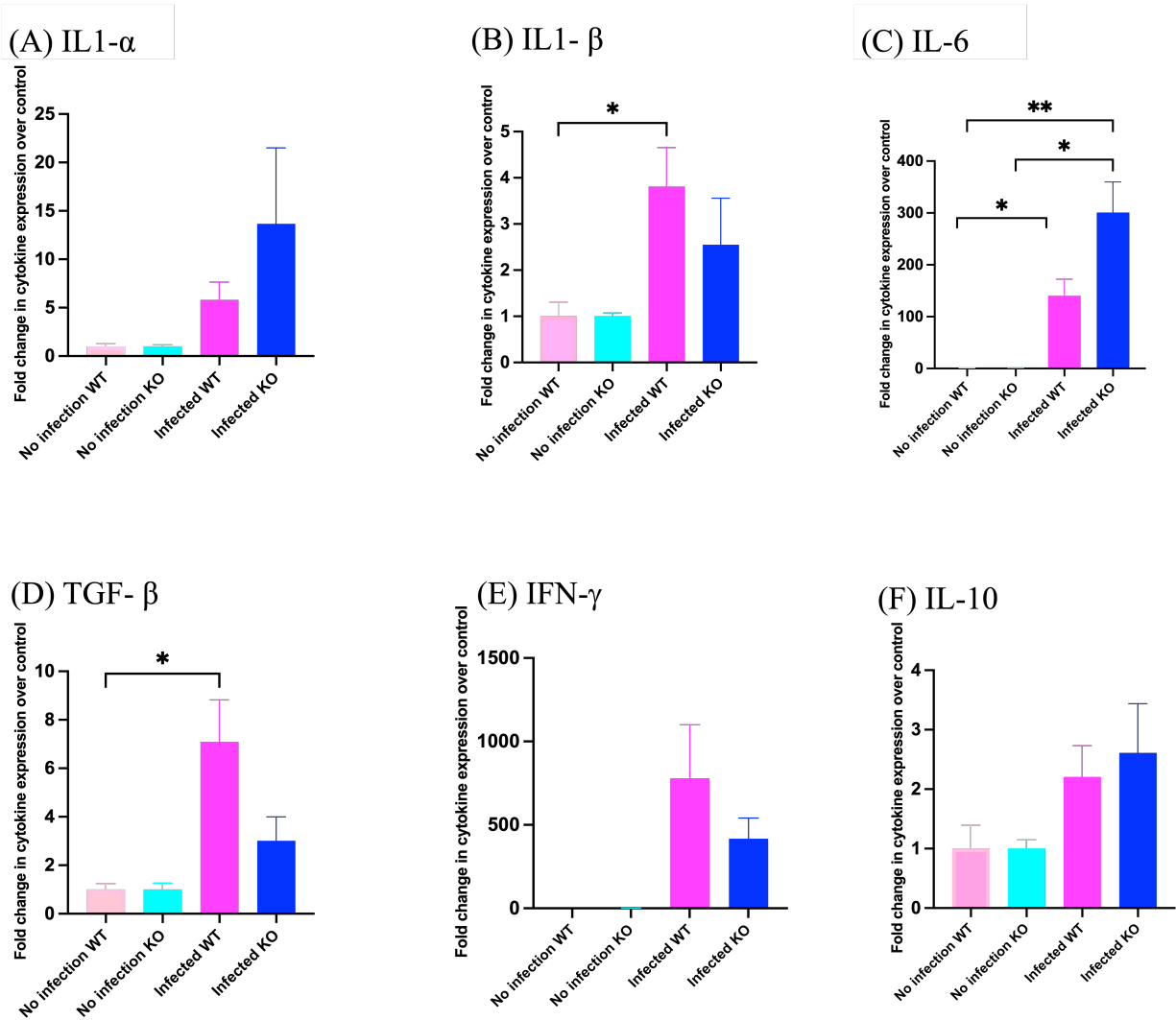


Figure 2.4. PR8 infection in the WT and KO mice. A, C, E: The body weight changes and survival curves (B, D, F) of WT and KO mice from three independent experiments. A, C, E, and B, D, F show body weight change and survival curves from experiments No.1, 2, and 3 respectively. Results were analyzed using GraphPad Prism version 10.2.2. Statistical significance for body weight changes was determined using student unpaired T-test and statistical significance for survival curves was determined using a log-rank (Mantel-Cox) test; $*P < 0.05$. Error bars represent the mean $\pm$ Standard Error of the means

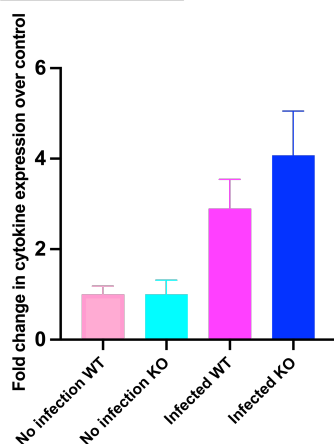
Lung viral titers of WT and KO mice

The lungs of the WT and KO mice in experiments 1 and 2, which were euthanized due to exceeding 20% weight loss on 3rd day post-infection, were collected for the determination of viral titers. The Lungs of no-infection mice euthanized on 3rd day after infection were used as the negative control. Influenza virus M gene mRNA levels in the lung were determined using qRT-PCR and the Ct values were converted to the TCID₅₀ values using a standard curve. Analysis of the results revealed no significant difference in the viral titers between the WT and KO mice in the lung tissue (Figure 2.5).

differences in the expression levels of IL-6, GM-CSF, IL-1 α , IL-10, IL-1 β , TGF- β , IFN- γ and TNF- α between the infected WT and KO mice (Figure 2.6).



(G) TNF- α



(H) GM-CSF

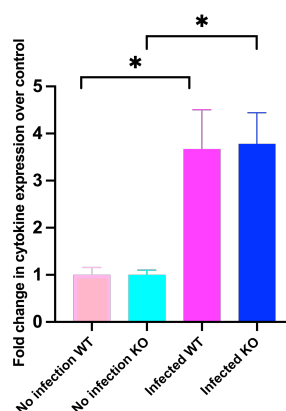


Figure 2.6 Fold changes of levels of mRNA expression of different cytokines (A-H) in the lungs of the WT and KO mice infected with PR8 compared to no infection control on the 3rd day post infection. Statistical significance was determined by using student unpaired T-test using GraphPad Prism software version 10.2.2; * $P < 0.05$, ** $P < 0.01$. Bars represent the mean $\pm$ standard error of the means.

