EXAMINING VIRUS INTERACTIONS WITH HOST SERINE HYDROLASES IN IMMUNOMETABOLISM

by

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Abstract

As obligatory intracellular parasites, viruses are in a constant battle with their host to establish infection. They can facilitate their propagation by modulating host immune or metabolic pathways. This modulation involves targeting various molecular factors such as microRNAs (miRNA), enzymes, or small molecules. Understanding how viruses alter the chemical makeup of a cell is crucial to identifying what pathways are being targeted, furthering our understanding of the virus life cycle, and may aid in identifying biomarkers of disease. Here, we examine host-virus interactions in the context of two viruses, hepatitis c virus (HCV) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). First, the modulation of serine hydrolases by a pro-viral microRNA, miRNA-122, is investigated using activity-based protein profiling (ABPP). This study identifies a downstream target of miRNA-122 that is differentially activated during HCV infection which can be targeted pharmacologically to reduce HCV infectivity. Second, we apply similar techniques to identify serine hydrolase changes associated with SARS-CoV-2 infection. Results point towards enrichment of endocannabinoid metabolism which may offer an alternative therapeutic avenue for combating SARS-CoV-2 infection. Together, the work presented in this thesis provides avenues for further investigation into miRNA-122 interactions during HCV infection and endocannabinoid metabolism in SARS-CoV-2 infection.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

AADAC	Arylacetamide deacetylase
ABHD10	Abhydrolase domain containing 10
ABHD12	Abhydrolase domain containing 12
ABHD2	Abhydrolase domain containing 2
ABHD6	Abhydrolase domain containing 6
ACOT2	Acyl-CoA thioesterase 1
APEH	Acylaminoacyl-peptide hydrolase
CES1	Carboxylesterase 1
CPVL	Carboxypeptidase vitellogenic like
CTSA	Cathepsin A
DPP4	Dipeptidyl peptidase 4
DPP8	Dipeptidyl peptidase 8
DPP9	Dipeptidyl peptidase 9
ESD	Esterase D
FASN	Fatty acid synthase
LIPC	Lipase C
LYPLA2	Lysophospholipase 2
LYPLAL1	Lysophospholipase like 1
MGLL	Monoglyceride lipase
PAFAH1B2	Platelet activating factor acetylhydrolase 1b catalytic subunit 2
PAFAH1B3	Platelet activating factor acetylhydrolase 1b catalytic subunit 3
PAFAH2	Platelet activating factor acetylhydrolase 2
PNPLA3	Patatin like phospholipase domain containing 3
PNPLA6	Patatin like phospholipase domain containing 6
PREP	Prolyl endopeptidase
PREPL	Prolyl endopeptidase like

Statement of Work

The entirety of this thesis is a product of my own work. Preliminary editing was carried out by me before being peer-reviewed by Dr. Magdalena Szuplewska. The idea for the project is largely credited to Dr. John Paul Pezacki. However, experimental design was a collaborative effort between Dr. John Paul Pezacki and I. Cell culture work including microRNA transfections, siRNA transfections and handling of the virus was performed exclusively by myself. Following sample preparation, all experiments including qPCR and ABPP were completed by myself except for the western blots presented in chapter 3 which were completed by Rachel Hausmen with supervision from myself.

Chapter 1: General Introduction

1.1 Immunometabolism and Host-Virus Interactions

Viruses are obligatory intracellular parasites that rely on the biochemical processes and regulatory pathways of host cells to facilitate their replication. Infection of the body by pathogens initiates a complex battle between the host and the invading organism. Upon infection, the innate and adaptive immune system mounts a defense response to eliminate the intruder and protect the body from harm (Janeway & Medzhitov, 2002; Medzhitov, 2007). This response involves the activation of various immune cells, the release of signaling molecules such as cytokines, and the initiation of specific immune pathways to recognize and destroy the pathogen. At the same time, pathogens deploy their own strategies to evade the host's immune system to establish a successful infection. They may evolve mechanisms to evade immune recognition, suppress immune cell activity, or hijack host cellular processes for their own benefit. These are just some examples of the complexities associated with host-virus interactions.

Immunometabolism is defined as the interplay between immunological and metabolic processes (Mathis & Shoelson, 2011). This broad term has been used to describe the study of metabolic pathways stimulated within immune cells, immune cell activation in metabolic disorders, and metabolic changes occurring due to the immune responses triggered by a pathogen (Diamond et al., 2010; Hotamisligil, 2017; Pearce & Pearce, 2013). Here, the primary focus will be on the latter, with the goal of understand how metabolism contributes to host-pathogen interactions at the cellular level. The study of host-virus interactions is crucial for understanding the molecular

mechanisms behind viral replication and pathogenesis (Aderem & Ulevitch, 2000; Randall & Goodbourn, 2008). It is known that viruses can alter their hosts' cellular metabolism to facilitate infection. One example is lipid metabolism. This is a hallmark of hepatitis C virus (HCV) infection which induces lipogenesis and alters lipid droplet dynamics, promoting lipid accumulation to facilitating viral assembly and release (Ogawa et al., 2009). Another recent example is the spike protein of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which was shown to impair lipid metabolism in host cells (Nguyen et al., 2022). These host dependencies can be used as potential therapeutic targets for developing effective antiviral agents. For this reason, a comprehensive analysis of enzymatic activity during infection can be useful and will provide a better understanding of how the disease state arises.

1.1.1 Proteomic Approach to Uncovering Host-Viral Interactions

In the last decade, there has been an increase in the application of proteomic approaches to studying viruses (Lum & Cristea, 2016). Various proteomic methods can be used to study host-viral protein interactions such as mass spectrometry-based techniques, affinity purification, and protein microarray analysis. They can help identify proteins that are differentially expressed during infection, providing insight into the host's response to infection. For instance, affinity purification coupled with mass spectrometry can identify protein-protein interactions between the virus and host. This is the case for Activity-Based Protein Profiling (ABPP) which can identify changes in enzyme activity caused by infection (Desrochers & Pezacki, 2019; Strmiskova et al., 2016). This is useful since abundance of a protein is not always reflective of the functional state of a cell (Figure 1.1). Post-translational modifications, such as phosphorylation, acetylation, and ubiquitination, can significantly impact signaling cascades.

That is why the application of these techniques has accelerated the impartial characterization of protein interactions between hosts and viruses during infection.

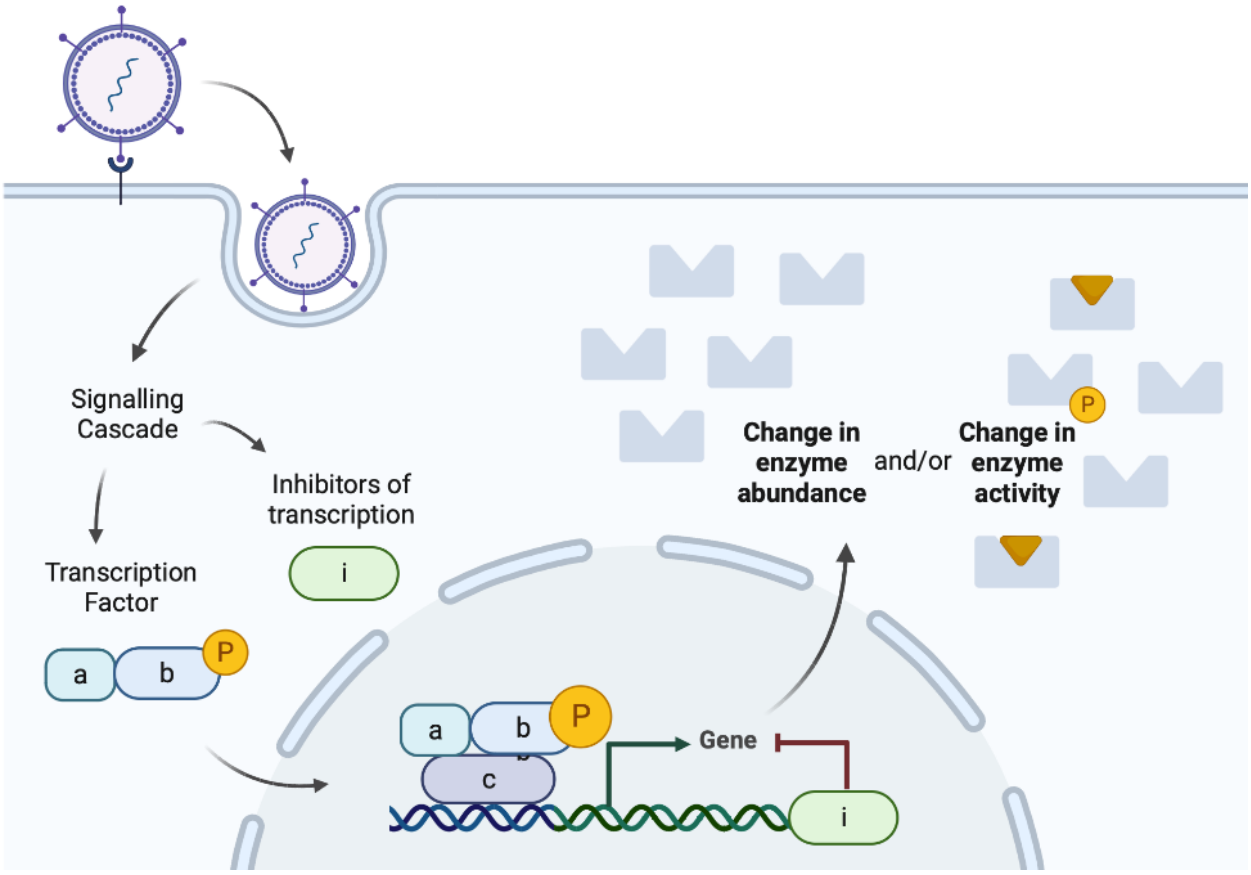


Figure 1.1 Scheme of host-virus interactions leading to change in enzyme abundance and activity. Endocytosis of a viral particle initiates a signaling cascade leading to an increase or decrease in gene expression. Downstream effects can be an increase or decrease in abundance or activity of enzymatic targets. Image created in Biorender.

1.2 Serine Hydrolases in Disease

Serine hydrolases are one of the largest enzyme families with more than 200 encoded in the human genome. Present in all domains of life, serine hydrolases encompass a wide range of functions. They are characterized by the presence of an active site serine used for the hydrolysis of ester, amide, and thioester bonds in small peptides or proteins (Long & Cravatt, 2011). The active site contains a nucleophilic serine residue, a general base histidine, and usually an acid-base aspartate or glutamate. While serine and histidine are often found in these triads, the acidic residue may be replaced by other amino acids in some cases (Ekici et al., 2008). Approximately half of the identified serine hydrolases fall into the category of proteases such as trypsin and chymotrypsin, while the remaining half are classified as "metabolic" enzymes. Some examples of metabolic serine hydrolase include lipases, which play a crucial role in lipid metabolic processes, peptidases, involved in the hydrolysis of peptide bonds in proteins, and amidases, for the hydrolysis of ester bonds (Bachovchin & Cravatt, 2012). Therefore, investigating the activity of these enzymes can provide insights into the status of metabolic pathways under different conditions.

Due to their involvement in many biological processes, the study of serine hydrolases continues to gather the attention of many scientists. Dysregulation of serine hydrolases can have serious implications on the progression of metabolic disorders and neurodegenerative diseases. For example, in the progression of non-alcoholic fatty liver disease (NAFLD), dysregulation of prolyl endopeptidase (PREP) and carboxylesterase 2 (CES2) has been shown to contribute to the progression of hepatic lipid accumulation and inflammation, making them a potential therapeutic target for NAFLD (Jiang et al., 2021; Kotowski et al., 2006; Ruby et al., 2017). PREP has also been linked to neurodegenerative disorders like Parkinson’s disease, Huntington’s disease, and

Alzheimer's disease; however, the underlying mechanism remains unclear (Becker et al., 2018; Myöhänen et al., 2012; Norrbacka et al., 2019). These studies highlight just a few examples of serine hydrolases and their implications in metabolic disorders and disease.

Serine hydrolases have become popular targets for drug development. Although some pharmacological inhibitors have shown promising results for treating various diseases, there remains much work to be done before their underlying mechanism can be discovered. It seems to be the case that many of these enzymes have not one distinctive role, but many, which depend on the milieu it is located in (García-Horsman, 2020). Further research in this field can shed light into the underlying mechanisms and help develop strategies for targeting these serine hydrolases in different diseases.

1.3 Activity-Based Protein Profiling (ABPP)

Established by Benjamin F. Cravatt *et al*, ABPP has become a powerful tool for characterizing enzymatic activity in different biological systems (Liu et al., 1999). It relies on the use of small molecule probes that covalently react with the active site of a target enzyme or enzyme family, allowing for their selective labeling and subsequent analysis. These probes often consist of three parts: a reactive warhead group, a recognition element, and a reporter tag (Figure 1.2A). The reactive warhead group is responsible for forming a covalent bond between the probe and target enzyme. Along with the recognition element, the reactive group can be optimized to select for a particular enzyme family such as serine hydrolases, tyrosine phosphatases, glycosidases, ATPases, threonine proteases and many more (Cravatt et al., 2008). Connected via a linker region, activity-based probes are also equipped with reporter tags to allow for detection or enrichment of labeled proteins. Examples of reporter tags include fluorophores, biotin, or click

handles for identification through techniques such as fluorescent imaging, gel electrophoresis, and mass spectrometry. This allows for detection of target proteins and the determination of their functional states and abundance in complex biological systems.

1.3.1 ABPP For Studying Host-Viral Interactions

ABPP has become an invaluable tool for bettering our understanding of host-viral interactions (Desrochers & Pezacki, 2019). To get a more comprehensive view of the human proteome, abundance-based and activity-based proteomic techniques should be used in combination. Traditional abundance-based proteomic technologies such as mass spectrometry (MS) and gene expression profiling have provided insight into many physiological processes, however, there remains some challenges. Primarily, they do not always reflect the true functional state of the cell because both active and inactivated enzymes are included in the quantitative analysis. For example, posttranslational events such as phosphorylation can regulate protein activity making an activity profile differ from a standard expression profile. This is why a chemical probe-based method which selectively targets enzymes in the active conformation, thereby excluding the inactive ones, can provide a more accurate 'fingerprint' of the functional state of the cell. ABPP is such a technique, and has been applied to various areas of research, including for discovering functions of proteins during viral infection (Blais et al., 2010; Desrochers et al., 2022; Filip et al., 2021; Müller et al., 2019; Shaw et al., 2018).

1.3.2 ABPP of Serine Hydrolases

One of the most used ABPP probes targets the serine hydrolase enzyme family. It uses fluorophosphate (FP) as the warhead because of its ability to selectively react with the

nucleophilic serine, forming a covalent bond, and thereby labelling only the active serine hydrolases (Figure 1.2B). The FP warhead is attached via the linker region to a biotin tag used for purification via streptavidin affinity chromatography. For this project, ABPP of serine hydrolases is combined with dimethyl labeling (DML). DML is a technique used to compare protein abundance between two samples in tandem. This is achieved by differentially labelling using stable isotopes of deuterium to create a mass difference between samples, allowing for quantification by mass spectrometry (MS). In DML-ABPP, target enzymes are first selected by activity-based probe assisted pull-down, after which digested samples are differentially labeled and analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS). A workflow is presented in figure 1.2C.