Discussion

Deciphering the roles of host factors that are important for virus infection may provide a better understanding of virus-host interactions and potential complementary therapeutic strategies to augment the existing control measures for influenza infections (Batool et al., 2023). Recently, the identification of a host receptor, CD163, for porcine reproductive and respiratory syndrome virus (PRRSV) successfully resulted in genetically modified pigs (CD163 knockout pigs) that are resistant to PRRSV. Some cellular chaperone proteins including PDIs have been reported to play important roles in influenza virus pathogenesis (Stertz & Shaw, 2011; Watanabe et al., 2010). PDIA3 is a major component in the glycoprotein folding pathway (Galligan & Petersen, 2012). The terminal two glucose residues on the nascent N-glycosylated glycoproteins in the ER are initially cleaved by glucosidase I and II, respectively, which allows the processing of the protein by two lectin-like chaperones, calnexin, and calreticulin, in the ER. PDIA3 interacts with calnexin and calreticulin to modulate the folding of proteins (Oliver et al., 1999). PDIA4 is also involved in glycoprotein folding by indirectly interacting with calreticulin via PDIA5 (PDIR) (Patel et al., 2020; Solda et al., 2006). PDI enzymes also catalyze the formation and reduction of disulfide bonds between proteins, facilitating oligomerization of proteins. For example, HIV envelope glycoprotein gp120 gains entry to host cells by reducing disulfide bonds of gp120 by PDI or other host oxidoreductase upon binding to the host receptor CD41 (Barbouche et al., 2003; Cantó et al., 2009; Gallina et al., 2002).

The role of PDIs in the pathogenesis of viral infections including IAV was reported in several studies (Auwerx et al., 2009; Jain et al., 2009; Wan et al., 2012). It has been reported that inhibition of PDIA3 by siRNA targeting PDIA3 or PDIA3 inhibitors in primary mouse lung cells decreased

viral replication via disrupting disulfide bonds and oligomerization of influenza HA and NA proteins (Chamberlain et al., 2022; Roberson et al., 2012; Solda et al., 2006). Reduction of influenza virus replication and inflammatory responses were also observed in the lungs of transgenic mice where lung-specific PDIA3 knockout is induced with doxycycline (Chamberlain et al., 2022). These previous reports demonstrate that inhibition of PDIA3 plays a crucial role in reducing influenza viral load and inflammation in the lungs of mice. However, constitutive knockout of PDIA3 was observed to lead to embryonic lethal in mice (Coe et al., 2010), which suggests that PDIA3 gene knockout may not be plausible to generate pigs resistant to IAV infection.

Our group has previously reported that the siRNA knockdown of PDIA1, PDIA3, or PDIA4 resulted in the inhibition of IAV infection in cells with PDIA4 inhibition being most effective (Kim & Chang, 2018). Unlike PDIA3 KO mice, PDIA4 KO mice do not exhibit noticeable phenotypes changes except for abnormality in blood clotting due to reduced platelet aggregation (Almeida et al., 2011b). Therefore, we determined the effect of PDIA4 on IAV infection using the constitutive KO mice and PDIA4-KO A549 cells in this study. The results showed that knockout of PDIA4 leads to a significant reduction in viral infection in KO A549 cells compared to WT A549 cells. However, the viral titers in the lungs of the KO mice were comparable to those of the WT mice in our experiments. The body weight loss at the time of euthanasia (3 days after virus infection) and the survival of mice infected with PR8 in two experiments were comparable between the KO and WT mice. In one experiment, the WT mice showed a higher survival rate and less body weight loss (on the day of euthanasia) compared to the KO mice, these results suggest that PDIA4 KO in vivo does not effectively reduce viral replication.

Increased levels of cytokines and chemokines, including IFN- γ , TNF- α , MIP-2, and MIP-1 α , and MCP-1, MIP-1 β , MIP-2, MIP-3 α , IL-1, IL-6, IL-12, IL-18, and G-CSF, were reported in the lungs of mice infected with influenza virus (Tumpey et al., 2005). When we measured the cytokine levels in the lungs of the mice at 3 days post-virus infection, we observed significant increased mRNA levels of IFN- γ , G-CSF between infected WT and KO mice compared to uninfected mice. Even though the infected WT and KO mice exhibited increased expression levels of TNF- α , IFN- γ , and IL-1 α compared to uninfected WT and KO mice, these differences did not reach statistical significance. However, IL-1 β and TGF- β showed a significant increase in expression in infected WT mice compared to uninfected WT mice, but there was no difference between virus-infected KO mice and uninfected KO mice.

These variations in the expression levels of cytokines may be due to the collection time of the lung samples after infection where some cytokines may be expressed highly in early infection while other cytokines may be expressed at a later time point after infection (Conn et al., 1995). Moreover, we did not observe a significant difference in the expression levels of IL-6, GM-CSF, IL-1 α , IL-10, IL-1 β , TGF- β , IFN- γ and TNF- α between the infected WT and KO mice, suggesting that the absence of PDIA4 did not change lung inflammation in mice. Although the underlying mechanism of this lack of effect of PDIA4 deletion in influenza virus replication in mice is unknown, one possible explanation is that constitutive PDIA4 KO, but not temporary knockdown of gene function, may induce compensation by other genes with similar activity. Compensation of a loss of function of genes by other genes with overlapping functions and expression patterns has been reported (Wang et al., 1995.; Nedvetzki et al., 2004). In addition to the PDIs, other proteins

including Hsp70 (BiP), Grp94, and ERp29t, form multiprotein chaperone systems in the ER that process unfolded protein substrates (Meunier et al., 2002). Since PDIA3, PDIA1 and several other ER resident proteins have been reported to play major roles in influenza infection, there is a possibility that knockout of PDIA4 may result in the activation of compensatory mechanisms with PDIs or chaperone proteins in the ER to counteract the loss of PDIA4 over time. Further studies need to elucidate the possible compensation mechanism for influenza infection in PDIA4 knockout models.

Chapter 3 - The effects of rabbit hemorrhagic disease virus 3C-like protease in cell death

Introduction

Classical RHDV is a highly contagious and deadly lagovirus of the family *caliciviridae* and primarily affects domestic and wild European rabbits (*Oryctolagus cuniculus*) (Ohlinger et al., 1990). It was first reported in China in 1984 among the rabbits imported from Europe, and later it spread rapidly around the world and became endemic in most parts of the world (Abrantes et al., 2012). Classical RHDV has high mortality rates ranging from 70% to 90% in adult rabbits while kits younger than 30 days old remain unaffected by the virus (Abrantes et al., 2012). A genetically and antigenically distinct variant of classical RHDV, known as GI.2/RHDV2/b (RHDV2), emerged in 2010 in France (Le Gall-Reculé et al., 2011) and expanded rapidly on a global scale, replacing classical RHDV in several regions in the world (Abrantes, Droillard, et al., 2020; Asin et al., 2021; Le Gall-Reculé et al., 2017; Rouco et al., 2019). RHDV2 can infect various lagomorphs, including European rabbits (*Oryctolagus cuniculus*), cottontail rabbits (*Sylvilagus spp.*), and some hare (*Lepus*) species (Dalton et al., 2014; Hall et al., 2017; Le Gall-Reculé et al., 2011). A variable mortality rate ranging from 5% to 70% is reported for RHDV2 infection, and RHDV2 can cause diseases in rabbits of all ages including kits as young as 11-15 days old (Capucci et al., 2017).

Several isolated cases of RHDV2 in domestic rabbits had been reported before 2020 in North America (Rouco et al., 2019). However, in 2020, RHDV2 infections in wild native North American lagomorphs, including wild desert cottontail (*Sylvilagus auduboni*) and black-tailed jackrabbit (*Lepus californicus*), have been confirmed in the State of New Mexico, USA (United States Department of Agriculture factsheet, 2020). Since then, it has been detected in at least 14 more states affecting wild lagomorph species and domestic rabbits (Animal and Plant Health Inspection Service, 2024) and RHDV2 has become endemic in several states. RHDV can spread through direct contact with infected rabbits, their carcasses, blood, urine, feces, food, and water or indirect transmission via fomites, clothing, cages, and equipment (Gleeson & Petritz, 2020). Mechanical vectors including fleas, blowflies, sandflies, and scavenging foxes and crows are also involved in disease transmission. The main routes of transmission of the virus include the fecal-oral route and nasal or conjunctival route. These caliciviruses have also shown remarkable environmental resilience where they can endure freezing conditions and survive in infected carcasses for extended periods (Abrantes et al., 2012).

The disease progression of classical RHDV and RHDV2 is similar in many aspects. In RHDV infection, excessive inflammation induces a procoagulant state, contributing to disseminated intravascular coagulation, characterized by widespread blood clot formation and severe bleeding tendencies (Trzeciak-Rydzek et al., 2015). The defining pathological feature of RHDV infection is acute hepatocellular necrosis, which results from extensive hepatocellular damage due to the combination of viral replication, inflammation, and immune responses (Lankton et al., 2021). RHD can be presented in three clinical forms; peracute, acute, or chronic forms. In the peracute form of RHD, rabbits may undergo sudden death without showing premonitory clinical signs (Bonvehí et

al., 2019). In the acute form, infected rabbits show depression, anorexia, neurological abnormalities, respiratory signs, and fever and may die within a few days of the onset of the symptoms. In the chronic disease, anorexia, generalized jaundice, and lethargy can be seen before recovery or death (Abrantes et al., 2012). Rabbits with subclinical infection continue to shed the virus for months without obvious clinical signs (Kerr & Donnelly, 2013). RHDV has a positive-sense, single-stranded genome of ~7.4 kb (Wu et al., 2020). The genome consists of two ORFs. ORF1 encodes a polyprotein that contains seven non-structural proteins (p16, p23, helicase, p29, VPg, 3CLpro, RdRp) and a major capsid protein (VP60) while ORF2 encodes the minor structural protein, VP10 (Droillard et al., 2021).

Recombination of RHDV mostly occurs at the junction between nonstructural gene RdRp and structural gene VP60. The recombinant RHDVs are designated as [RdRp genotype] P-[capsid genotype] (Le Pendu et al., 2017). Viruses that encode 3C or 3CLpro are found in several virus families including *Picornaviridae* (3Cpro), *Coronaviridae* (3CLpro), and *Caliciviridae* (3CLpro). Picornavirus and calicivirus 3Cpros have a catalytic triad (Cys, His, Glu/Asp) while coronavirus 3CLpro contains a catalytic dyad (Cys, His). The 3Cpro and 3CLpro has Cys residue serving as the nucleophile in the active site (Kim et al., 2012). RHDV 3CLpro processes most cleavage sites on the expressed viral polyproteins to produce functional non-structural proteins, that are crucial in viral replication (Meyers et al., 2000). RHDV 3CLpro is comprised of 143 amino acids and is notably smaller than picornavirus 3C or coronavirus 3CLpro, which are generally larger than 180 amino acids (Wirblich et al., 1995). RHDV 3CLpro has a catalytic triad of Cys104, His27, and Asp44, and the cleavage occurs between Glu (or Gln) and Gly residues on the virus polyproteins (Boniotto et al., 1994)

Some viral 3Cpro and 3CLpros were found to play additional roles in modulating host cell processes and inducing apoptosis. The rhinovirus and poliovirus 3Cpros induce apoptosis by cleaving transcription and translation initiation factors, which is important in cell survival and death pathways. Enterovirus 71 3Cpro is shown to be involved in apoptosis in human neural cells via activation of caspases (Li et al., 2002). SARS-CoV 3CLpro was reported to induce caspase-dependent apoptosis, but conflicting results have been published on SARS-CoV-2 3CLpro's ability to induce apoptosis (Komissarov et al., 2022; Lin et al., 2006). The 3Cpro and 3CLpros of several viruses, including FMDV, EV, SARS-CoV-2, and PEDV, have been shown to inhibit type I interferon (IFN) response and the nuclear transcription factor kappa B (NF- κ B) signaling pathways by cleaving the key proteins in these pathways (de los Santos et al., 2007; Wang et al., 2016; Zhang et al., 2022). Moreover, picornavirus 3Cpro and calicivirus 3CLpro are involved in the cleavage of host poly(A)-binding protein, which is important for cellular mRNA translation (Joachims et al., 1999; Kerekatte et al., 1999; Kuyumcu-Martinez et al., 2004). It has been reported that overexpression of classical RHDV 3CLpro resulted in inducing apoptosis in cells (Chen et al., 2019).

In this study, we investigated the cytotoxic effects and mechanism of cell death of RHDV2 3CLpro and the roles of the catalytic domain of 3CLpro in mediating cell death and cytokine production in HEK293T cells.

Materials and Methods

Cell culture and maintenance

Human Embryonic Kidney (HEK293T) cells were cultured in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) and penicillin (250 U/ml) and streptomycin (250 µg/ml) and 2 mM L-glutamine. Cells were maintained in a humidified incubator at 37°C with 5% CO₂.

Generation of active and inactive RHDV2 3CLpro.

The full-length codon-optimized RHDV2 3CLpro sequence obtained from GenBank (GenBank accession number: QDF46325.1) was cloned into the pLVX-EF1α-IRES-Puro plasmid and was designated as pLVX RHDV2 3CLpro (Active RHDV2 3CLpro). An inactive RHDV2 3CLpro was generated by mutating cysteine to alanine in the catalytic site of 3CLpro using site-directed mutagenesis (Agilent QuickChange mutagenesis kit, Agilent, Santa Clara, CA) and was designated as inactive pLVX RHDV2 3CLpro. Empty pLVX-EF1α-IRES-Puro (pLVX) plasmid was used as a control in all experiments.

Determination of pLVX RHDV2 3CLpro activity

The activity of pLVX RHDV2 3CLpro was determined using a dual luciferase reporter assay (Dual-Luciferase kit, Promega, Madison, WI). The pGloSensorTM 30F plasmid (Promega) which contains a circular, permuted firefly luciferase gene with a caspase 3/7 cleavage site (DEVDG) and Renilla luciferase as an internal control was purchased from Promega and pGloSensor-RHDV was generated by replacing the caspase 3/7 cleavage site with RHDV 3CLpro cleavage site

(ASFEG) using site site-directed mutagenesis as reported previously by our lab (Perera et al., 2022). HEK293T cells at 70-80% confluency in 48 -well plate was co-transfected with active or inactive RHDV2 3CLpro (50 ng/well) with pGloSensor-RHDV2 plasmid (50 ng/well) using lipofectamine 0.5 μ l/well. At 24 hrs. after transfection, the cells were lysed, and a dual luciferase reporter assay (Dual luciferase kit, Promega, Madison, WI) was conducted to measure firefly and Renilla luciferase activities on a luminometer (GloMax® 20/20 Luminometer, Promega, Madison, WI) following the manufacturer's protocol. The firefly luciferase was normalized with Renilla luciferase for the determination of the activity of the expressed active RHDV2 3CLpro and inactive RHDV2 3CLpro in the cells.