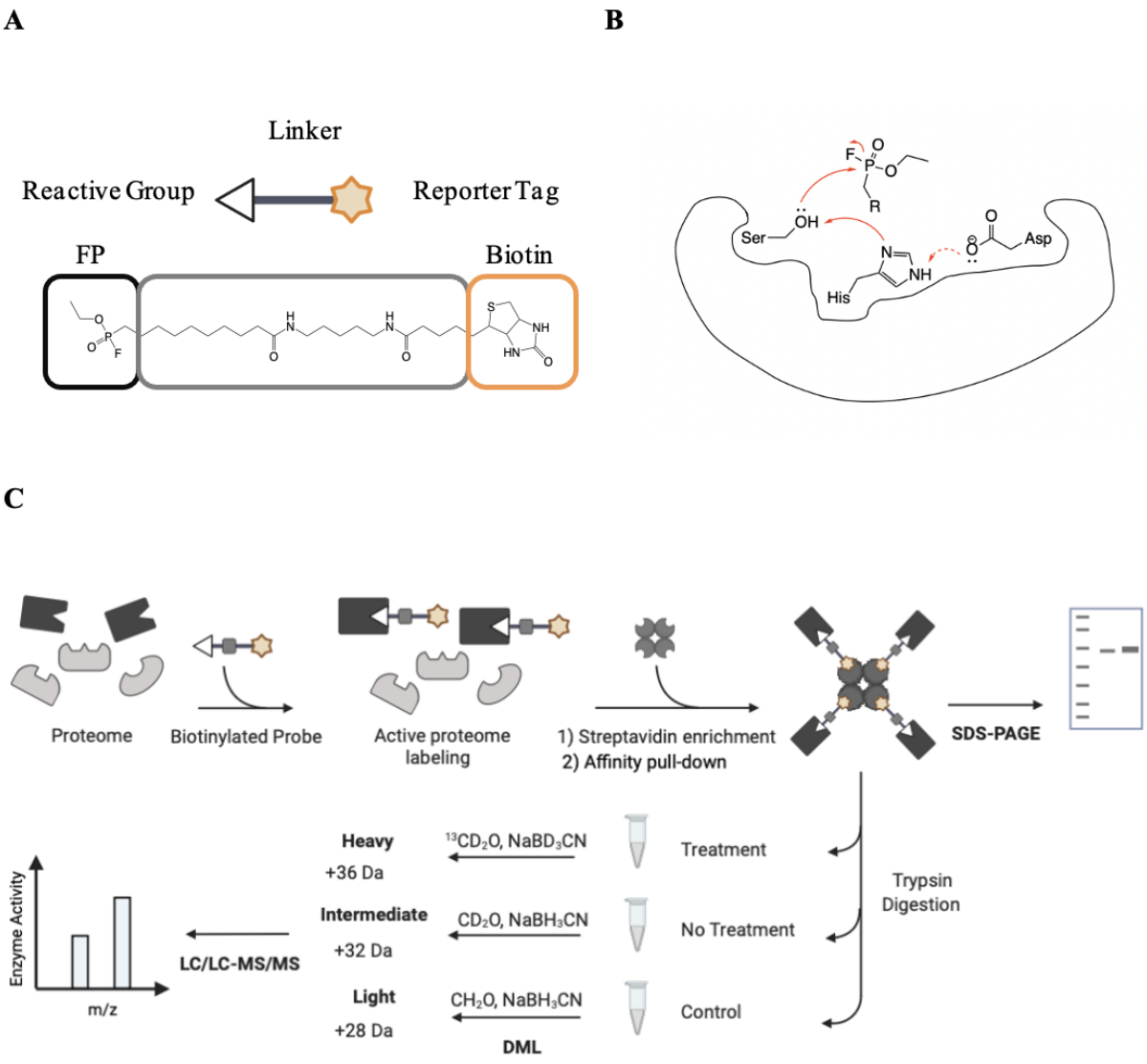


Figure 1.2: ABPP for western blot and mass spectrometry. (A) Schematic of generic ABPP probe and structure of FP-Biotin. (B) Simplified mechanism of FP labeling of serine hydrolases. (C) Workflow of ABPP for western blot and liquid chromatography- tandem mass spectrometry (LC/LC-MS/MS). Active serine hydrolases are labelled with the FP-Biotin probe. Following streptavidin enrichment, the active proteome is isolated by affinity pull-down and digested. Activity changes can be visualized by SDS-PAGE or dimethyl labelling (DML) can be used to isotopically label samples for analysis by LC/LC-MS/MS. Graphic created in Biorender.

1.4 Rationale and chapter objectives

The work presented in the thesis lives at the interface of chemistry and biology. It aims to better our understanding of immunometabolism with the common goal of providing a better understanding of complex interactions in metabolic pathways. The second chapter investigates the transcriptional and activity changes which occur within liver cells following hepatitis C virus infection (HCV). It is known that HCV RNA functionally sequesters miRNA-122 which reduces miRNA binding on endogenous mRNA targets (Luna *et al.* 2015). However, the impact this has on the activity of these cellular targets has yet to be studied in depth. Given that traditional abundance-based profiling methods are not always reflective of the functional state of the cell, ABPP will be employed. Providing a global view of these effects by targeting a large enzyme family, the serine hydrolases, this work contributes to our overall understanding of the role miRNA-122 plays during the immunometabolic response to HCV infection. The third chapter of this thesis similarly makes use of ABPP to investigate host-virus interactions taking place during SARS-CoV-2 infection. This study demonstrates changes in enzymatic activation following transfection with the membrane protein of SARS-CoV-2 and following infection with a surrogate virus model, HCoV-229E. These findings set the stage for future research into the role of MGLL and ABHD12 during SARS-CoV-2 infection, two serine hydrolases involved in endocannabinoid metabolism. Finally, the last chapter discusses significance of the methodology and future directions for each project.

Chapter 2: miRNA-122 mediates differential activation of Prolyl Endopeptidase during HCV infection

2.1 Statement of contributions

As the sole author of this thesis, I made significant experimental and intellectual contributions to this project. The original idea for this project is accredited to Dr. John Paul Pezacki, but was a collaborative effort between Tyler Shaw, Dr. John Paul Pezacki and I. Cell culture work including microRNA transfections, siRNA transfections and handling of the virus was performed exclusively by myself. Following sample preparation, all experiments including RT-qPCR, ABPP and data analysis was completed by myself. Raw mass spectrometry results were provided by the John L. Holmes Mass Spectrometry Facility at the University of Ottawa. I carried out the preliminary editing of this thesis before being peer-reviewed by Dr. Magdalena Szuplewska and Dr. John Paul Pezacki.

2.2 Abstract

Viruses will alter their hosts' cellular metabolism to facilitate infection. This requires the interaction of numerous host and viral factors that can affect enzymatic activity both directly and indirectly. For this reason, a comprehensive analysis of enzymatic activity during infection is needed to better understand pathogenesis of diseases. Activity-based protein profiling (ABPP) has become a powerful proteomic technique for characterizing enzymatic activity in different biological systems. It employs an active site-directed chemical probe which selectively reacts with a protein family to allow for a global view of cell state. Here, ABPP is combined with

differential isotopic labelling by reductive dimethylation (DML) to study the downstream effects of hepatitis C virus (HCV) infection on metabolic regulatory enzymes. Additionally, the effects of microRNA-122 dysregulation on the activity of these metabolic enzymes are examined. It has been previously demonstrated that microRNA-122 is sequestered during the immunometabolic response to HCV infection (Luna et al., 2015); however, the downstream effects on the cell's microenvironment remains unclear. The Prolyl Endopeptidase (PREP) is identified as being differentially activated under microRNA-122 knockdown conditions. Experiments in HCV FJH1$_T$-infected cells also reveal enhanced enzymatic activity of this target. Similarly, siRNA-induced knockdown of PREP resulted in lower levels of HCV expression. Using activity-profiling we were able to identify PREP as being differential activated during HCV infection, a possible downstream effect of the microRNA-122 interactome.

2.3 Introduction

2.3.1 microRNAs

The discovery of non-coding RNAs introduced a new way of thinking about signal transduction. Their ability to affect cell regulation to a great enough extent that they have therapeutic potential has made non-coding RNAs central for the topic of gene expression and regulation. A subset of these short RNAs are microRNAs (miRNAs), which are derived from the processing of longer precursor mRNAs that act as inhibitors of translation. Once overlooked, miRNAs have now become recognized for their role in regulating cellular function.

Generally spanning ~20-25 nucleotides, miRNAs act to silence gene expression through canonical base pairing with a target mRNA (Lee et al., 1993). A seed sequence spanning

nucleotides 2-7 provides specificity and facilitates proper interaction of the miRNA to the 3'UTR of its mRNA target (Lewis et al., 2005). Having a short region conferring specificity is advantageous because it allows a single miRNA to have several mRNA targets. Downregulation of gene expression can be mediated by reducing translation efficiency or decreasing mRNA levels by degradation. An additional mechanism exists whereby binding of the miRNA leads to destabilization of the mRNA and premature deadenylation.

Although most miRNA research focuses on its role in gene inhibition, select cases of miRNA activation of protein expression exist (Ramchandran & Chaluvally-Raghavan, 2017; Truesdell et al., 2012). These select cases of miRNA activation were thought to require destabilization of mRNA lacking a 5'-cap, typical of non-dividing cells; however, emerging evidence suggests miRNA can upregulate protein expression in proliferating cancer cells (Chaluvally-Raghavan et al., 2016; Jame-Chenarboo et al., 2022). The exact mechanism of miRNA-mediated RNA activation (RNAa) remains unclear. In some cases, it is caused by the direct interaction of the miRNA to the promotor region of target mRNA that initiates the recruitment of transcription factors needed for gene transcription (Tuz Zohora et al., 2023). Further investigation is needed to better understand what factors contribute to RNAa. Nevertheless, this emerging field in RNA biology is expanding the way we think about miRNA regulation.

Studying miRNA function has been made possible with the development of RNA interference tools such as miRNA inhibitors, miRNA mimics and silencing RNAs. Inhibiting specific miRNAs proves valuable in studying the functions of individual miRNAs in cellular processes (Krützfeldt et al., 2005). For this miRNA inhibitors have been designed to specifically block the activity of endogenous miRNAs. This can be achieved using single-stranded synthetic RNA

oligonucleotides or antagomirs that are complementary to the target miRNA sequence. Chemical modification made to the RNA antagomir including methylation of the 2'oxygen of the ribose sugar, adding stability and reducing susceptibility to RNase degradation. Other chemical modifications also include a 3' end cholesterol-moiety to facilitate cellular update (Lima et al., 2018). These differ from miRNA mimics, also referred to as agomirs, which act to enhance the endogenous effects of a particular miRNA. These double-stranded RNA oligonucleotides contain a guide strand with the same sequence as the endogenous miRNA, enabling it to scan for mRNA seed sequence complementarity in a similar fashion. While miRNA mimics have multiple mRNA targets, a silencing RNA (siRNA) can be used for selective targeting of a single mRNA. This can be useful for studying the effects of knocking down a particular protein of interest. Structurally similar to miRNA mimics which are also short double-stranded RNA oligonucleotides, siRNAs differ in that they have perfect sequence complementarity to its mRNA target. This characteristic allows for selective targeting, unlike endogenous miRNAs and miRNA mimics which contain mismatches to accommodate multiple mRNA targets .

2.3.2 microRNA Biogenesis

MicroRNA biogenesis begins in the nucleus with transcription by RNA polymerase II. These transcripts fold into their secondary structure forming primary miRNA. Drosha, an RNase III-type endonuclease cleaving enzyme in the nucleus cuts primary miRNA into a ~65 nucleotide stem loop precursor miRNA with a 5' phosphate and a two nucleotide 3' overhang. It is important to note that Drosha mediated processing requires an essential cofactor DGCR8. Precursor microRNA can be exported out of the nuclease via Exportin 5 mediated transport. Once in the cytosol, the precursor microRNA is further processed by the Dicer RNase III, resulting in a ~20-25 nucleotide miRNA duplex. Following release from Dicer, the miRNA

duplex is incorporated into the RNA-induced silencing complex (RISC) through association of the Argonaute protein. The miRNA duplex is imperfect in its pairing which promote unwinding and guide RNA selection. The passenger strand is lost and the Argonaute protein remains associated to the guide strand. Thermodynamic features of the duplex appear to play an important role in deciding the guide strand versus passenger strand. The strand with the weakest binding at its 5'-end is more likely to become the guide strand. Other key characteristics of human miRNA guide strands are a U-bias at the 5'-end, whereas the passenger strands have a C-bias. Other proteins are also known to play a role in strand selection (TRBP and PACT). Together, the Argonaut-miRNA complex will scan for sequence complementarity, silencing as many as several hundred mRNA (O'Brien et al., 2018). A summary of the miRNA biogenesis pathway is illustrated in Figure 2.1.

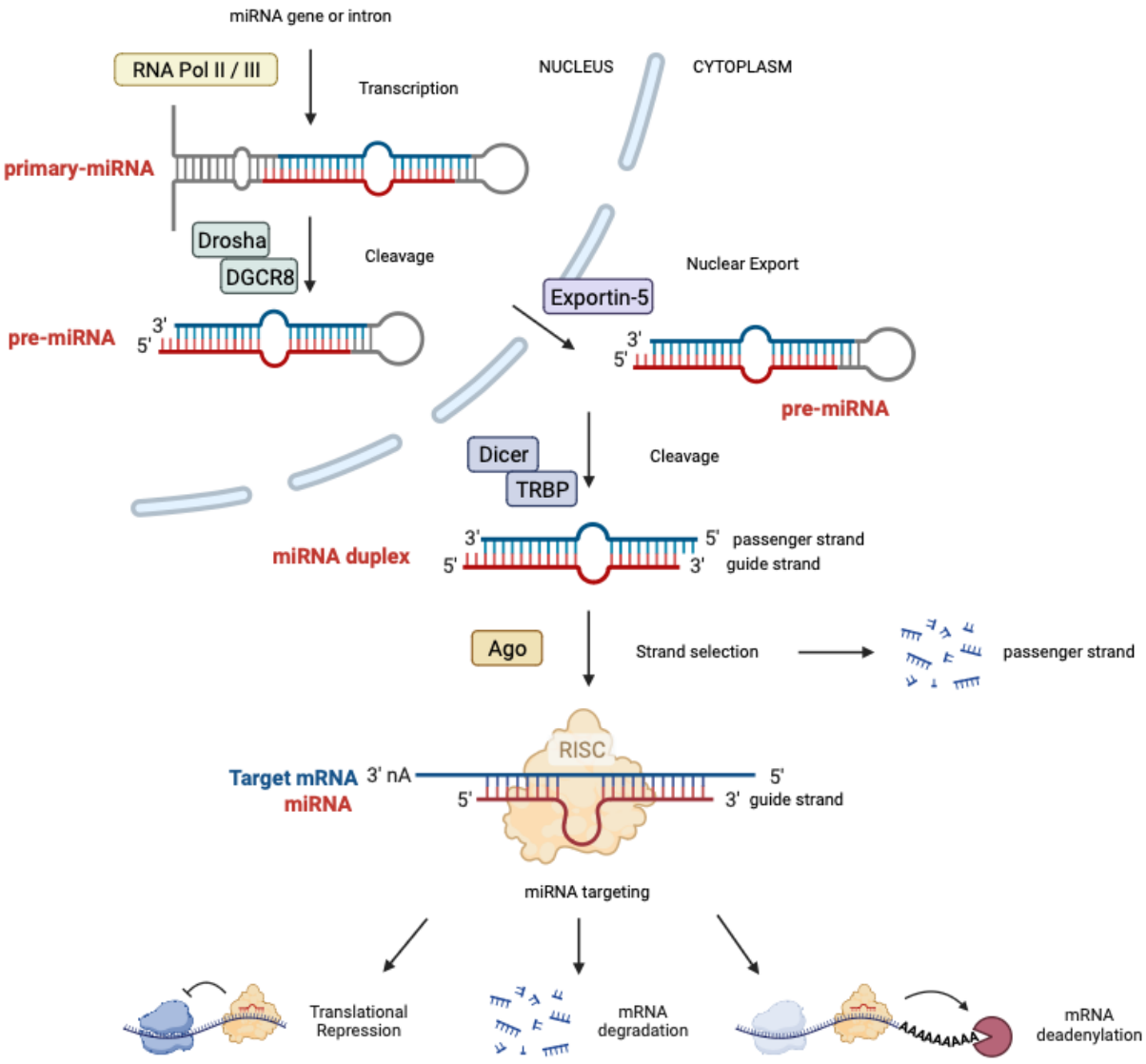


Figure 2.1: microRNA biogenesis pathway. Primary-miRNA is transcribed in the nucleus and is processed by the Drosha RNase III endonuclease resulting in a ~65 nucleotide stem loop precursor miRNA (pre-miRNA). Pre-miRNA exits the nucleus mediated by Exportin-5. Further processing of the pre-miRNA into a ~20-25 nucleotide long miRNA duplex is mediated by Dicer. Association of the Argonaute protein (Ago) and other enzymes not specified here form the RISC complex which carries out strand selection. The guide RNA remains associated to the Ago and scans for sequence complementarity. Silencing of gene expression occurs through target cleavage and degradation, deadenylation or repression of translation. This figure was adapted from (Winter et al., 2009) and made using BioRender.