Western blot analysis of active and inactive pLVX RHDV2 3CLpro

HEK293T cells at 70-80% confluency in 6-well plates were transfected with pLVX RHDV2 3CLpro or inactive pLVX RHDV2 3CLpro (1 μ g/well) using 4 μ l/well lipofectamine. At 24 hrs post-transfection, cell lysates were prepared by adding Invitrogen NuPAGE 1X LDS lysis buffer, followed by sonication on ice to ensure complete lysis of cells. SDS-PAGE was conducted using 12% Tris-glycine gel under reducing and denaturing conditions. After electrophoresis, the proteins were transferred to a nitrocellulose membrane. The membranes were blocked with 2% non-fat dry milk in TBS-T (Tris-buffered saline with 0.05% Tween-20) for 1 hr at room temperature. The membranes were then incubated overnight at 4°C with primary antibodies to strep tag (Invitrogen, Carlsbad, CA) diluted 1: 3000 in TBS-T with 2% non-fat dry milk. After washing with TBS-T, the membranes were incubated with secondary antibodies conjugated with horseradish peroxidase (Invitrogen) diluted 1:1000 in TBS-T with 2% non-fat dry milk for 2 hrs at room temperature. β -actin was used as a loading control. The proteins were visualized using the Pierce ECL Western

Blotting Substrate (Thermo Scientific, Rockford, IL) and imaged with a chemiluminescence detection system.

Determination of cell viability and cell death by pLVX RHDV2 3CLpro

The effect of active RHDV2 3CLpro on cell viability and cell death was determined by transfecting 70-80% confluent HEK293T cells with pLVX RHDV2 3CLpro, inactive pLVX RHDV2 3CLpro or empty pLVX (50 ng/well) in a 96 well plate using lipofectamine 0.2 µl/well. Cell viability was determined using RealTime-Glo™ MT cell viability assay (Promega) at 24, 36, and 48 hrs after transfection., and cell death was determined using RealTime-Glo™ Annexin V necrosis assay (Promega) at 24, 48, and 72 hrs after transfection., following the manufacturer's protocol. Fold changes of cell viability and cell death in the HEK293T cells transfected with change to plasmid names over empty pLVX vector transfected cells were calculated.

Cytotoxicity assay

The 70-80% confluent HEK293T cells in a 48-plate were transfected with 100 ng of pLVX RHDV2 3CLpro, inactive pLVX RHDV2 3CLpro or pLVX using 0.7 µl/well of lipofectamine. At 36 hrs. post-transfection, the supernatant was collected, and CyQUANT™ LDH Cytotoxicity Assay was conducted to determine LDH (Invitrogen) according to the manufacturer's instructions. Lactate dehydrogenase (LDH) is an enzyme that serves as a marker for cell membrane integrity and cytotoxicity. LDH release is proportional to the cell membrane damage and subsequent cell death. LDH levels in the cells transfected with pLVX RHDV2 3CLpro, inactive pLVX RHDV2 3CLpro, or pLVX were normalized by subtracting background LDH in the media, and the percent

cytotoxicity was calculated by dividing the LDH released from transfected cells by maximum LDH released from cells during complete lysis

Caspase 3/7 activity

The 70-80% confluent HEK293T cells in a 48-plate were co-transfected with 50 ng of active pLVX RHDV2 3CLpro, inactive pLVX RHDV2 3CLpro or pLVX with 50 ng of pGloSensorTM 30F plasmid containing the caspase 3/7 cleavage site (DEVDG) using 0.4 µl/well of lipofectamine. At 2 hrs. post-transfection, staurosporine (0.03 µM in final concentration) was added the cells, which was used as the positive control for inducing apoptosis via activation of caspase 3/7. At 24 hrs. post-transfection, cells were lysed, and a dual luciferase reporter assay (Dual luciferase kit, Promega, Madison, WI) was conducted to measure firefly and Renilla luciferase on a luminometer (GloMax® 20/20 Luminometer, Promega, Madison, WI), following the manufacturer's protocol. Caspase 3/7 activity in the cells transfected with pLVX RHDV2 3CLpro, inactive pLVX RHDV2 3CLpro, or pLVX was determined by normalizing firefly luciferase over Renilla luciferase. Fold change of caspase 3/7 luciferase activity of active RHDV2 3CLpro, inactive RHDV2 3CLpro over pLVX was calculated.

Cytokines assays

The reporter plasmids which are composed of promoter gene of cytokines and luciferase gene including pIFNβ-Luc, pGAS-Luc, pISRE-Luc, pNFκβ- Luc, pSTAT3-Luc, and pAP1-Luc were used for this study. The 70-80% confluent HEK293T cells in a 48-well plate were co-transfected with each reporter plasmid and pLVX RHDV2 3CLpro (100 ng/well), inactive pLVX RHDV2 3CLpro (100 ng/well) or pLVX (100 ng/well) using lipofectamine 0.7 µl/well. pRL-CMV (0.05

ng/well) was used as a transfection control. At 24 hrs post-transfection of HEK293T cells with pLVX, 3 μ l/well Sendai virus (2000 units/ml) was added, and this served as the positive control. At 48 hrs after transfection, the cells were lysed, dual luciferase reporter assay (Dual luciferase kit, Promega,) was conducted to measure firefly and Renilla luciferase according to the manufacturer's protocol on a luminometer (GloMax® 20/20 Luminometer, Promega). The promoter activity of each reporter plasmid was determined by normalizing firefly luciferase activity over Renilla luciferase activity. Fold change of promoter activity of cytokines with pLVX RHDV2 3CLpro, and inactive pLVX RHDV2 3CLpro over pLVX was calculated.

Real-time qPCR to determine IFN- β mRNA

The 70-80% confluent HEK293T cells in a 48-plate were co-transfected with 100 ng of pLVX RHDV2 3CLpro or 100 ng pLVX, 100 ng of pTRIP-SFFV-EGFP-FLAG-cGAS plasmid (Addgene, Watertown, Massachusetts) and 100 ng of pTRIP-SFFV-mtagBFP-2A STING plasmid (Addgene, Watertown, Massachusetts) using 0.7 μ l lipofectamine per well. At 24 hrs. post-transfection, cell lysates were collected by three freeze-thawing cycles, and RNA was extracted from the lysates using the RNeasy kit according to the manufacturer's instructions (Qiagen, Valencia, CA). RT-qPCR (GoTaq® 2 step RT qPCR, Promega) was conducted to detect the mRNA levels of IFN- β , according to the manufacturer's protocol. The Primers of IFN- β are, forward primer- 5' AAATCATGAGCAGTCTGCA 3' and reverse primer 5' AGGAGATCTTCAGTTTCGGAGG 3'. The reaction conditions for RT-qPCR were 1 cycle of 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 1 min, and elongation at 72°C for 30 s. The $\Delta\Delta C_t$ method was employed to calculate the relative mRNA levels of IFN- β . The beta-actin gene was used as a housekeeping gene. Fold change in

IFN- β levels of active RHDV2 3CLpro with or without cGAS (cyclic GMP–AMP synthase) and STING (stimulator of interferon genes) plasmids over pLVX was calculated.

Statistical Analysis

Data from all experiments were analyzed using the Prism 10.0 software version 10.2.2 (GraphPad, San Diego, CA) using unpaired t-test or one-way ANOVA. All experiments were repeated at least two times independently.

Results

Expression and activity of RHDV2 3CLpro in HEK293T cells

On western blot analysis, a single band of about 30 kDa, which corresponds to dimers was observed for active RHDV2 3CLpro, and double bands band of 16 kDa and 30 kDa, which corresponds to a monomer and dimer, respectively, of inactive RHDV2 3CLpro (Fig 3.1B). The activity of the expressed RHDV2 3CLpro was confirmed using dual luciferase assay (Fig 3.1A). The mutated 3CLpro where nucleophilic Cys was replaced with Ala in the active site showed minimal activity (3500-fold lower than active 3CLpro). The expression levels of 3CLpro were similar to each other.

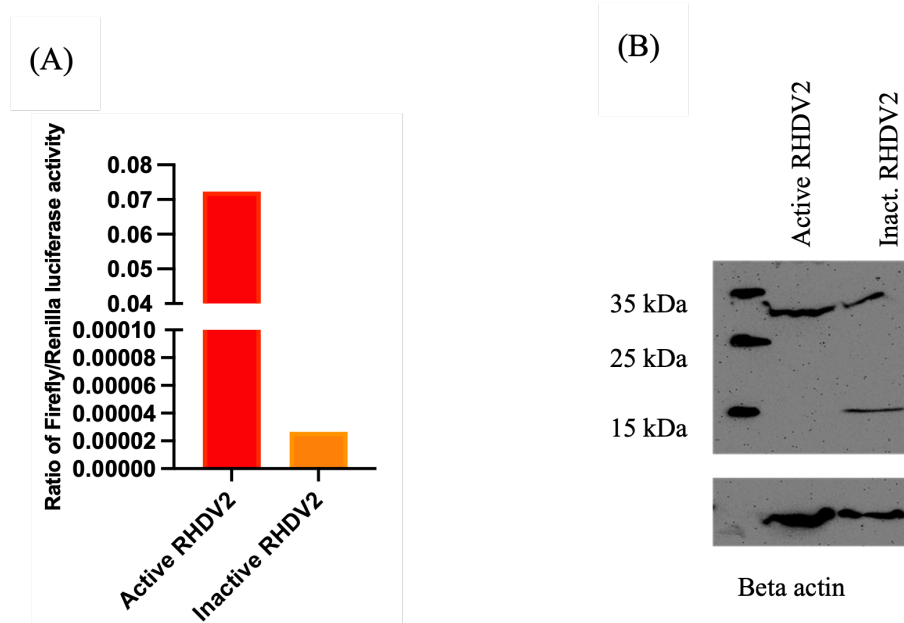


Figure 3.1. Activity and expression of RHDV2 3CLpro in HEK293T cells. (A) Firefly luciferase activity of pLVX RHDV2 3CLpro or inactive pLVX RHDV2 3CLpro co-transfected with pGloSensor-RHDV in HEK293T cells are shown. (B) Western blot analysis of active and inactive RHDV2 3CLpro expressed in HEK293T cells. A single band corresponding to a dimer (30 kDa) for active RHDV2 3CLpro and double bands around 15 kDa and 30 kDa corresponding to monomer and dimer for inactive RHDV2 3CLpro, respectively, are shown. Beta-actin was used as the housekeeping gene.

The effect of RHDV2 3CLpro on cell viability

The RealTime-Glo™ MT Cell Viability Assay measures the reduction potential of cells to generate a luminescent signal proportional to the number of metabolically active cells. The metabolically active cells can reduce the viability substrate diffused into the cells, which results in the generation of a luminescent signal. The cell viability test results showed a significant reduction in cell viability in HEK293T cells expressing active RHDV2 3CLpro in comparison to those expressing the

inactive RHDV2 3CLpro at 36 hrs and 48 hrs post-transfection while a significant decrease in cell viability over pLVX was observed at 24 hrs, 36 hrs and 48 hrs of transfection. However, there was no significant difference in cell viability between inactive RHDV2 3CLpro and empty vector at 24 hrs, 36 hrs, and 48 hrs after transfection (Figure 3.2).

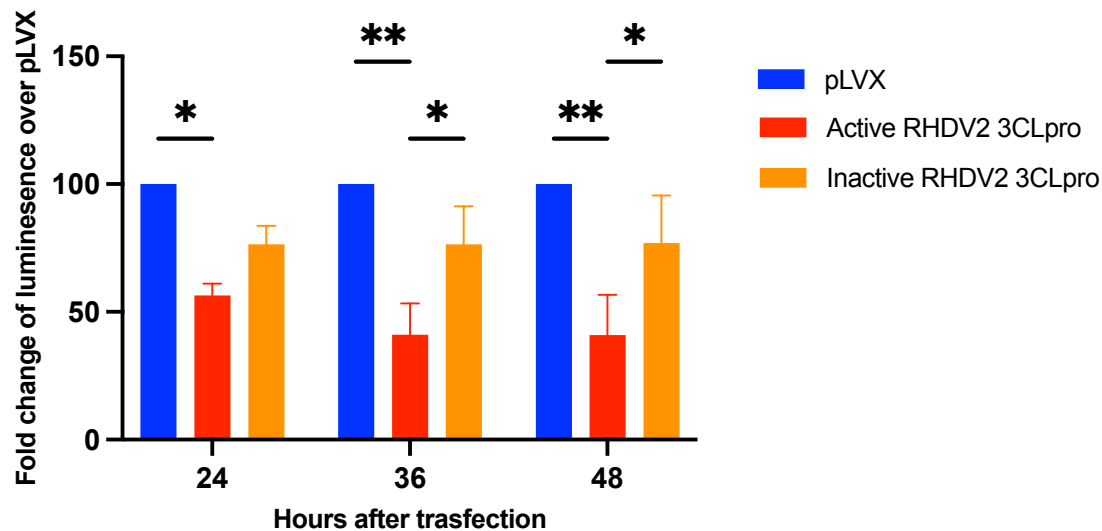


Figure 3.2. Fold change in cell viability over control (pLVX) at different time points in HEK293T cells expressing active, inactive RHDV2 3CLpro or empty vector. Statistical significance was determined by using one-way ANOVA using GraphPad Prism software version 10.2.2. ; * $P < 0.05$, ** $P < 0.01$. Bars represent the mean $\pm$ standard error of the means.