2.3.3 microRNA-122

As the predominant miRNA in the liver, miRNA-122 accounts for approximately 70% of its total miRNA content (Lagos-Quintana et al., 2002). It is one of the first examples of a tissue-specific miRNA, regulating numerous aspects of liver metabolism (Jopling, 2012). The microRNA Database suggests 5′-UGGAGUGUGACAAUGGUGUUUG-3′ as a consensus sequence of miR-122-5p in both humans and mice. Though many hepatic functions undergo rhythmic changes under circadian regulation, it has been noted that levels of mature miRNA-122 remain relatively constant (Gatfield et al., 2009). This is most likely due to its high stability and central role in maintaining liver homeostasis. Some crucial functions performed by miRNA-122 in the liver include regulating cholesterol and triglyceride metabolism as well as maintaining iron homeostasis (Esau et al., 2006). One target of miRNA-122 is fatty acid synthase (FASN), a serine hydrolase responsible for the denovo synthesis of fatty acids.

Due to its regulatory function, some viruses have evolved ways to dysregulate miRNAs to facilitate infection. For example, promoting lipogenesis can be advantageous to viruses that travel as lipo-viral particles in order to evade immune detection. This is the case for hepatitis C virus (HCV) which uses miRNA-122 during its replication (Luna et al., 2015). miRNA-122 dysregulation is also implicated in the progression of certain disease states. Abundance of miRNA-122 has been shown to be downregulated in human hepatocellular carcinoma (HCC) patients, a common form of liver cancer (Coulouarn et al., 2009). Several knockdown studies using antisense oligonucleotides or antagomirs showed reduced levels of total cholesterol, low-density lipoproteins, and triacylglycerol levels (Chun, 2022). Additionally, the first miRNA targeting antiviral, Miravirsen, was developed as a novel anti-hepatitis C virus therapeutic and

completed Phase II clinical trials (H. L. A. Janssen et al., 2013). This highlighting the potential of anti-miRNA-122 therapeutics for treating metabolic disorders.

2.3.4 Hepatitis C Virus (HCV)

Hepatitis C virus (HCV) is a major cause of liver disease worldwide. The World Health Organization (WHO) estimates an approximate 58 million individuals are affected globally, with about 1.5 million new infections occurring annually. Infection can cause acute or chronic hepatitis, with severity ranging from mild to lifelong illness. Chronic infection can ultimately lead to health complications such as liver failure, cirrhosis, and hepatocellular carcinoma (Perz et al., 2006).

Belonging to the *Flaviviridae* family, HCV is a single stranded RNA virus whose genome encodes a single polypeptide. Replicating in hepatocytes, the virus enters the body mainly through blood-blood transmission. Virus particles travel associated to lipoproteins which facilitates membrane attachment and host immune evasion(Popescu et al., 2014). Receptor-mediated contact of viral and lipoprotein components allows for entry into the target cell. Once in the cytosol, the viral particle is transported to the endoplasmic reticulum where the translated polyprotein is processed by viral proteases (NS2 and NS3/NS4a) into the mature proteins. Further processing mediated by a host signal peptide peptidase occurs at the C terminus of the capsid protein (McLauchlan et al., 2002). Structural proteins include core and envelope glycoproteins E1/E2. The viral encoded replicase components NS3-4A, NS4B, NS5A and NS5B are sufficient to support viral genome replication (Tabata et al., 2020). There remains some debate around the mechanism by which new viral particles are released; however, it is thought that lipid droplets are involved in the production of new viral particles (Miyanari et al., 2007).

2.3.5 HCV Sequesters miRNA-122

HCV replication requires the liver's most abundant miRNA for viral replication. The viral RNA possesses complementarity to the seed site of miRNA-122, indicating possible target recognition. High-throughput sequencing and crosslinking immunoprecipitation (HITS-CLIP) has revealed Argonaute binding on the 5' UTR of HCV RNA at known miRNA-122 sites (Luna et al., 2017; Moore et al., 2015). This binding not only promotes viral replication by stabilizing the 5' uncapped HCV genome, preventing its degradation, but simultaneously sequesters miRNA-122 from binding and repressing its endogenous mRNA targets. This sponging effect can lead to increased translation of miRNA-122 direct mRNA targets as well as increased or decreased translation of downstream targets. This sponging effect is summarized in Figure 2.2. Analysis of the human transcriptome during HCV infection has revealed de-repression of miRNA-122 target mRNA which supports the sponging effect hypothesis. One example of a protein that is post-transcriptionally regulated by miRNA-122 and is de-repressed during HCV replication is Patatin-like phospholipase domain-containing protein 2 (PNPLA2) (M. C. H. Janssen et al., 2013; Yang et al., 2015). This serine hydrolase is involved in the hydrolysis of triglycerides to free fatty acids. During HCV infection, PNPLA2 expression is increased which leads to a rise in free fatty acids, facilitating HCV replication (Eguchi et al., 2022).

Most research focuses on direct mRNA targets of miRNA-122, however; less is known about the impact sequestering miRNA-122 has on its downstream targets. Moreover, miRNA-122 regulates the translation of enzymes involved in post-translationally modifying other enzymes. This can lead to increased or decreased activity of certain enzyme populations with no change to their abundance. For example, activity-based protein profiling has identified Carboxylesterase 1

(CES1) as being differentially activated during HCV infection (Blais et al., 2010). This suggests altering levels of a miRNA can have a cascading effect on enzyme abundance as well as enzyme activity (Figure 2.2). Understanding the miRNA target interactions taking place during HCV replication is needed for the development of effective therapeutics. For example, CES1 is required for the activation of Sofosbuvir, a therapeutic for chronic HCV infection (Bhatia et al., 2014; Li et al., 2021). This treatment is primarily offered to later stage HCC patients, but there remains the need to develop early-stage treatment options or vaccines. This will require a better understanding of all aspects of viral replication as well as miRNA target interactions and their downstream effects.

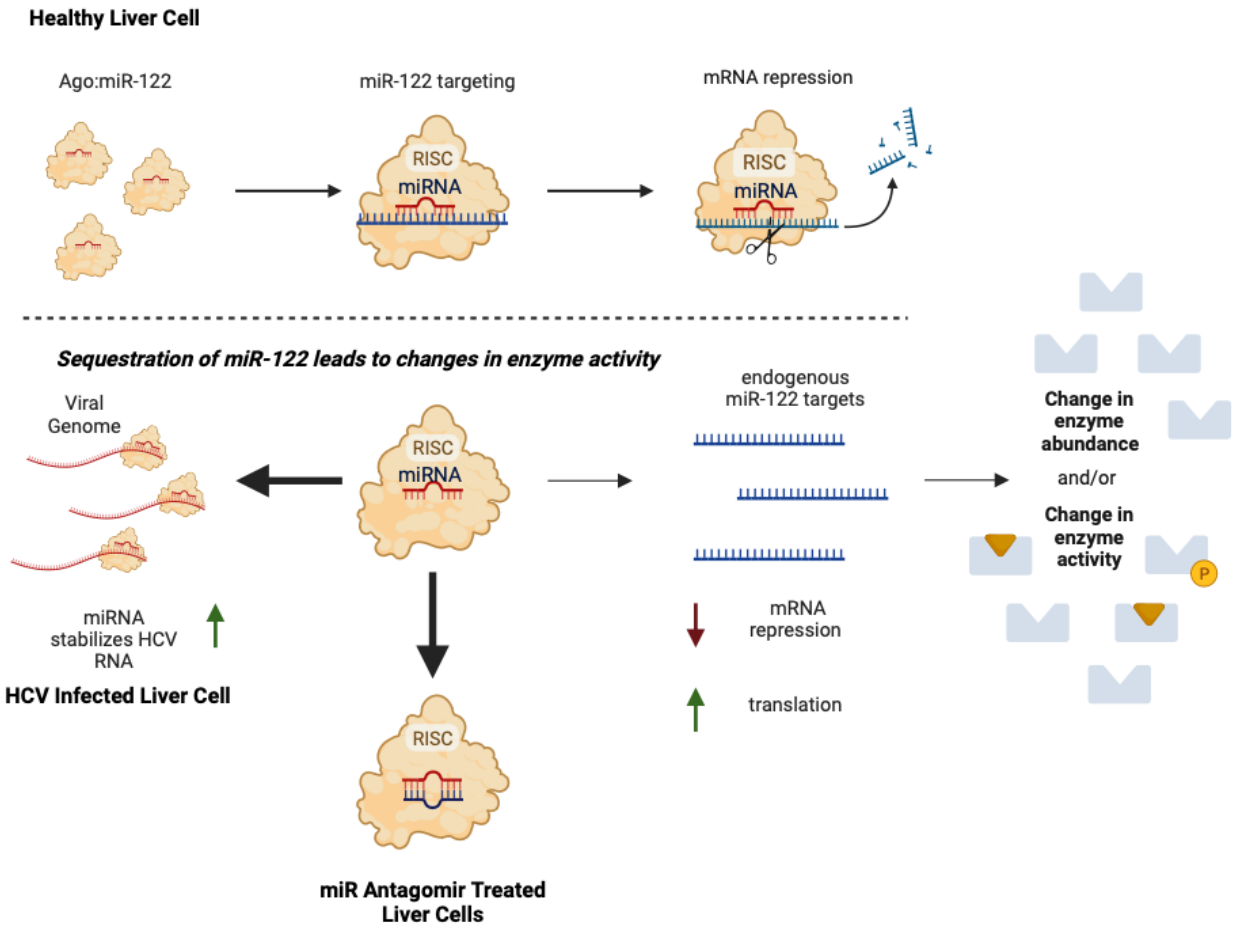


Figure 2.2: miRNA-122 binds the HCV genome. In healthy liver cells, miRNA-122 associated with the Argonaute protein (Ago:miR-122) is free to bind and repress translation of its direct mRNA targets. However, in the presence of the HCV genome miR-122 will bind the viral RNA. This action stabilizes the viral genome and simultaneously reduces the available pool of miR-122 to bind its endogenous targets, leading to reduced repression of direct targets of miR-122. The use of a miR-122 antagomir yields similar effects. Figure was adapted from (Luna et al., 2015) and was created in Biorender.

2.4 Objectives

The overarching theme of this thesis is immunometabolism. In this chapter we aim study the immunometabolic response to HCV infection by identifying host-factors which are dysregulated during infection. Specifically, we are interested in miRNA-122 and identifying direct and indirect miRNA-modulated proteins during infection. With this information we aim to better our understanding of the metabolic pathways affected by the virus in hopes of identifying potential pharmacological targets. Finally, we aim to achieve these goals by working at the interface of chemistry and biology, using chemical tools for tackling complex biological questions. We apply activity-based protein profiling (ABPP), solidifying it as a useful tool in studying host-viral interactions.

2.5 Results

2.5.1 HCV infection alters activity of several metabolic enzymes

Due to miRNA-122 being sequestered by the viral RNA during HCV infection, it was initially believed that knock-down of this miRNA would result in altered activity of its direct and downstream targets. To assess weather knock-down of miRNA-122 affects metabolic pathways in the liver, Huh7.5 cells were treated with an inhibitor (also referred to as an antagomir) of miRNA-122 and activity was assessed by ABPP. The use of a miRNA inhibitor versus a miRNA mimic was intentional given that miR-122 is highly abundant in the liver, therefore the effects of a miRNA mimic may be masked due to saturation of its targets. For this reason, reducing levels of endogenous miRNA-122 using a miRNA-122 inhibitor may be more informative. Additionally, depleting miRNA-122 levels emulates the sponging effect observed during HCV infection. A fluorophosphonate (FP)-Biotin probe was used to select for the serine hydrolase

family. ABPP was combined with dimethyl isotopic labelling to allow for comparisons to be made between samples by liquid chromatography with tandem mass spectrometry (LC-MS/MS). Activity change can be deduced by comparing the abundance of a peptide in the treatment sample over your control sample. By using an activity probe and isolating the active proteome, the dimethyl ratio can report on changes in activity. The use of differential isotopic labeling also reduces the impact of inter-run variability, a common pitfall in comparative mass spectrometry experiments. This approach revealed modest change in numerous serine hydrolases (Figure 2.3A). Next, we sought to investigate whether similar changes were observed in an HCV model. We infected Huh7.5 cells with the $JFH1_T$ strain of HCV and forty-eight hours post-infection, performed in situ labelling of the enzyme using the FP-Biotin probe (Figure 2.3B). As shown in Figure 2.3C, HCV infection resulted in a similar activity profile as observed in the miRNA-122 knock-down samples. PREP, PREPL, ESD, CES1 and CTSA showed modest increase or unchanged levels under both miRNA-122 knock-down and HCV infected conditions. On the other hand, the following serine hydrolase showed a modest decrease under both conditions: PAFAH2, LIPC, LYPLAL1, LYPLA2, MGLL, LYPLA1, PAFAH1B3, FASN and AADAC. CES1 has previously been shown to be differentially active during HCV replication (Blais et al., 2010). Additionally, LIPC was downregulated during HCV infection which supports previous findings by our group, although believed to be under miR-27b regulation (Desrochers et al., 2022). PREP, a prolyl endopeptidase, also showed modest increased (although variable) activity when subjected to HCV and miRNA-122 knock-down conditions.

Next, to validate some of the activity changes observed by mass spectrometry ABPP was repeated, but this time visualized by western blotting (WB). A workflow for ABPP-WB is laid

out in Figure 2.3D. While miRNA-122 inhibition resulted in decreased activity of LIPC and MGLL when compared to a control miRNA inhibitor, we observed increased band intensity of PREP when treated with a miRNA-122 inhibitor (n=3) (Figure 2.3E). These finding are consistent with the mass spectrometry results. Work by Jiang *et al*. illustrates that PREP inhibitors can reduce hepatocyte lipid accumulation and may even be participating in the progression of nonalcoholic fatty liver disease (Jiang et al., 2021; Jiang et al., 2020). Therefore, PREP seems to be an important factor in HCV infection and was included for further investigation.

2.5.2 Gene ontology analysis reveals metabolic pathways regulated by miRNA-122

To investigate the hepatic pathways regulated by miRNA-122 in the context of HCV infection, gene ontology analysis of all serine hydrolases identified in the pulldowns was performed. Gene ontology analysis revealed that all serine hydrolases identified in the pulldown were metabolic serine hydrolases (Figure 2.4A). Gene cluster analysis via Panther and ToppGene Suite was performed to identify pathways being affected by miRNA-122 knock-down (Figure 2.4B). Interestingly, inflammatory pathways involving PREP were also enriched. Of the top hits, LIPC, ESD, MGLL, CES1, PREP, PREPL and AADAC were selected for further investigation. Additionally, to identify potential direct targets of miRNA-122 in hepatocytes, we examined seed-sequence complementarity between predicted targets of miR-122. A full list of predicted miRNA-122 direct serine hydrolase targets can be found in Table 6.3. ABHD11 was the only serine hydrolase we detected in the pulldown with miRNA-122 complementarity in its 3'UTR, therefore it was also selected for further investigation.

2.5.3 miRNA-122 inhibition dysregulates abundance and activity of serine hydrolases

To determine if miRNA-122 inhibition was leading to post-translational changes in activity or simply an increase or decrease in abundance, we performed gene expression profiling by qRT-PCR. We analyzed the expression of LIPC, ESD, MGLL, CES1, PREP, PREPL, ABHD11, and AADAC (Figure 2.4C). Consistent with the activity profile results (Figure 2.3A-B), LIPC showed decreased expression when Huh7.5 cells were treated with a miRNA-122 inhibitor. This indicates that the observed decrease in LIPC is most likely a result of translational repression and not a post-translational change affecting activity. ESD, CES1, ABHD11 and PREPL showed increased expression and activity when treated with a miRNA-122 inhibitor, suggesting there is an increase abundance of the enzymes and most likely no effect on enzyme activity (Figure 2.4D). PREP on the other hand showed decreased expression, although unsignificant, while having increased activity under the same miRNA-122 knock-down conditions. Although unexpected, this result is possibly explained by the existence of a pre-existing pool of inactive PREP which is post-translationally activated under miRNA-122 knock-down conditions. Collectively, these observed changes could be a result of canonical translation inhibition or other downstream effects such as allosteric regulation of pre-existing enzymes. Further investigation will be needed to determine the exact mechanism.

2.5.4 siRNA knock-down of PREP results in decreased HCV replication

Overall, the change in expression or activities of LIPC, ESD, MGLL, CES1, PREP, PREPL, ABHD11 and AADAC further confirms the role of miRNA-122 in modulating various intracellular metabolic pathways. Next, to determine if these serine hydrolases are advantageous to HCV replication, we used small interfering RNAs (siRNA) to specifically knock-down a particular enzyme and observe its effect on HCV-IRES expression by qRT-PCR (Figure 2.5).

Infection of CES1, MGLL, and PREP siRNA-transfected cells with HCV JFH1$_T$ lead to significant reduction in viral RNA after a 48-hour incubation. CES1 siRNA knockdown leading to reduced HCV levels is consistent with previous findings by our lab (Blais et al., 2010). Similarly, work by Filip et al., 2021 showed that infection of MGLL siRNA-transfected cells with HCV JFH1$_T$ lead to a significant reduction in viral RNA as well. Interestingly, knock-down of PREP showed a similar reduction in viral load which, to our knowledge, has not been thoroughly studied in the literature. All other targets showed little to no effect on HCV-IRES expression levels. Given that CES1 and MGLL have previously been studied and that PREP is potentially a novel downstream target of miRNA-122, we selected PREP to investigate further.

2.5.5 Inhibition of PREP by Pikromycin decreases HCV levels

PREP is recognized as a peptidase relevant in neuropeptide metabolism (Toide et al., 1996). While the exact role of PREP in liver metabolism is not as extensively studied as its role in the brain, there is some evidence to suggest its involvement. PREP disruption was shown to reduce triglyceride accumulation in mice hepatocytes and reduce expression of pro-inflammatory factors p65 and ERK (Jiang et al., 2021; Jiang et al., 2020). Also, serine hydrolases have been proven to be promising drug targets, with several being targets of approved drugs for treatment of type 2 diabetes, Alzheimer's disease (Bachovchin & Cravatt, 2012). Therefore, we were interested to see if PREP has therapeutic potential given the observed increased in activity during miRNA-122 knockdown and HCV infection. First, to validate the antiviral effect of PREP siRNA knockdown, we used a pharmacological inhibitor of this serine hydrolase. Using Pikromycin, a PREP inhibitor, on Huh7.5 cells infected with the JFH1$_T$ strain of HCV resulted in a potentially antiviral effect of approximately 75% with an IC50 value of 0.0001945 μM (Figure 2.6).

Therefore, upregulation of PREP in the liver via miR-122 dysregulation during HCV infection may be an important part of its pro-viral effect.

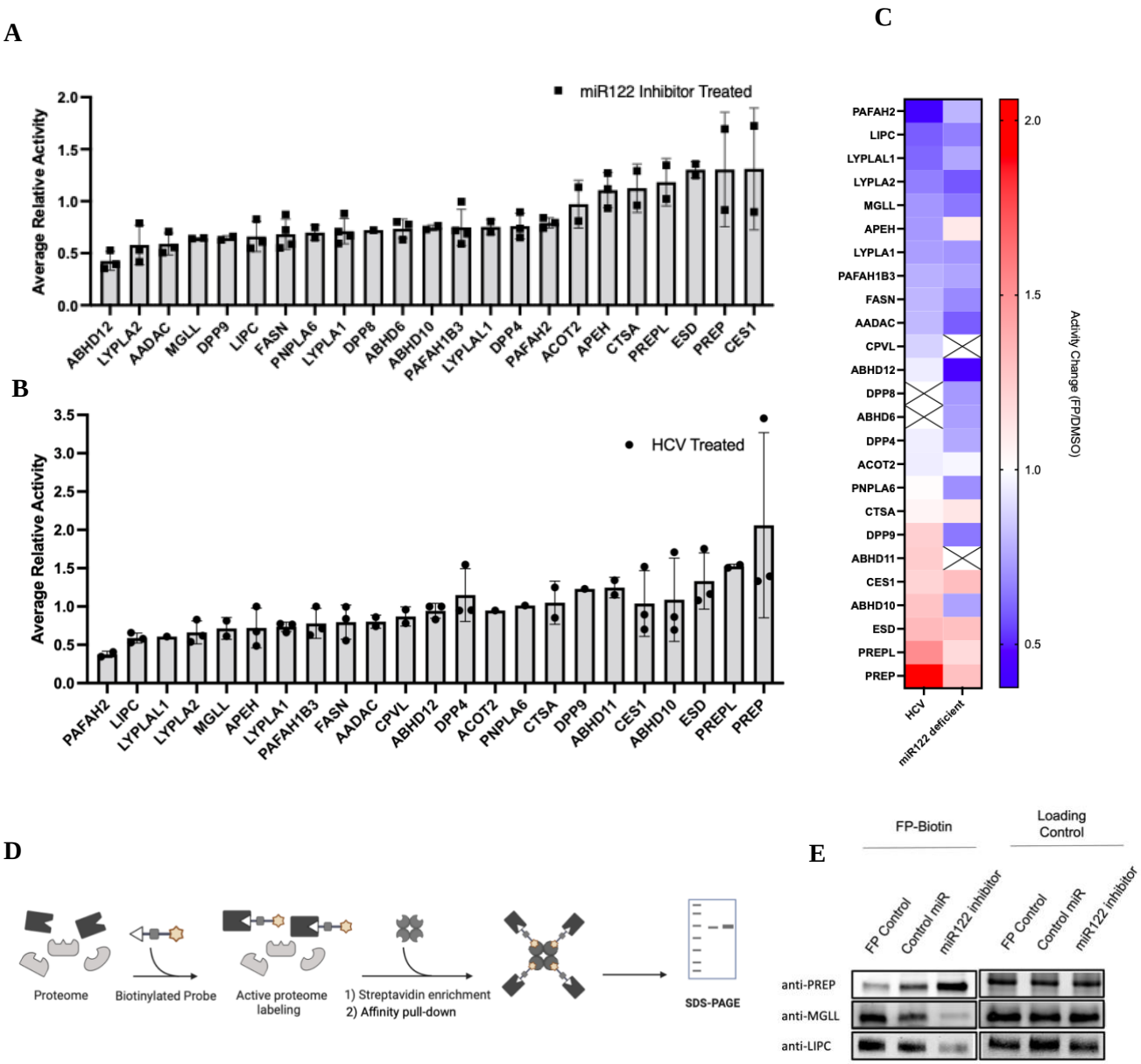


Figure 2.3: Activity-Based Protein Profiling (ABPP) of serine hydrolases.

*(*A) Huh7.5 cells were transfected with inhibitor of miR-122 or a negative control inhibitor at a final concentration of 100 nM. Total proteomes were labelled with FP-biotin (2µM) before ABPP-DML and LC-MS/MS analysis. DML ratio of FP labeled relative to DMSO negative control is shown (Average Relative Activity). (*B*) Huh7.5 cells were infected with HCV $JFH1_T$ strain (MOI = 0.1) for 5h. 48 hours post-infection, the effects of HCV infection on serine hydrolase activity were assessed by ABPP-DML and LC-MS/MS analysis. In *B* and *C*, data are represented as the mean ± S.E. of four biological replicates (n=2-4). (*C*) Heat map comparing average relative activity between Huh7.5 cells treated with anti-

miR122 and HCV infected samples. If a peptide was not detected by MS, the box is marked by an X. (*D*) Workflow of ABPP for western blotting (ABPP-WB). (*E*) Huh7.5 cells were transfected with a miR-122 inhibitor or a miRNA inhibitor control and ABPP-WB was performed. Non-treated Huh7.5 cells were also labelled with the probe and served as an FP Control. An aliquot of each sample prior to FP-Biotin mediated pulldown served as a loading control. Data representative of n=3.

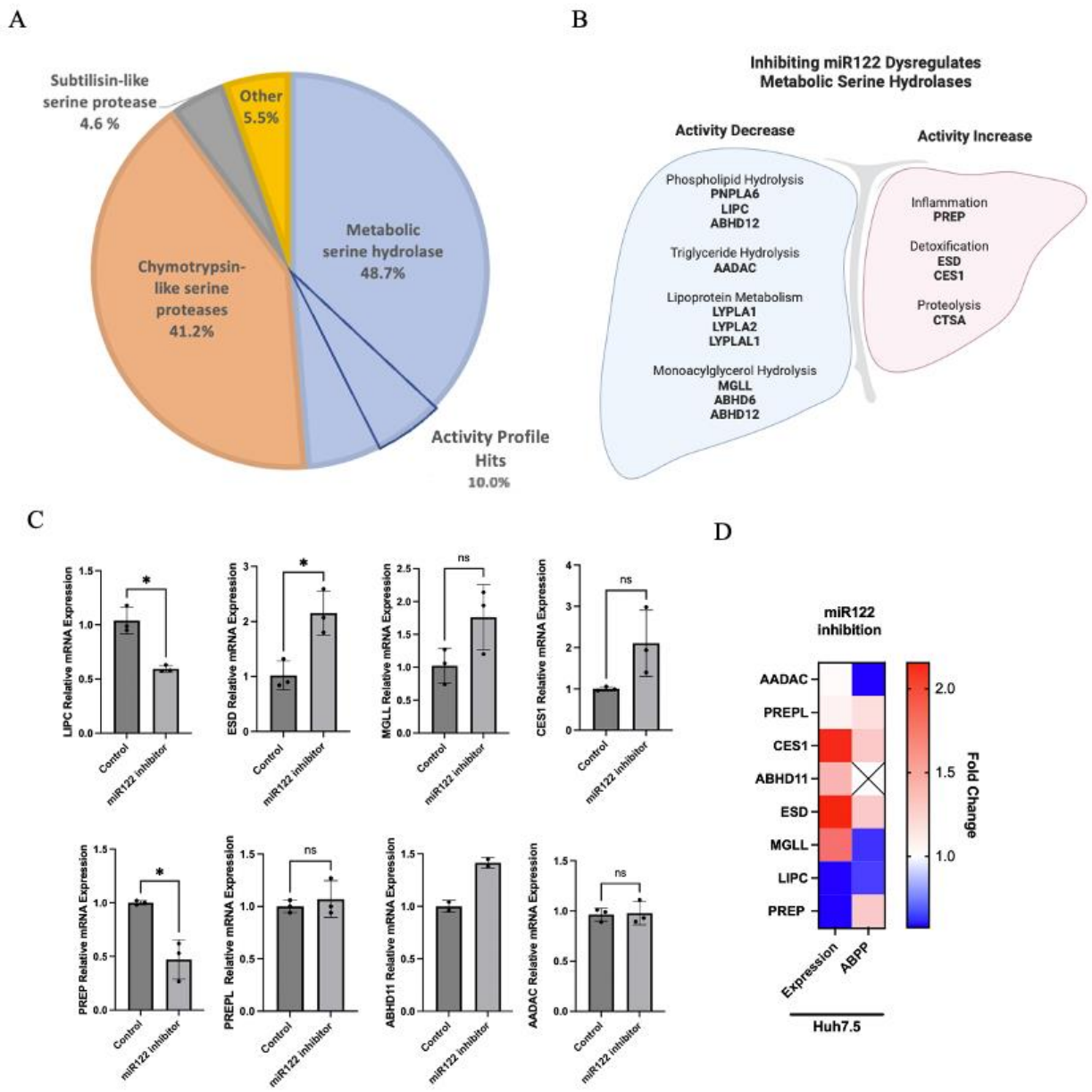


Figure 2.4: miRNA-122 down-regulates metabolic serine hydrolases. (A)Pie-chart depicting classification of the known serine hydrolases based on sequence relatedness (Bachovchin and Cravatt, 2012). All serine hydrolases detected in the pulldown are metabolic. (B) Biological function of serine hydrolases detected in ABPP pulldown organized by increase or decrease in activity following treatment with a miRNA-122. (C) Huh7.5 cells were transfected with inhibitors of miRNA-122 relative to a negative control inhibitor (100 nM). The effects of the miRNA-122 inhibitor on serine hydrolase expression were assessed by qRT-PCR; ns = not significant. (D) Heat map compare activity change by ABPP vs expression change of select serine hydrolases following miRNA-122 inhibitor treatment. Data are represented as the mean ± S.E. (n=2-3). Two-tailed student t-test (*$p<0.05$).