The effect of RHDV2 3CLpro on cell death

RealTime-Glo™ Annexin V necrosis assay results showed an increased fluorescence indicative of cell death in the presence of active RHDV2 3CLpro compared to inactive RHDV2 3CLpro. A significant increase in fluorescence from the expression of active RHDV2 3CLpro compared to inactive RHDV2 3CLpro or pLVX was observed at 24 hrs, 48 hrs, and 72 hrs after transfection,

while no significant difference in fluorescence was seen between inactive RHDV2 3CLpro and pLVX (figure 3.3).

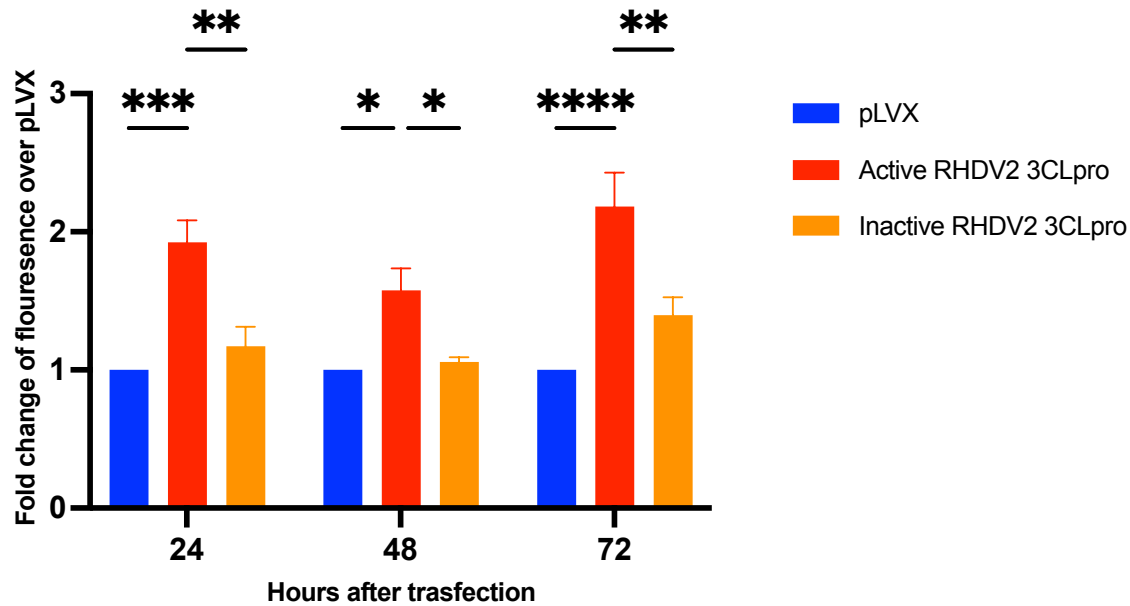


Figure 3.3. Fold change in fluorescence from pLVX RHDV2 3CLpro, inactive pLVX RHDV2 3CLpro over control (pLVX) at different time points in HEK293T cells. Statistical significance was determined by using GraphPad Prism version 10.2.2 using one-way ANOVA; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. Bars represent the mean $\pm$ standard error of the means.

The effect of RHDV2 3CLpro in cytotoxicity

Cell death was also determined by CyQUANT™ LDH cytotoxicity assay at 36 hrs. after transfection of HEK293T cells with pLVX RHDV2 3CLpro, inactive pLVX RHDV2 3CLpro, or pLVX. The CyQUANT™ LDH cytotoxicity assay measures the amount of LDH released from lysed cells by producing a fluorescent signal proportional to the number of damaged cells. Percent

cytotoxicity of the cells expressing active RHDV2 3CLpro was significantly higher compared to that of inactive RHDV2 3CLpro or pLVX at 36 hrs. post-transfection. No significant difference in percent cytotoxicity was observed between inactive RHDV2 3CLpro and pLVX (Figure 3.4).

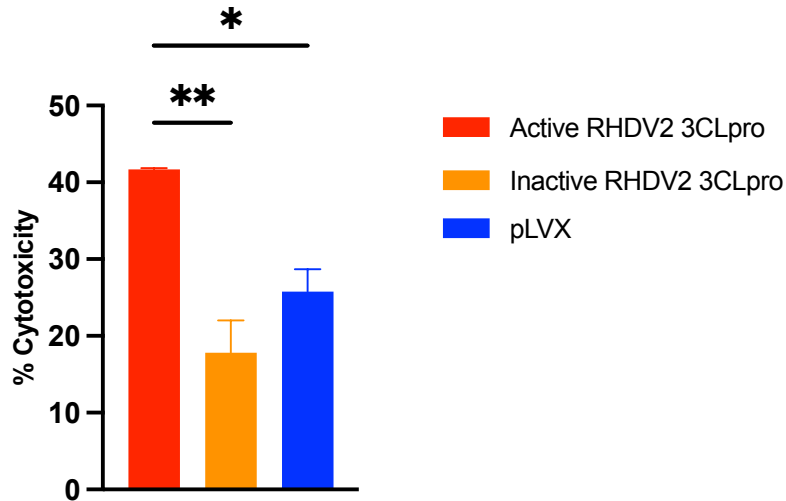


Figure 3.4. Percent cytotoxicity of cells expressing active RHDV2 3CLpro, inactive RHDV2 3CLpro and pLVX at 36 hrs. post-transfection. Statistical significance was determined using one-way ANOVA by GraphPad Prism software; $*P < 0.05$, $**P < 0.001$. Bars represent the mean $\pm$ standard error of the means.

Activation of caspase 3/7 activity by RHDV2 3CLpro

Fold change in caspase 3/7 activity in the HEK293T cells co-transfected with pLVX RHDV2 3CLpro, inactive pLVX RHDV2 3CLpro or pLVX and pGloSensorTM 30F was determined over pLVX at 24 hrs. post-transfection. Staurosporin was used as the positive control and induced strong activation of caspase 3/7 in the cells at 24 hrs. after treatment. Significant upregulation in caspase 3/7 activity was seen in cells expressing active RHDV2 3CLpro compared to inactive RHDV2 3CLpro or pLVX at 24 hrs post-transfection. However, no significant difference in caspase 3/7 activity was observed between inactive RHDV2 3CLpro and pLVX (Figure 3.5).