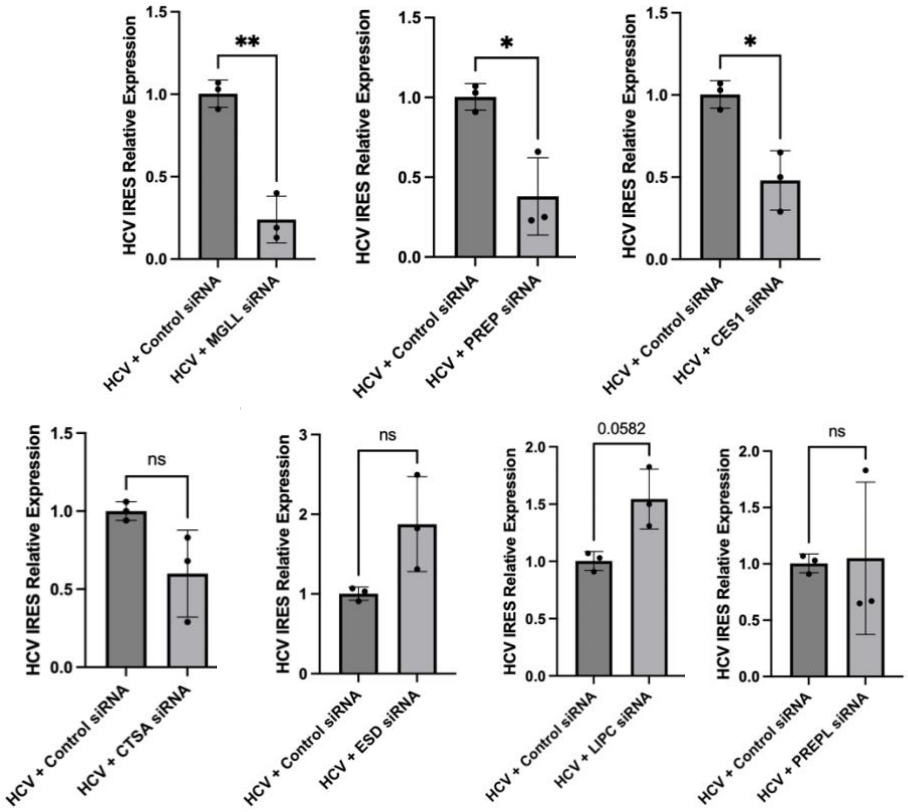
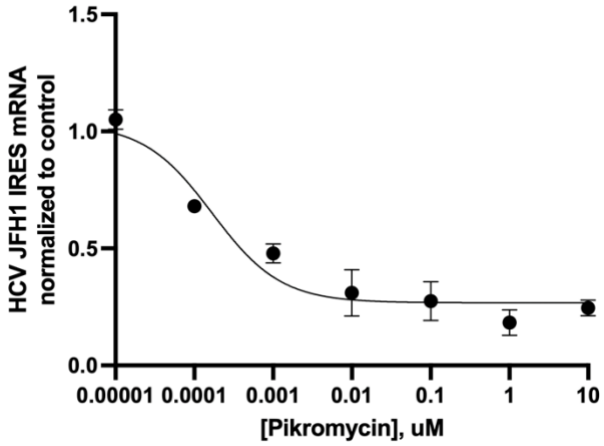


Figure 2.5. siRNA knock-down of metabolic serine hydrolases dysregulates HCV. Relative HCV-IRES expression was quantified by qRT-PCR. Huh7.5 cells were infected with HCV JFH1T strain (MOI = 0.1) and transfected with siRNA (siR) of MGLL, PREP, CES1, CTSA, ESD, LIPC or PREPL relative to a siR Control. 72 hours post-infection, intracellular RNA was harvested for analysis. HCV-IRES expression was normalized to an 18S control. Data are represented as the mean ± S.E. of 3 biological replicates (n=3). ns = not significant; *$p<0.05$; **$p<0.01$

A B



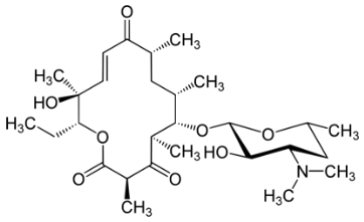


Figure 2.6: Pikromycin decreases HCV-IRES expression in Huh7.5s. (A) Relative HCV-IRES expression was quantified by qRT-PCR and normalized to a DMSO control. Huh7.5 cells were infected with HCV $JFH1_T$ strain (MOI = 0.1) and treated with Pikromycin ranging from 0 to 10 μM. 48 hours post-infection, intracellular RNA was harvested for analysis. HCV-IRES expression was normalized to an 18S control. Data are represented as the mean ± S.E. (n=3). IC50 = 0.0001945 μM. (B) Chemical structure of pikromycin.

2.6 Discussion

Among the serine hydrolases upregulated during HCV infection, prolyl endopeptidase (PREP) demonstrated the largest increase (Figure 2.3A-C). PREP, also known as post-proline cleaving enzyme or prolyl oligopeptidase, abbreviated PO, PE, PEP, or POP was first described in 1971 as a peptidase able to cleave short peptides at the C-side of an internal proline (Walter et al., 1971, 1980). Present in all organs and tissues studied, some findings suggest the highest enzyme activity is in the brain, with modest activity in the liver, heart, kidney, and spleen (Yoshimoto et al., 1979). It was soon regarded as a peptidase relevant in neuropeptide metabolism, with a great drug target potential for neurological disorders' therapy. Unfortunately, there remains uncertainty on PREPs role in the body which has impeded its progress as a therapeutic candidate. It seems to be the case that PREP has multiple roles which depend on the milieu it is located: in or out of the cell, the type of cell or tissue, or the metabolic or pathological conditions in cells. Here, we sought to identify serine hydrolases that are up- or down-regulated during HCV infection with focus on the miRNA-122 interactome. For the first time, we identified PREP as being differentially activated under miRNA-122 depleted conditions (Figure 2.3E). To our knowledge, this is a novel discovery and PREP activation has yet to be studied in the context of miRNA-122 dysregulation. Furthermore, we were able to use siRNAs and a selective pharmacological inhibitor of PREP to confirm its role in HCV replication (Figure 2.5-2.6). Sequence alignment revealed no predicted miRNA-122 seed sites in the PREP mRNA which suggests the observed increase in activity is most likely a downstream effect of miRNA-122 inhibition. Moving forward, efforts will be placed on elucidating the signaling cascade leading to PREP activation as well as the molecular mechanism by which PREP leads to decreased HCV production.

Liver diseases, such as non-alcoholic fatty liver disease (NAFLD), often involve inflammation as a key component and is a hallmark of chronic HCV infection. Recent work from Jiang et al. 2021 illustrates that PREP inhibitors can reduce hepatocyte lipid accumulation and may even be participating in the progression of nonalcoholic fatty liver disease in mice (Jiang et al., 2020). Further work by the same group demonstrated PREP inhibition prevented phosphorylation and activation of ERK and p65 while also reducing the levels of proinflammatory cytokines (Jiang et al., 2021). Since PREP activity was increased in both ABPP profiles (Figure 2.1A-B), this led us to believe HCV sponging of miRNA-122 is contributing to PREP-mediated liver inflammation. Furthermore, we speculate if this surge in PREP activity is functioning as a molecular switch, contributing to the liver inflammatory environment changing from early-stage steatosis to late-stage chronic inflammation; often associated with the progression of HCV infection (Figure 2.7). Transitioning between different stages of NAFLD most likely involves many factors. However, there is consensus that the first hit of steatosis gives rise to excess free fatty acids (FFAs) in liver, making it vulnerable to aggressive factors of the second hit such as oxidative stress and proinflammatory cytokines (TNF-a, IL-6, IL-8) (Bujanda et al., 2010). All to say, it is plausible that de-repression of known miRNA-122 targets is leading to excess FFAs and lipotoxicity, acting as a positive regulator of PREP activity.

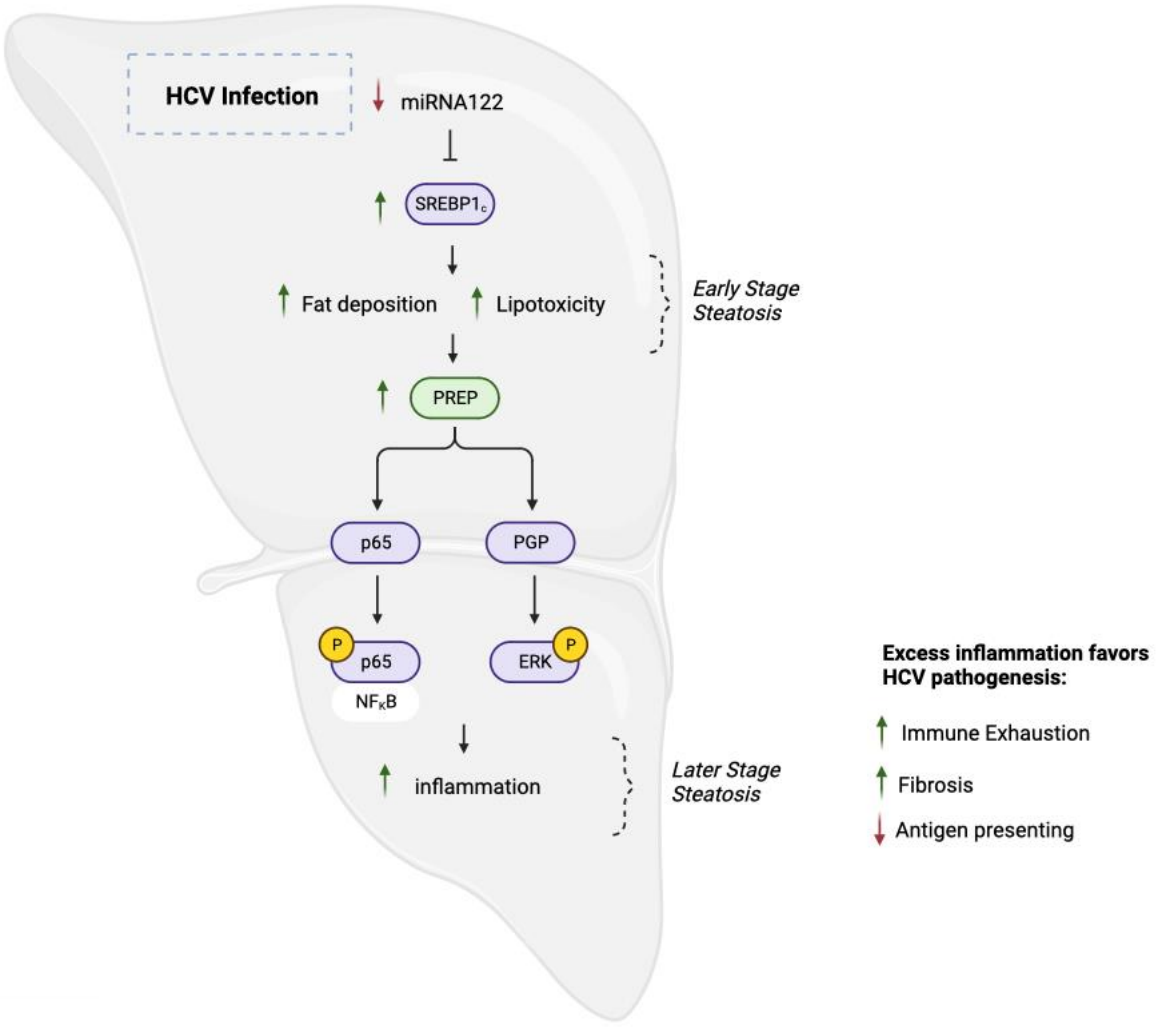


Figure 2.7: Scheme of PREP activation functioning as a molecular switch in the progression of steatosis. Decreased levels of functional miRNA-122 by HCV infection derepresses known direct target the sterol regulatory element-binding protein 1c (SREBP1c) transcription factor contributing to early-stage steatosis. Changes in the local microenvironment of the liver during infection leads to increased PREP activity, resulting in increased NFKB signaling, contributing to long-term inflammation, and potentially inducing the switch from early to late-stage chronic steatosis. Adapted from Jiang et al., 2021 .Image created in BioRender.

2.7 Conclusion

Overall, this study brought forth a new enzyme involved in HCV pathogensis. Activity-based protein profiling showed up-regulation of PREP activity during miRNA-122 knock-down conditions and during HCV infection. Additionally, our findings highlight the crucial role of miRNA-122 in regulating hepatocellular pathways. The identification of PREP as a key player in HCV replication provides a novel avenue for further research of liver diseases. As we move forward, uncovering the signaling cascade leading to PREP activation and elucidating its downstream effects on HCV production will be needed. Moreover, we touched on exploring the broader implications of PREP dysregulation in liver inflammation and its possible role as a molecular switch in disease progression. This presents many exciting avenues for future research as discussed in chapter 4. In conclusion, this study not only advances our knowledge of miRNA-122 but also identifies PREP as having a role in liver metabolism and HCV pathogenesis. It underlines the potential of serine hydrolases as promising therapeutic targets and emphasizes the importance of continued investigation in this field.

2.8 Materials and Methods

Cell culture and treatments

Huh-7.5 human hepatocellular carcinoma cells were grown in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 1X non-essential amino acids (Gibco) and 10 % fetal bovine serum (FBS; Gibco). Uninfected cells were seeded in 6-well plates at a density of 9 x 10^4 cells/well. Following a 24-hour incubation period, uninfected cells were transfected with the miRNA-122 inhibitor or control. Transfections were done with Lipofectamine™ RNAiMAX

Transfection Reagent (Thermo Fisher Scientific) in Opti-MEM for a final miRNA inhibitor concentration of 100 nM per well.

The HCV JFH1T strain was used for HCV infection of Huh7.5 cells. This strain is adapted from the classical JFH-1 strain and is a cell culture model for infection (Wakita, 2009). Naturally occurring patient-derived HCV differs in its ability to infect human hepatoma cells in comparison to the JFH-1 and cell-culture adapted JFH1T strains (Sarhan et al., 2012). The JFH1T strain is highly infectious to Huh-7.5 cells in contrast with patient-derived HCV which are not. Huh7.5 cells were infected at an MOI of 0.1 for 5 hours and then the virus was removed. Following a 48-hour infection period, cells were lysed for RNA extraction in RLT Plus Lysis Buffer (RNeasy Plus Mini Kit; Qiagen).

RTq-PCR

Following cell lysis in RLT Plus Lysis Buffer (RNAeasy Plus Mini Kit; Qiagen), the RNeasy Plus Mini Kit (Qiagen) protocol was followed for RNA isolation. RNA concentration and purity was quantified using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). Reverse transcription (RT) of 250 ng of RNA was performed using iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocols on a T100 thermal cycler (Bio-Rad).

Quantitative real-time PCR was performed on a CFX Connect Real-Time PCR Detection System (Bio-Rad) according to the manufacturer's protocol following reverse transcription. SSOAdvanced Universal SYBR Green Supermix (Bio-Rad) was used to prepare qPCR reaction mixtures for each gene tested. 18S housekeeping gene was used to normalize all the results. A

list of all primer sequences used can be found in the supplemental data Table S.1. Controls for each experiment include no reverse transcriptase and no cDNA template. Fold change in expression relative to the normalized samples were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Data is presented as the mean of replicates. Significance assessed with two-tailed, unpaired student's t-test where $p < 0.05$ was considered significant.

Western Blotting

Cells harvested for western blotting were collected 48 hours post-transfection in 1X SDS lysis buffer (50 mM Tris-HCl [pH 6.8], 2 % SDS, and 10 % glycerol). Protein quantification was done using a DC Protein Assay (Bio-Rad) following the manufacturer's protocol. Readings were obtained on a SpectraMax i3 plate reader (Molecular Devices). Samples containing 20 μg of protein from cell lysate were loaded onto a 12% polyacrylamide gel and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Gels were cast using the TGX Stain-Free FastCast Acrylamide Solutions (Bio-Rad), following manufacturer's protocol. The proteins were transferred from the gel to a PVDF membrane using the Trans-Blot Turbo Transfer System (Bio-Rad). To ensure gel to membrane protein transfer, an image of both the gel and the blots were taken using a ChemiDoc MP (Bio-Rad). Visualizing protein load served as a control. Following membrane blocking in 5% Bovine Serum Albumin (BSA) in TBST (pH 7.4) for 1 hour, the designated primary and secondary antibodies were used to probe for their respective protein. Details regarding the dilutions and antibodies used can be found in table S.2. Visualizing the blots was done using Clarity ECL Western blotting reagents (Bio-Rad) on the ChemiDoc MP (Bio-Rad).

ABPP labelling

Harvesting of proteins for ABPP was done by first discarding the culture medium and the treated Huh-7.5 cells were washed twice with 5 mL 1XPBS. Cells were lysed in 1% Triton X-100 lysis buffer in 1X PBS followed by sonication (15 1s pulses). Quantificaiton of protein abundance was carried out by DC Protein Assay following the manufacturer's protocol on a SpectraMax i3 (Molecular Devices). A total of 1.5 mg of protein was labeled with a FP-Biotin probe (Santa Cruz Biotechnologies) for a final concentration of 1 μM. No probe controls contain DMSO in place of the FP-Biotin. Samples were incubated at 37 °C for 1 hour. Labelled proteins were precipitated out with 5X volume of cold acetone and stored at -80 °C. Following centrifugation at 14000 rpm for 15 minutes at 4°C, the acetone was decanted, and the pellet washed in cold methanol two times. The supernatant was removed and 2.5% SDS in PBS was added followed by sonication (15 pulses) and heating of the samples at 60 °C for 5 minutes. Lastly, the samples were spun 4 minutes at 6500 x g at RT and the supernatant collected. An aliquot was taken to serve as the loading control. Samples were stored in 1X PBS at -20 °C.

Streptavidin pulldown and trypsin digestion

A 50 % streptavidin-agarose bead slurry (Thermo Fisher Scientific) was washed 3 times with 1X PBS in a Bio-Spin column (Bio-Rad) at 1000 x g for 1 minute. Using PBS, the beads were transferred to the protein sample and put on a rotator for 1.5 hours at RT. Pelleted beads were transferred to Bio-Spin columns and washed 3 times with 1 % SDS, 3 times with fresh 6M urea, once with 1X PBS, 5 times with 50 mM ammonium bicarbonate (ABC), and transferred to microfuge tubes using 50 mM ABC. ABC was removed and 10 mM DTT in 50 mM ABC was

added to pelleted beads. Samples were heated to 65 °C for 15 minutes and iodoacetamide was added for a final concentration of 25 μM. They were left to rotate for 30 minutes in the dark before being centrifuged 2 minutes at 1400 x g to pellet the beads. Beads were washed 3 times with TEAB (pH 8.5) before adding 0.01 mg/mL Trypsin. Digestion occurred overnight at 37 °C on a rotator. Supernatants containing trypsin-digested peptides were then transferred to a Bio-Spin column and centrifuged for 1 minute at 1000 x g to collect the digested peptides.

Dimethyl labelling

Trypsin-digested peptides were reconstituted in 100 mM TEAB. Samples were differentially labeled with either light formaldehyde, intermediate ^{13}C formaldehyde or heavy ^{13}C- deuterated formaldehyde (0.16% v/v). Sodium cyanoborohydride was added to light and intermediate samples, and sodium cyanoborodeuteride to the heavy sample for a final concentration of 0.022M. All samples were incubated in a fume hood for 1 hour at RT before ammonia (0.13% v/v) quenched the reaction. To further quench the reaction and acidify the samples, formic acid (0.30% v/v) was added. Differentially labelled samples were sent to the John L. Holmes Mass Spectrometry Facility at the University of Ottawa for analysis by LC-MS/MS.

Chapter 3: MGLL Inhibition downregulates HCoV-229E

3.1 Statement of contributions

The initial idea for this project was a collaborative effort between Dr. John Paul Pezacki and I, Tiffany Stern. Experimental design, sample preparation, RT-qPCR and infections were done by me. ABPP was performed by myself with raw data produced by Zoran Minic at the Mass Spectrometry Facility at the University of Ottawa. A special thanks to Rachel Hausman for her contributions in the wet lab as a summer student. Western blotting was performed by Rachel Hausman as part of her honours research project. I would also like to thank Dr. Magdalena Szuplewska her contribution to the editing of this thesis.

3.2 Abstract

As the world continues to deal with the challenges posed by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), comprehensive investigations into its virology and host interactions continue to be necessary. Investigating the molecular characteristics of SARS-CoV-2 infection is crucial for shedding light on the health implications in some individuals who have recovered from the acute phase of SARS-CoV-2 infection, but still experience lingering symptoms. Here, we employ activity-based protein profiling (ABPP) to identify serine hydrolases that are differentially upregulated or downregulated during infection with a SARS-CoV-2 surrogate virus, HCoV-229E. We observe MGLL and ABHD12 activity as being up-regulated when transfected with SARS-CoV-2 membrane protein and when infected with HCoV-229E. Upon siRNA-knock down of these two enzymes, HCoV-229E abundance is shown to decrease, suggesting MGLL and ABHD12 are potentially pro-viral enzymes. A pharmacological inhibitor of MGLL, MJN110, was also shown to decrease viral replication in vitro. Overall, this study contributes to a better understanding of the roles serine hydrolases play during SARS-

CoV-2 infection and provides insight into some metabolic changes occurring during infection. Further work will be needed to identify long-term effects of these changes and if they have an impact on any physiological systems. Additionally, more studies will be needed to see if there is any connection to the lingering symptoms observed in some SARS-CoV-2 patients.

3.3 Introduction

3.3.1 Sars-CoV-2

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is a novel coronavirus that is responsible for the 2019 pandemic. This global pandemic not only impacted public health, but also had a profound impact on global economies and mental health. As most of the world returns to everyday life, scientists remain engaged in understanding this virus. Most of the efforts were directed towards understanding its pathogenicity to develop effective interventions and implement public health strategies; however, there remains uncertainty around the long-term effects of this virus (Raveendran et al., 2021).

Belonging to the beta-coronavirus genus, SARS-CoV-2 is an enveloped, positive-sense, single-stranded RNA virus. The viral genome encodes several structural and non-structural proteins including the Spike (S) protein, Membrane (M) protein, Envelope (E) protein, and Nucleocapsid (N) protein (Wu et al., 2020). The S protein houses the receptor-binding domain (RBD) which binds the angiotensin-converting enzyme 2 (ACE2) receptor on host cells, necessary for viral entry (Shang, Ye, et al., 2020). Unlike Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV), cell entry of SARS-CoV-2 is preactivated by proprotein convertase furin, reducing its dependence on target cell proteases for entry (Shang, Wan, et al., 2020; Walls et al., 2020). Together the increased ACE2 binding affinity and furin preactivation of spike makes this virus

more efficient at cell entry and contributes to its pathogenicity. Following entry, the viral RNA is released into the cytoplasm and replication is mediated by the viral RNA-dependent RNA polymerase (RdRp). Viral subgenomic RNAs are then translated into viral proteins, including the replicase-transcriptase complex and the structural proteins, within the endoplasmic reticulum-Golgi intermediate compartment before being packaged (Saraste & Prydz, 2021). Lastly, mature virions are released from the host cell through exocytosis, resulting in the spread of infection to neighboring cells.

Similarly to other coronaviruses like SARS-CoV and Middle East Respiratory Syndrome Coronavirus (MERS-CoV), SARS-CoV-2 transmission takes place primarily through respiratory droplets; although, aerosols, direct contact with contaminated surfaces, and fecal–oral transmission were also reported during the SARS epidemic (Otter et al., 2016; Yu et al., 2004). Upon entry into the respiratory tract, SARS-CoV-2 infects epithelial cells in the upper and lower airways. Symptoms can vary from mild cold-like symptoms to more severe symptoms like pneumonia and acute respiratory disease syndrome, in some cases leading to death (Huang et al., 2020). To date, several pharmacological interventions have been explored and approved for the management of SARS-CoV-2 infection, aiming to alleviate symptoms, reduce severity, and curb transmission (Beigel et al., 2020; Najjar-Debbiny et al., 2023; Patel et al., 2022). These therapeutic approaches range from vaccines to repurposed drugs, each with distinct mechanisms of action. Despite significant progress in therapeutic development, there remain critical gaps in our understanding of the host-virus interactions and the optimal treatment strategies to mitigate the long-term impact of this virus.

Long Covid refers to the persistent symptoms experienced by individuals following the acute phase of infection (Guziejko et al., 2022; Raveendran et al., 2021). approximately 35% of people reporting lingering effects. Common symptoms include fatigue, difficulty breathing, joint pain, and chest pain. The underlying pathophysiological mechanisms involve exacerbated comorbidities, physical damage to the lungs, and psychological symptoms stemming from an intensive recovery process (Huang et al., 2021; Richardson et al., 2020; F. Zhou et al., 2020). Researchers are particularly focused on a subset of non-hospitalized individuals without comorbidities, whose immune and inflammatory responses remain elevated for eight or more months after infection clearance. The connections between these prolonged responses and symptoms like heart palpitations, pins and needles, and brain fog are unclear, prompting further investigation into potential genetic factors. Notably, the expression of ACE2 does not seem to predict lingering symptoms, but individuals with chronically elevated levels of interferon B may be more susceptible (Phetsouphanh et al., 2022).

3.3.2 Models for studying SARS-CoV-2

The Centre for Disease Control outlines airborne transmissible diseases like SARS-CoV-2 as Biosafety Level 3 (BSL-3). These viruses can pose significant risk to researchers and the surrounding environment which is why they are contained in specialized facilities (BCL 3 or 4). The use of a surrogate virus model can offer an alternative, safer approach to studying these viruses that is also accessible to more laboratories. One example of a surrogate virus model used to study upper respiratory tract infection in humans is Human Coronavirus 229E (HCoV-229E). Although the symptoms of HCoV-229E are less severe, it still shares key similarities with SARS-CoV-2, making it a valuable tool for understanding coronavirus pathogenesis.

Previous research using HCoV-229E and other Coronaviruses have provided insights into viral entry mechanisms, intracellular trafficking, and proteolytic activation of the viral spike protein (Bertram et al., 2013; Perrier et al., 2019; Shirato et al., 2018). Additionally, studies on HCoV-229E have contributed to our understanding of the host immune response to coronavirus infections, including the release of pro-inflammatory cytokines and induction of the interferon responses (Duncan JKS et al., 2023; Liu et al., 2021). By comparing the similarities between HCoV-229E and SARS-CoV-2, researchers can use this model to explore molecular mechanisms, antiviral strategies, and drug targets, potentially accelerating new scientific discoveries.

3.3.3 Host-viral interactions of SARS-CoV-2

The time-sensitive nature of the SARS-CoV-2 pandemic has resulted in substantial efforts being placed on the development of vaccines, new drugs as well as the repurposing of existing drugs (Liu et al., 2020; Zhou et al., 2023). Through this process it became clear that efforts to develop these therapeutics were hindered by the limited knowledge surrounding SARS-CoV-2 host-viral interactions and their downstream effected molecular pathways. In what was a collaborative effort, researchers came together to address some of these issues. One key paper published by Gordon *et al*. mapped protein-protein interactions between SARS-CoV-2 and human proteins. They identified 332 SARS-CoV-2 protein-human interactions that relate to multiple biological processes, including protein trafficking, translation, transcription, and regulation of ubiquitination (Gordon et al., 2020). With this information they were able to identify promising antiviral drugs targeting specific host factors, like translation inhibitors Zotatifin and Ternatin 4, highlighting the usefulness of studying host-viral interactions. In another paper by Liu *et al.*, they

looked at gene signatures of SARS-CoV-2-infected ferret lungs in both short- (3 days) and long-term (14 days) models. Some highly expressed genes in both short- and long-term models played a crucial role in the progression of SARS-CoV-2 infection, including serine hydrolases DPP4, MGLL, and ABHD6 (Liu et al., 2020). They also observed differences in what pathways were enriched, suggesting that different biological processes are affected in short versus long-term infection. These studies represent just a fraction of the extensive research within this rapidly evolving field.

3.3.4 Serine Hydrolases MGLL, ABHD12 and ABHD6

The endocannabinoid system (ECS) is an important signaling network that regulates key biological processes including the immune response, pain sensation, mood, and appetite. Found in all mammals, the ECS is made up of endocannabinoids as the ligand, the cannabinoid receptors, primarily CB1 and CB2, and the enzymes that synthesize and degrade these molecules to maintain homeostasis, including MGLL, ABHD12 and ABHD6. The main signaling lipids involved in the ECS are anandamide (AEA) and 2-arachidonoylglycerol (2-AG). Both are synthesized on-demand and released from post-synaptic neurons to then bind the CB1 and CB2 receptors. These receptors are found all over the central nervous system and can control the release of neurotransmitters. CB1 receptors are predominantly found in the brain while CB2 receptors are found in immune cells and are involved in immunomodulation and inflammation regulation (Lu & MacKie, 2016).

One of the main enzymes involved in ECS is a serine hydrolase monoglyceride lipase (MGLL). MGLL is well characterized and is responsible for regulating the production of a variety of signaling lipids. It is the rate-limiting enzyme in the degradation of monoacylglycerols. Most

notably it accounts for approximately 85% of 2-AG hydrolysis into arachidonic acid (AA) and glycerol, which is the major enzymatic route for 2-AG inactivation. AA is a precursor for prostaglandin synthesis which are involved in regulating inflammation, blood flow and immunity. Additionally, more is now known about MGLL regulation, which is thought to be under PPARα transcriptional control (Filip et al., 2021; Rakhshandehroo et al., 2007). PPARα is activated by free fatty acids and regulates the transcription of genes involved in fatty acid oxidation, lipoprotein and lipid droplet metabolism, triglyceride synthesis and breakdown, and gluconeogenesis (Bougarne et al., 2018). Given MGLL's involvement in these diverse physiological processes, there has been a surge in the research and development of MGLL inhibitors in the last few years, as evidenced by the over 20 patents since 2018 alone (Bononi et al., 2021). This spotlight on MGLL can be attributed to its potential implications in a variety of health domains including: the treatment of inflammatory diseases, pain management, depression and addressing metabolic disorders.

Similarly to MGLL, although not as well characterized, Abhydrolase domain containing 12 (ABHD12) and abdydolase domain containing 6 (ABHD6) are two other serine hydrolases involved in the ECS. Together, the three serine hydrolases MGLL, ABHD12 and ABHD6 govern about 99% of all 2-AG hydrolase activity in the brain; 9% of which can be attributed to ABHD12 and about 4% by ABHD6 (Blankman et al., 2007). There is still much to learn regarding ABHD12 physiological substrates and functions, however, it does participate in the hydrolysis of 2-AG into ethanolamine. Unlike MGLL which is a soluble enzyme, ABHD12 and ABHD6 are integral membrane enzymes with its active site oriented toward the lumenal/extracellular compartments of the cell (Blankman et al., 2007). There have been some links between ABHD12 dysfunction and disease. Inactivation of the ABHD12 gene have been

connected to the neurodegenerative disease PHARC (polyneuropathy, hearing loss, ataxia, retinitis pigmentosa, and cataract) (Fiskerstrand et al., 2010). Unfortunately, development of therapeutics will require further characterization of this enzyme which is impeded by the lack of selective inhibitors.

3.3.5 MGLL pharmacological inhibitor MJN110

Many pharmacological inhibitors targeting MGLL have been identified (Bononi et al., 2021). The interest in developing MGLL inhibitors is evidently driven by its significance in various physiological and pathological processes such as pain, inflammation, mood, metabolic disorder, cardiovascular disease, and cancer (Gil-Ordóñez et al., 2018). Among these inhibitors, MJN110 emerges as a promising candidate due to its remarkable potency and comparable selectivity when compared to previously developed MGLL inhibitors such as KML29 (Niphakis et al., 2013). The compound's selectivity for MGLL suggests a reduced likelihood of off-target effects, enhancing its therapeutic profile.

3.4 Objectives

This chapter investigates the activity changes that occur in the cell following exposure to SARS-CoV-2 structural proteins as well as in response to HCoV-229E infection. This study ties HCoV-229E infection to increased activity of serine hydrolases MGLL and ABHD12, two key players in endocannabinoid metabolism. This chapter is yet another example of how chemical-biology techniques such as activity-based protein profiling (ABPP) can be applied to the study of host-viral interactions. Here, we aim to use ABPP to identify direct and indirect enzymatic changes that arise as a result of SARS-CoV-2 structural proteins transfection. This information will help

us understand what metabolic pathways are being dysregulated during infection with the ultimate goal of identifying potential pharmacological targets.