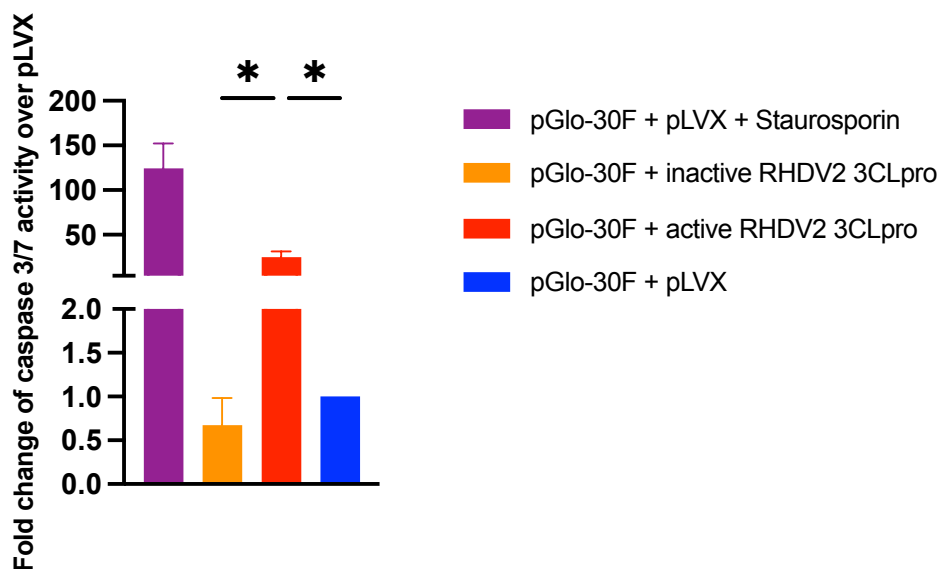


Figure 3.5. Fold change of caspase 3/7 luciferase activity in cells expressing active or inactive RHDV2 3CLpro over the control (pLVX) vector at 24 hrs. after transfection. Staurosporin was used as a positive control. Statistical significance was determined using one-way ANOVA by GraphPad Prism software; $*P < 0.05$. Bars represent the mean $\pm$ standard error of the means.

The effect of RHDV2 3CLpro on the cytokine promoters in cells

The activation of several cytokine promoters was determined using a dual luciferase reporter assay. In active RHDV2 3CLpro expressing cells, a significant increase in the luciferase activity of pIFN β -Luc and pGAS-Luc was observed compared to inactive RHDV2 3CLpro or pLVX. However, significant differences in pISRE-Luc, pNF κ B-Luc, pSTAT3-Luc, and pAP1-Luc promoter activity were not observed in cells expressing active RHDV2 3CLpro or inactive RHDV2 3CLpro compared to pLVX (Figure 3.6). Sendai virus is a potent inducer of interferon antiviral responses and was used as the positive control. In sendai virus-treated cells, increased luciferase activities were observed in cells transfected with pIFN β -Luc (by about 50-fold) or pISRE-Luc (by about 7-fold) compared to pLVX-transfected cells (Figure 3.6).

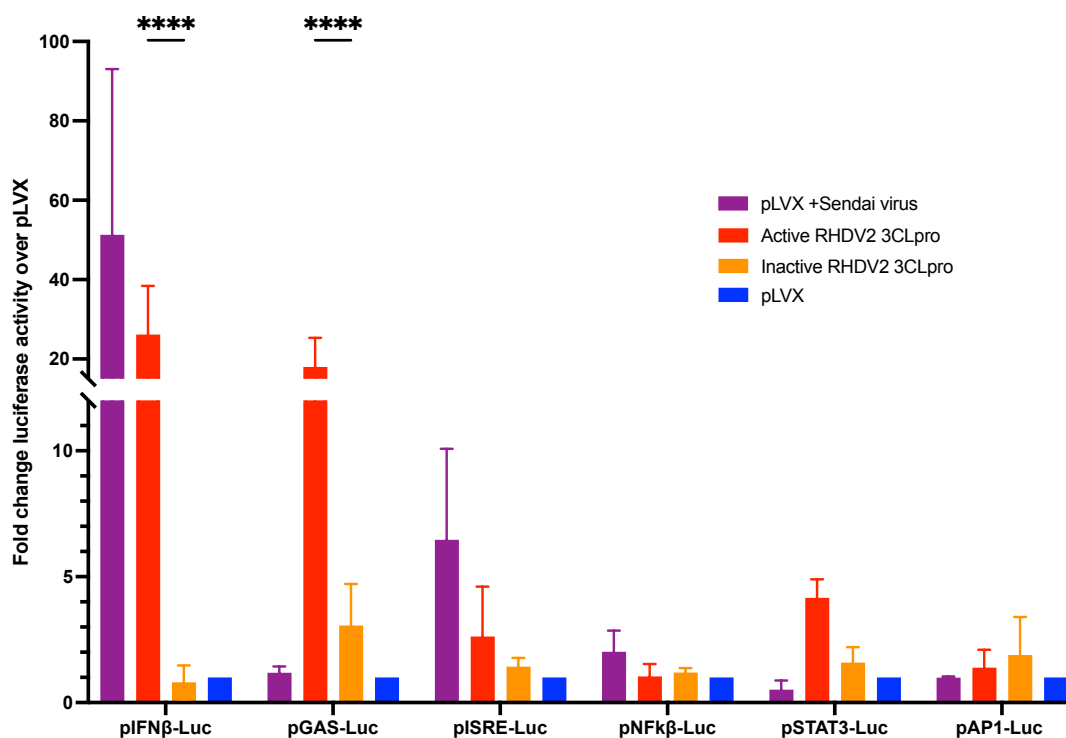


Figure 3.6. Fold change in luciferase activity of the reporter plasmids in HEK293T cells transfected with pLVX RHDV2 3CLpro, inactive pLVX RHDV2 3CLpro over the pLVX. Sendai

virus was used as a positive control. Statistical significance was determined using one-way ANOVA by GraphPad Prism software; **** $P < 0.0001$. Bars represent the mean $\pm$ standard error of the means.

Effect of RHDV2 3CLpro and cGAS and STING on IFN- β mRNA expression levels

We determined the expression levels of IFN- β mRNA in HEK293T cells transfected with active RHDV2 3CLpro with or without co-transfection of cGAS and STING plasmids. This experiment was done to determine whether active RHDV2 3CLpro is involved in the activation of the cGAS/STING pathway to induce the expression of IFN- β . When cGAS and STING plasmids were co-transfected with active RHDV2 3CLpro, we observed a moderate increase of 20-30% of IFN- β mRNA levels compared to the pLVX, but the increase was not statistically significant ($p < 0.05$). We also did not observe a significant change in IFN- β mRNA when cGAS and STING plasmids were co-transfected with pLVX. Moreover, significant increase in IFN- β mRNA levels were not observed in HEK293T cells transfected with active RHDV2 3CLpro only or pLVX (Figure 3.7).