3.5 Results

3.5.1 SARS-CoV-2 M Protein alters serine hydrolases involved in endocannabinoid metabolism.

In order to identify differences in gene signatures during SARS-CoV-2 infection, A549 cells were transfected with the SARS-CoV-2 membrane (M) protein and activity changes were assessed by ABPP. The FP-Biotin probe was employed once again to select for the serine hydrolase enzyme family and dimethyl isotopic labeling (DML) allowed for simultaneous analysis by LC-MS/MS of M protein treated samples versus a control vector. Serine hydrolases that appeared in the pulldown are shown in Figure 3.1. To our surprise, the presence of the M protein alone was sufficient to cause changes in enzymatic activity. Modest changes to enzymatic activity were observed in several serine hydrolases. The three most upregulated serine hydrolases identified in the pulldown were MGLL, ABHD12 and ABHD6, the three main enzymes involved in ECS. Similarly, these serine hydrolases showed increased activity when A549 cells were transfect with the accessory protein ORF6 (Figure 6.2). Given that mass spectrometry (MS) can notoriously give rise to various inconsistencies, we sought to validate these activity changes by ABPP- WB. We transfected A549 cells with the sample plasmid containing the M protein and used FP-Biotin to isolate the active serine hydrolases before visualizing by SDS-PAGE. Results are presented in Fig 3.2. Consistent with the MS results, ABPP-WB revealed increased MGLL and ABHD12 activity when treated with the M protein compared to an empty vector control. The M protein vector also containing an additional 3xFlag tag to allow us to monitor transfection efficiency. It should be noted that we were not able to obtain a functioning ABHD6 antibody and therefor have omitted it from further investigation.

3.5.2 Infection with HCoV-229E increased MGLL and ABHD12 activity

To distinguish differences in gene signatures during SARS-CoV-2 progression, we wanted to see if similar changes in serine hydrolase activity were observed in an infectious model. Given the lack of accessibility to BSL-3 facilities we opted to use a surrogate virus model. A549 cells were infected with HCoV-229E and ABPP-WB was performed to see if MGLL and ABHD12 activity were affected in a similar manor. Consistent with the M protein transfection, infection with HCoV-229E also resulted in an increase in MGLL and ABHD12 activity (Figure 3.3). These results point towards possible enriched endocannabinoid metabolism taking place during coronavirus infection. Next, to determine if MGLL and ABHD12 are advantageous to viral replication, we used siRNAs to specifically knock them down and observe its effect on HCoV-229E glycoprotein expression by qRT-PCR. As shown in Figure 3.4, infection of MGLL and ABHD12 siRNA-transfected A549 cells with HCoV-229E resulted in significant reduction in viral RNA after a 48-hour incubation.

3.5.3 Inhibition of MGLL by MJN110 decreases HCoV-229E levels

While cannabinoid-based drugs do exist and act as a target for the treatment of various diseases, there remains room to investigate their potential application towards studying SARS-CoV-2 infection (Klein, 2005; Lowe et al., 2021; Nagarkatti et al., 2009). Also, gene signature studies of SARS-CoV and SARS-CoV-2-infected animal lungs showed elevated MGLL expression levels 14-days post-infection (Lowe et al., 2021). Therefore, we were interested to see if MGLL has therapeutic potential given the observed increased in activity during HCoV-229E infection.Using MJN110, an MGLL pharmacological inhibitor, A549 cells were infected with HCoV-229E before receiving varying concentrations of the drug (Figure 3.5). We observe a dose-dependent

decrease in HCoV-229E glycoprotein expression with increasing concentration of MJN110 as determined by RT-qPCR. The half maximal inhibitory concentration (IC_{50}) of the drug is 0.02759 μM. Cell morphology did appear to change at the highest dose of 10 uM with no other changes observed at the other concentrations (data not shown). In the future, a cell viability assay will be needed to determine the toxicity threshold of the drug. We hypothesis that MGLL inhibition by MJN110 may exert its antiviral effects through upregulation of 2-AG and downregulation of its degradation into proinflammatory metabolite AA. Future efforts will be placed on understanding functional outcomes of chronic endocannabinoid signaling modulation and whether targeting 2-AG at the stage of hydrolysis may have a restorative effect.

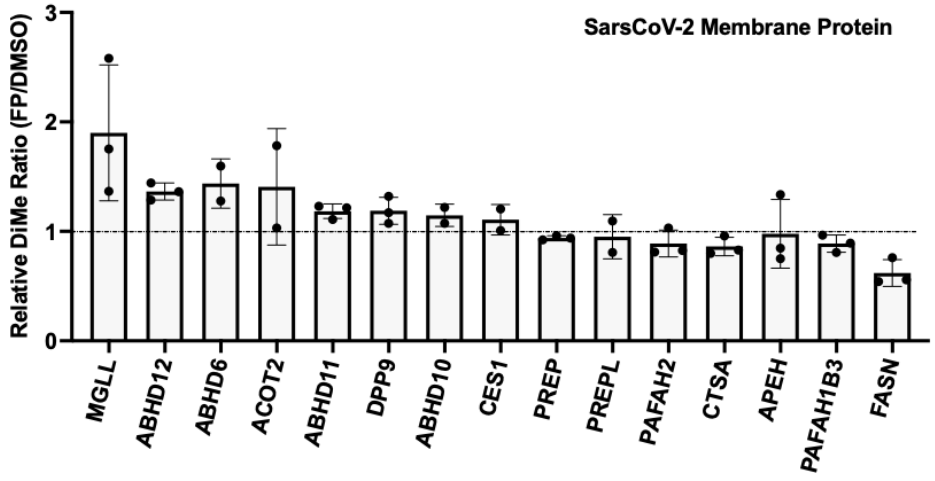


Figure 3.1: Serine hydrolase activity changes in A549 transfected with SARS-CoV-2 membrane (M) protein (ABPP analysis). A549 cells were transfected with the SARS-CoV-2 membrane (M) protein or an empty 3xFlag vector control before being labelled with the FP-Biotin probe (2µM) and isolated by streptavidin pulldown. Samples were then isotope dimethyl labelled for quantitative proteomics. Dimethyl ratio of FP labeled relative to DMSO negative control is shown (Relative DiMe Ratio). Data are represented of at least two biological replicates (n=2-3).

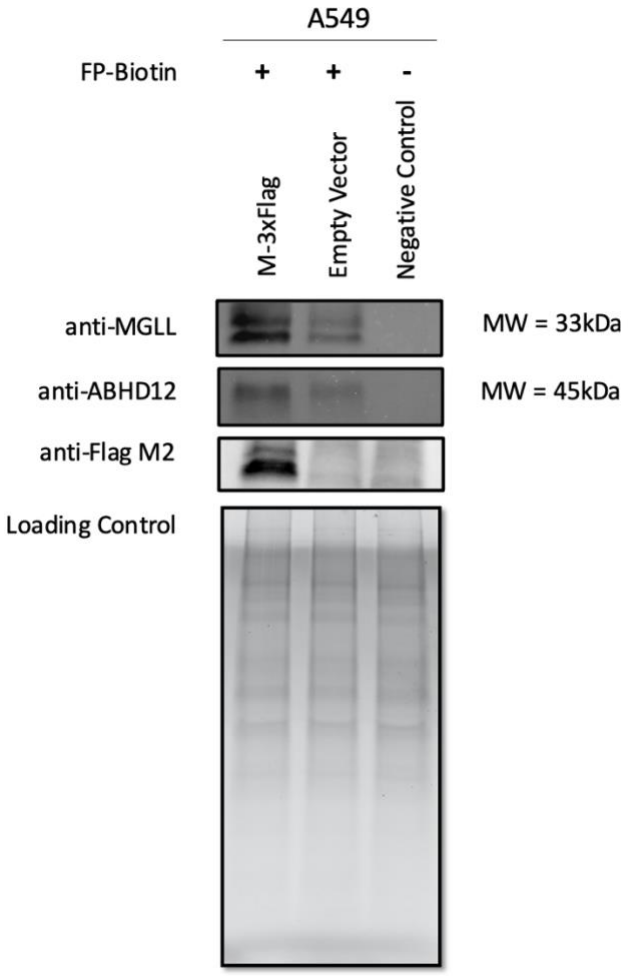


Figure 3.2: Up-regulation of MGLL and ABHD12 in A549 cells transfected with SARS-CoV-2 M protein. A549 cells were transfected with SARS-CoV-2 membrane (M) protein or an empty pTwist vector control before being labelled with the FP-Biotin probe (2μM) and isolated by streptavidin pulldown. Samples were then visualized by western blotting. Blot is representative of three biological replicates (n=3).

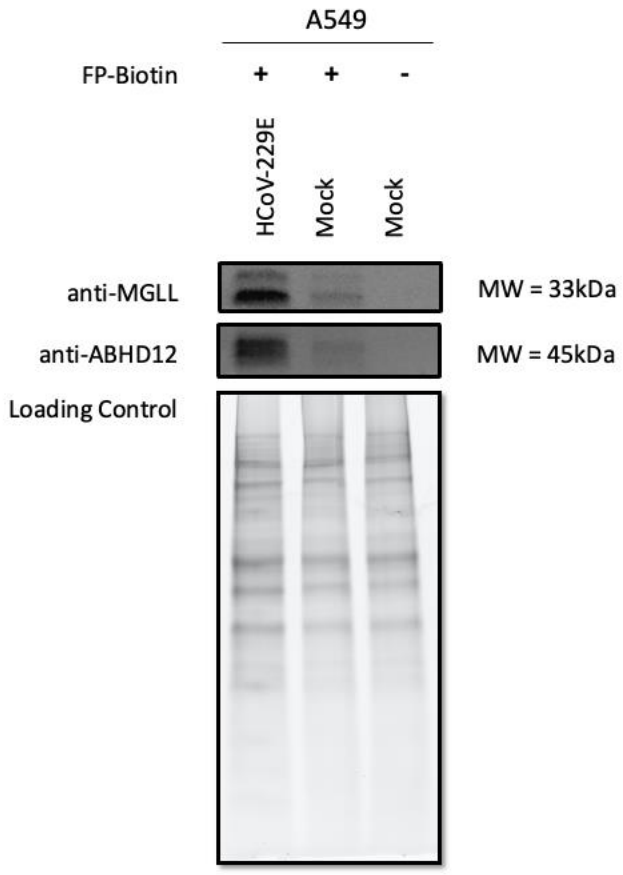


Figure 3.3: Up-regulation of MGLL and ABHD12 during HCoV-229E infection. A549 cells were infected with HCoV-229E (MOI=0.05) then lysed in 1X Triton X-100 before being labelled with the FP-Biotin probe (2µM) and isolated by streptavidin pulldown. One mock infection control was also labelled with the FP-Biotin probe and a second negative control was not labelled. Samples were then visualized by western blotting where they were probed for MGLL and ABHD12 using the designated primary and secondary antibodies. Blot is representative of three biological replicates (n=3).

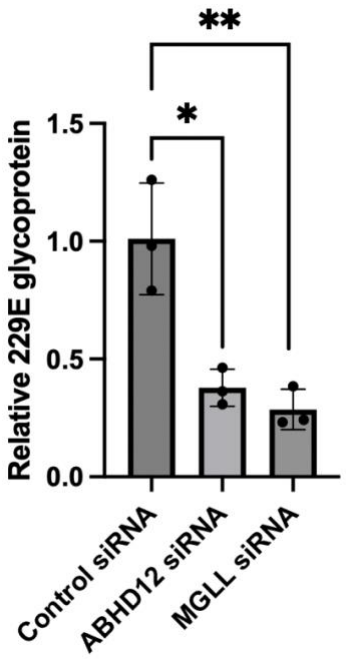
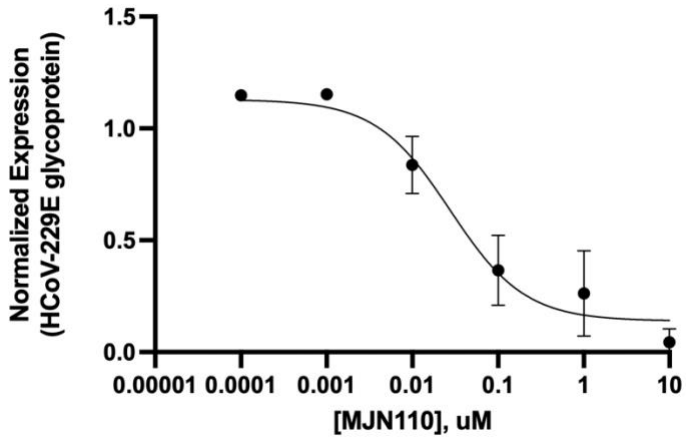


Figure 3.4: Relative expression of HCoV-229E glycoprotein in infected A549 cells during knockdown of MGLL and ABHD12 expression. A549 cells were infected with HCoV-229E (MOI=0.05) and treated with either a control siRNA, MGLL siRNA, or ABHD12 siRNA. 48 hours post-infection, intracellular RNA was harvested for analysis. HCoV-229E spike glycoprotein expression was normalized to an 18S control. Data are represented as the mean ± S.E. of 3 biological replicates (n=3). *p<0.05; **p<0.01

A



B

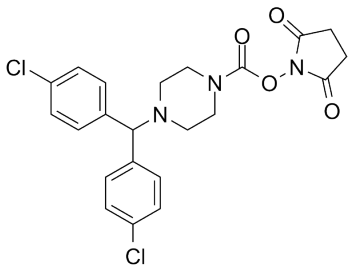


Figure 3.5: MGLL inhibitor, MJN110, decreases HCoV-229E in A549s. (A) Relative HCoV-229E glycoprotein expression was quantified by qRT-PCR. A549 cells were infected with HCoV-229E (MOI=0.05) and treated with MJN110 ranging from 0 to 10 μM. 48 hours post-infection, intracellular RNA was harvested for analysis. HCoV-229E glycoprotein expression was normalized to an 18S control. (B) Chemical structure of MJN110 is shown. Data are represented of 3 biological replicates (n=3). IC_{50}=0.02759 μM.

3.6 Discussion

The endocannabinoid system is present in various systems of the human body including the immune system. It has previously been demonstrated that activation of this pathway may have beneficial outcomes towards fighting infection such as reducing viral entry, decreased pro-inflammatory cytokines, and increasing anti-inflammatory cytokines like IL-10 (Klein, 2005). Taken together, the cannabinoid system shows promise in potentially mitigating pulmonary inflammation, attenuating viral replication, and controlling the cytokine storm. On the other hand, it would be advantageous to the virus to increase the abundance or activity of enzymes involved in the hydrolysis of endocannabinoids, resulting in decreased cannabinoid receptor binding. This could potentially diminish the system's capacity to exert its beneficial effects, including decreasing viral entry and replication, as well as modulating pro-inflammatory cytokines.

Here we show two key players in endocannabinoid metabolism, MGLL and ABHD12, as being differentially activated during HCoV-229E infection. ABPP revealed increased activity of both enzymes when exposed to the SARS-CoV-2 M protein and in the presence of the surrogate virus, HCoV-229E. Additionally, siRNA-induced knock-down of MGLL and ABHD12 resulted in significantly reduced expression levels of HCoV-229E (Figure 3.4). When A549 cells were treated with increasing amounts of MJN110, an MGLL inhibitor, we observed a dose-dependent decrease in HCoV-229E, with an EC50 of 0.02759 μM. This represents a potential targeting strategy for the battle against human coronaviruses (Figure 3.5). However, it will be necessary to conduct a cell viability assay in the future to determine LC50 values. While the cannabinoid system holds various mechanisms that could potentially offer benefits in the treatment of SARS-

CoV-2 infection, further research in this area is crucial for a more understanding the impact of cannabinoids in this context.

New studies funded by the National Institute of Health have revealed SARS-CoV-2 infection results in prolonged inflammation in the nervous system even a month after viral clearance, acting as a driver for long COVID; however, the molecular mechanism to how this arises remains unclear (Frere et al., 2022). It is well established that endocannabinoids mediate inflammation by regulating cytokines at different steps throughout the inflammatory response, and that pharmacological agents acting on various components of the system have already been adopted for their anti-inflammatory properties; however, they have yet to be applied to SARS-CoV-2 'long-haul' treatment (Nagarkatti et al., 2009). Now, we speculate if overstimulation of the ECS may play a contributing role to these lingering effects observed in some recovering SARS-CoV-2 patients. It is plausible that chronic dysregulation of this pathway could contribute to the persistence of symptoms in some individuals. Furthermore, our findings regarding the activation of key enzymes MGLL and ABHD12 during HCoV-229E infection could hint towards a potential mechanism through which the virus manipulates the ECS to its advantage. Of course, this would require further investigation.