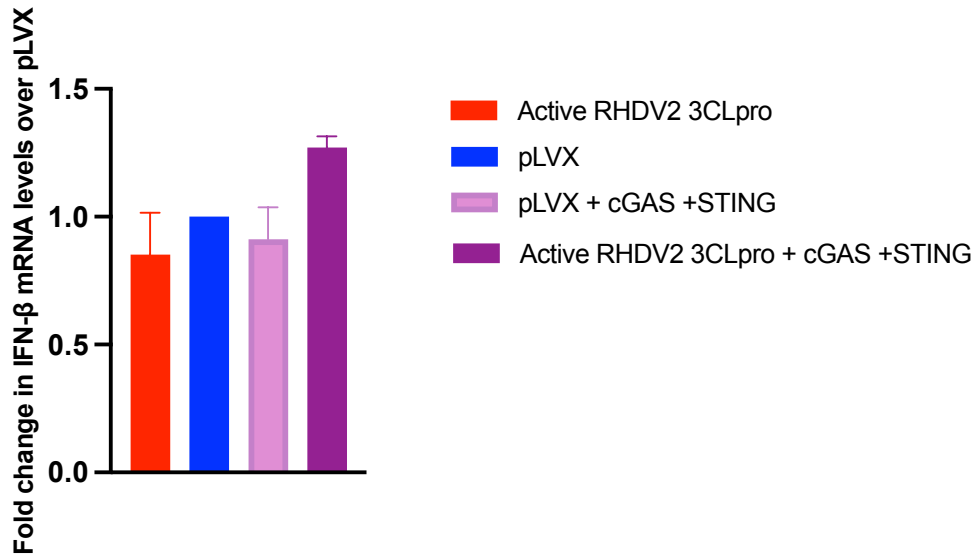


Figure 3.7. Fold change of IFN- β mRNA levels in HEK293T cells transfected with pLVX RHDV2 3CLpro or pLVX or co-transfection of these vectors with cGAS and STING compared to pLVX. Statistical significance was determined using the unpaired T-test by GraphPad Prism software; $*P < 0.05$. Bars represent the mean $\pm$ standard error of the means.

Discussion

Several studies have reported that apoptosis is the predominant form of cell death associated with RHDV infection in rabbits (Jung et al., 2000; Niedźwiedzka-Rystwej et al., 2013). The liver was found to be the major organ where many cells are undergoing RHDV-induced apoptosis, and the other organs, such as kidneys, peripheral blood leukocytes, and lungs were also reported to have cells showing apoptosis during RHDV infection in rabbits (Alonso et al., 1998; Niedźwiedzka-Rystwej et al., 2013). Therefore, it was suggested that RHDV-induced apoptosis in multiple organs likely contributes to pathogenesis. Even though apoptosis has been reported as the mode of cell death associated with RHDV infection, there also are reports of RHDV causing necrosis and autophagic response in liver cells (Teifke et al., 2002; Vallejo et al., 2014). In one of the reports, it was suggested that RHDV infection initially induces a rapid autophagic response in liver cells, which may decline with ongoing infection leading to the apoptosis of the liver cells (Vallejo et al., 2014).

RHDV2 3CLpro processes most cleavage sites on the expressed viral polyproteins to produce functional non-structural proteins and is crucial in viral replication. Beyond their primary role in cleaving virus polyproteins during replication, 3C protease (3Cpro) of rhinovirus, poliovirus, enterovirus 71, and 3CLpro of SARS-CoV have been shown to have additional functions such as inducing apoptosis and modulating host immunological processes and transcription (Barco et al., 2000; Li et al., 2002; Shubin et al., 2015). In the case of SARS-CoV 3CLpro, contradicting results have been reported about the ability of 3CLpro to induce apoptosis (Komissarov et al., 2022; Lin et al., 2006). The roles of RHDV2 3CLpro in addition to processing viral polyproteins have not been fully understood. However, a study has reported that overexpression of classical RHDV 3CLpro plays a key role in inducing apoptosis in cells (Chen et al., 2019). In our study, we

observed a significant decrease in cell viability, increase in cell death, and cytotoxicity effects in HEK293T cells expressing RHDV2 3CLpro compared to those expressing inactive RHDV2 3CLpro where the nucleophilic Cys was mutated to Ala at the active site. This result indicates that the RHDV2 3CLpro induces cell death and its activity is mediated through its catalytic activity.

Then we determined whether the cell death was mediated by the activation of Caspase 3/7, which are key executioner enzymes in the programmed cell death pathway (Ai et al., 2024). Caspase 3 and 7 activation is a central event in the apoptotic pathway that leads to the proteolytic cleavage of cellular substrates and ultimately results in apoptosis (Lakhani et al., 2006). We observed in our experiment that overexpression of active RHDV2 3CLpro led to a significantly increased caspase 3/7 activity compared to inactive RHDV2 3CLpro in HEK293T cells. This suggests that active RHDV2 3CLpro plays a significant role in inducing apoptosis through the activation of caspase 3/7, and this is mediated by the catalytic activity of RHDV2 3CLpro.

In our study, we observed a significant increase in the promoter activity of pIFN β -Luc and pGAS-Luc genes when RHDV2 3CLpro was expressed in HEK293T cells. The pGAS-Luc construct contains the gamma-activated sequence (GAS) elements, which are specific DNA sequences that respond to interferon gamma (IFN- γ) signaling. When IFN- γ binds to its receptor, it activates the JAK-STAT signaling pathway, leading to the phosphorylation and dimerization of STAT1 and STAT2 proteins. These STAT dimers translocate to the nucleus, where they bind to GAS elements, promoting the transcription of downstream genes. The observed increase in pGAS-Luc activity indicates that RHDV2 3CLpro expression may enhance the activation of the IFN- γ -induced signal transduction pathway.

When we determined the mRNA expression levels of IFN- β by transfecting RHDV2 3CLpro and pLVX without cGAS and STING plasmids, we did not observe a significant increase in IFN- β mRNA levels compared to those of empty pLVX vector. However, transfection of RHDV2 3CLpro with cGAS and STING plasmids increased IFN- β mRNA expression compared to pLVX although it was not significant. This indicates that the activation of IFN- β promoter by RHDV2 3CLpro did not result in marked IFN- β mRNA expression.

Picornaviral 3Cpros of poliovirus, coxsackievirus, FMDV, and encephalomyocarditis virus can disrupt the stress granules involved in regulating IFN- β expression (Lötzerich et al., 2018; Sun et al., 2016). Stress granules act as an important antiviral stress response that stimulates the host immune system, and 3Cpro of these viruses mediate the cleavage of stress granule core protein G3BP1 to disrupt host defenses by decreasing IFN- β expression (Mondal et al., 2023; Sun et al., 2016). The recognition of viral RNA by the host immune system primarily occurs through Toll-like receptor 3 (TLR3) and melanoma differentiation-associated protein 5 (MDA5). These receptors detect viral RNA and trigger signaling pathways that lead to the production of interferons. TLR3 activates interferon production via the MyD88/TRIF pathway, while MDA5 activates it through Interferon Promoter Stimulated Protein 1 (IPS-1) (Men et al., 2023). A recent study has found that the classical RHDV 3CLpro inhibits interferon signaling by cleaving IPS-1 in the MDA5 pathway (Men et al., 2023). Therefore, there is a possibility that RHDV2 3CLpro may be able to interrupt IFN- β expression via cleaving key proteins in the MDA5 pathway. To gain insight into the detailed mechanism of RHDV2 3CLpro, further studies are needed to evaluate the pathways and factors associated with RHDV2 3CLpro-mediated IFN- β mRNA expression suppression.

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