3.7 Conclusion

Overall, our findings revealed significant alterations in serine hydrolase activity in response to SARS-CoV-2 M protein. Particularly, ABPP revealed upregulation of MGLL and ABHD12 in response to SARS-CoV-2 M protein and in the presence of the surrogate virus HCoV-229E. Subsequent siRNA knockdown experiments also suggest these two enzymes are necessary in the

viral replication process. Together, these finding point towards a potential mechanism through which the virus may be manipulating the ECS to its advantage. Additionally, the use of MJN110, an MGLL inhibitor, demonstrated a dose-dependent decrease in viral glycoprotein expression, offering a potential therapeutic avenue for combating human coronaviruses. Although promising, this serves as a starting point for deeper investigation into elucidating the molecular mechanisms through which MGLL mediates its pro-viral effects.

3.8 Material and Methods

Cell culture, transfections, and viral infections

A549 cells were grown in DMEM (Invitrogen) and supplemented with 10% Fetal Bovine Serum (FBS; Wisent) and seeded in 6-well plates at a density of $2x10^5$ cell per well. Cells were incubated for 24 hours before being transfected with either MGLL siRNA (ThermoFisher-SciQuest), ABHD12 siRNA (ThermoFisher-SciQuest), or the control siRNA. Transfections were performed as dictated by the manufacturer's protocol using Lipofectamine™ RNAiMAX Transfection Reagent (Thermo Fisher Scientific). The transfection solution contained 7.5uL RNAiMax and 2.5uL of the appropriate siRNA for a final concentration of 25uM of siRNA. A549 cells were infected with viral strain HCoV-229E at a multiplicity of infection of 0.05 in Fetal Bovine Serum-free DMEM. Cells were incubated for 2 hours at 37°C with shaking every 30 minutes. Cells were then washed in 1X PBS and kept in DMEM supplemented with 10% FBS. After an infection period of 48 hours cells were lysed.

ABPP labelling and streptavidin pulldown

Proteins were harvested from treated A549 cells by discarding the culture medium and washing twice with 5mL of PBS before lysing with 1% Triton X-100 in PBS. Samples were then sonicated for 15s (15 1s pulses). Protein quantification was measured using DC assay following the manufacturer's protocol on SpectraMax i3 (Molecular Devices). 1.5mg of protein was labelled with the activity-based fluorophosphonate-biotin probe (Santa Cruz Biotechnologies) and incubated at 37°C for 1 hour before adding 5X volume of cold acetone and left to precipitate at -20°C overnight. Samples were centrifugated at 14,000 rpm for 15 minutes and the acetone was then decanted. Protein pellets were washed with methanol and sonicating before being centrifugated at 6500 x g and 4 °C for 5 minutes. 2.5% SDS in PBS was then added before sonicating for 15 pulses. Samples were then heated for 5 minutes at 60 °C and centrifuged for 4 minutes at 6500 x g. A 20uL aliquot of each sample was taken as a loading control for Western Blotting. PBS was then added to the samples to bring the final volume to 8mL. 100uL of 50% streptavidin-agarose bead 3X slurry (Thermo Fisher Scientific) was added to a Bio-Spin Column (Bio-Rad) and washed 3 times with PBS by centrifugation at 1000g for 1 minute. Streptavidin beads were then transferred to the protein samples in PBS and rotated at room temperature for 1.5 hours. Centrifugation at 1400 x g for 2 minutes was used to pellet the beads and the supernatant was removed before using PBS to transfer the beads and proteins back to the Bio-Spin columns where they were washed 3 times with 1% SDS and 3 times with fresh 6M urea.

Dimethyl labelling and mass spectrometry

Streptavidin-labelled protein samples were washed once with PBS and then washed 5 times with 50mM ammonium bicarbonate. Samples were then transferred to microfuge tubes using 50mM

ammonium bicarbonate and pelleted via centrifugation at 1400 x g for 2 minutes then the ammonium bicarbonate was removed and 10mM DTT in 50mM ammonium bicarbonate was added. Samples were heated at 65°C for 15 minutes and iodoacetamide was added to a final concentration of 25uM. Samples were then rotated in the dark for 30 minutes before being centrifuged at 1400 x g for 2 minutes. The supernatant was removed and the samples washed 3 times with TEAB, pH 8.5, and then 0.01mg/mL of Trypsin was added. Samples were then left to incubate with the Trypsin overnight at 37°C. The beads were then pelleted and the supernatant was transferred to a new Bio-Spin column and centrifuged at 1000 x g for 1 minute. The flow-through is kept for Dimethyl-labeling as it contains the digested peptides.100mM TEAB was added to the trypsin-digested samples which were then differentially labelled with either light formaldehyde, intermediate $_{13}$C formaldehyde or heavy $_{13}$C-deuterated formaldehyde (0.16%v/v). Light and medium samples were treated with sodium cyanoborohydride and the heavy sample received cyanoborodeuteride with a final concentration of 0.022M. All samples were incubated for 1 hour and then the reaction was quenched with ammonia (0.13%v/v) and then formic acid (0.30%) was added to completely quench the reaction as well as acidify it. The light, intermediate, and heavy samples were then sent for analysis by LC-MS/MS at the University of Ottawa's John L. Holmes Mass Spectrometry Facility.

Western Blotting

Samples used for Western Blot were first labelled with the FP-Biotin probe and pulled down using streptavidin. These samples were then washed and resuspended in PBS and centrifuged at 5000 x g for 5 minutes to pellet the beads before pipetting off the remaining PBS. Two 12% polyacrylamide gels were cast using the manufacturer's protocol for TGX Satin-Free FastCast Acrylamide Solutions (Bio-Rad). SDS-PAGE loading buffer was added to both ABPP and

loading control samples at a final concentration of 1X. Samples were then incubated at 95 °C for 15 minutes. 40uL of each sample, including the loading control samples, were loaded onto their respective gels and ran at 120V for 1 hour. Proteins from the gels were then transferred onto PVDF membrane using the Trans-Blot Turbo Transfer System protocol (Bio-Rad). Using the ChemiDoc MP (Bio-Rad), images of both gels and blots were taken to confirm protein transfer. Visualizing of the protein loading control gel served as a control for gel loading. Membranes were then blocked in 5% Bovine Serum albumin (BSA) in TBST (pH 7.4) overnight. The respective primary antibody for the designated protein is diluted in 2.5% BSA in TBST and added to the blots for 1 hour. The blots are then washed 3 times with TBST before adding the respective secondary antibody. The blots are then washed again 3 times with TBST before visualizing blots with Clarity ECL Western Blotting Reagents (Bio-Rad) on the ChemiDoc MP.

RTq-PCR

A549 cells were lysed in RLT Plus lysis buffer (RNeasy Plus Mini Kit, Qiagen) and the protocol for the RNeasy Plus Mini Kit (Qiagen) was followed for RNA isolation. NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific) was used to quantify the RNA. 250ng of RNA was used for reverse transcription using iScript cDNA Synthesis Kit (Bio-Rad) following the manufacturer's protocol on a T100 thermal cycle. CFX Connect Real-Time PCR Detection System (Bio-Rad) was used after cDNA synthesis for quantitative real-time PCR following the manufacturer's protocol. 18S housekeeping gene was used to normalize all results and 229E glycoprotein was used to measure the amount of virus in cells. qPCR reaction mixtures for both genes were prepared using SSO Advanced SYBR Green Supermix (Bio-Rad). 0.5uM of each the forward and reverse primer was added to each well for a total reaction volume of 10uL. No

reverse transcriptase and no cDNA template controls were used. Fold change in glycoprotein expression was normalized to control siRNA samples and calculated using the 2-ΔΔCt method (Livak & Schmittgen, 2001). Data is presented as the mean +/- standard error of 3 replicates.

Chapter 4: General Discussion and Future Directions

There exists a constant battle between viruses and their host organism to establish or eliminate infection. Understanding how viruses impact metabolic pathways and immune responses is crucial for uncovering host-viral interactions and achieving the long-term goal of developing therapeutic agents. The work presented in this thesis falls into this category, looking at the immunometabolic response to infection. The work sits at the interface of chemistry and biology, using chemical probes for studying complex biological problems. Both chapters made use of a chemical biology tool, activity-based protein profiling (ABPP), to better understand the roles of a particular virus in the body. The first experimental chapter employs ABPP to study the miRNA-122 interactome and its dysregulation in response to hepatitis C virus (HCV) infection. In the second experimental chapter, we use ABPP as a starting point to better understand the impact SARS-CoV-2 has on the serine hydrolase enzyme population. The following discussion summarizes the key findings of each chapter, acknowledges limitations, and explores future directions for each project.

4.1 Functional effects of miRNA-122 determined by activity-based protein profiling

Chapter 2 of this thesis uses ABPP to determine the effects of miRNA-122, the liver's most abundant miRNA, on the regulation of serine hydrolases. Overall, the results provide a deeper understanding of how HCV-induced miRNA-122 sequestration reshapes the cellular environment in the liver, promoting infection. This is done through the regulation of expression and/or activity of various serine hydrolases including monoacylglyceride lipase (MGLL), carboxylesterase 1 (CES1), lipase C (LIPC), and notably prolyl endopeptidase (PREP). PREP,

who has been well documented for its roles in neuropeptide metabolism in the brain has in the last few years re-emerged as a peptidase with anti-inflammatory properties and important roles in liver metabolism (García-Horsman, 2020). It has also been applied towards the study of non-alcoholic fatty liver disease (Jiang et al., 2020). To our knowledge, PREP has not previously been studied in the context of miRNA-122. Here, we were able to determine that miRNA-122 inhibition increased PREP activity by ABPP. Interestingly, we also identified PREP as a new pharmacological target whose inhibition and downregulation hinder HCV propagation. From here, there are numerous paths for deeper exploration into how metabolic pathways operate during HCV infection, along with uncovering their regulatory mechanisms. First, our study primarily used the Huh7.5 cell line. While this provided valuable insights, it may not fully represent the complexity of in vivo liver metabolism. Future studies might consider using primary hepatocytes or an animal model. Second, while miRNA-122 knockdown led to changes in metabolic serine hydrolases, it's important to acknowledge that miRNA knock-down can have off-target effects. Additionally, ABPP analyzed by liquid-chromatograph tandem mass spectrometry can notoriously produce variable results. For these reasons, the specificity of the observed effects of miRNA-122 should be confirmed. This can be done by ABPP analyzed by western blot, which was only done for PREP, MGLL and LIPC due to time constraints. The other serine hydrolases detected in the pulldowns should be validated as well in the future. Thirdly, pharmacological inhibition and siRNA knock-down of PREP showed a potent antiviral effect against HCV. While Pikromycin has shown promise as a PREP inhibitor, further investigations are needed to assess its specificity and potential toxicity. Conducting additional experiments, including cell viability assays, will be crucial in the future. In the upcoming sections, we discuss the benefits of using proteomic techniques like ABPP to study miRNA

function, and we explore the possible connection between hepatitis C virus and PREP's role in inflammation.

4.1.1 ABPP as a tool for studying microRNAs

As described in the general introduction, a range of proteomic approaches can be applied to investigate interactions between host and viral proteins. These include techniques like mass spectrometry, affinity purification, and protein microarray analysis. They play a crucial role in identifying proteins that exhibit altered expression levels during infection, providing valuable insights into the host's response to infection. Among these techniques, ABPP stands out as an invaluable tool for studying carbohydrates, lipids, and proteins in biological processes. So much so that creator Benjamin Cravatt was named winner of the 2022 Wolf Prize in Chemistry for his contributions towards understanding cellular communication (Weerapana & Hacker, 2023). In our examination of miRNA-122, we use the fluorophosphonate activity-based probe to assess the activity of serine hydrolases. While the fluorophosphonate (FP)-based probe has been instrumental in characterizing serine hydrolase activity, it's important to note that ABPP encompasses a wide range of probes tailored for diverse applications. For instance, diazirine-based probes have proven invaluable in studying photo-crosslinking events, allowing for precise identification of enzyme-substrate interactions (Shigdel et al., 2008). Other probes include the Wortmannin which our research team has previously used to identify kinases that exhibit varying activity when subjected to the full genomic replicon of HCV (Desrochers et al., 2016). Many of these probes have been applied to the study of miRNA function. In the past, the study of miRNAs has often relied on microarrays or high-throughput sequencing of crosslinked protein-RNA complexes to uncover miRNA direct targets. However, these methods often overlook biomolecular interactions and post-translational modifications that can lead to functional

outcomes. For these reasons, ABPP offers a promising alternative because it can pick up on some of these post-translational modifications. For example, in two studies lead by Roxana Philips and Geneviève Desrochers in our group, ABPP revealed that miRNA-185 and miRNA-27b downregulated MGLL and LIPC respectively, both central to the miRNA's effects on lipid metabolism (Desrochers et al., 2022; Filip et al., 2021). Additionally, PAFA1B3 was identified as being differentially activated by miRNA-27b, although no change in abundance was observed. After further characterization by Parrish Evens in our lab, PAFAH1b3's increase in activity seems to be a result of increased s-palmitoylation. This would have gone unnoticed with traditional abundance based proteomic techniques since there is no overall change in the abundance of the enzyme. These studies not only solidify ABPP as an indispensable tool for studying miRNA function, but also highlight its applicability to the field of chemical biology.

4.1.2 Role of PREP in inflammation

A surprising result of our investigation into miRNA-122's regulation of serine hydrolases was its modulation of PREP, a peptidase involved in the metabolism of peptide hormones and neuropeptides (García-Horsman, 2020). Additionally, when subjected to HCV infection we saw increase in its activity. Furthermore, siRNA mediated inhibition as well as the use of a pharmacological inhibitor, Pikromycin, showed potent antiviral effects against HCV. Although some links between PREP function and liver metabolism have been made, the mechanism through which upregulation of PREP is proviral is not clear. In the literature, PREPs involvement in liver disease was brough to light by the work of Jiang *et al.,* where they demonstrated that PREP inhibitors can reduce hepatocyte lipid accumulation. Further work by this group showed that PREP inhibition prevents p-ERK and p-p65, reducing the levels of proinflammatory cytokines (Jiang et al., 2021). They also propose that PREP disruption hinders the buildup of

hepatic macrophages by influencing the production of the peptide PGP and the movement of neutrophils in response to HFD stimulation. In recent work by a different group, Zhang *et al.*, they also looked at high fat diet (HFD) induced liver disease but at different timepoints and discovered PREP activity and expression is increased in HFD mice compared to control mice only after 12 weeks (Zhang et al., 2023). Based on this information and our findings, we propose a mechanism through which miRNA-122 sequestration during HCV infection leads to downstream activation of PREP (Figure 2.7). It is important to mention that this is just a piece of the puzzle and does not encompass the totality of PREP mediated inflammation during HCV infection. Investigating whether the decrease in inflammatory response is primarily attributed to the absence of PREP or to the reduced accumulation of hepatic fat in these mice could be a potential hypothesis for subsequent research. Finally, investigating the potential long-term effects of PREP inhibition on liver function and metabolism will be interesting to assess its potential function as a molecular switch from early-stage steatosis to late stage chronic hepatosteatosis.

Moving forward, elucidating the intricate signaling cascade leading to PREP activation and its subsequent downstream effects on HCV production would provide insights into the underlying molecular mechanism. Exploring the potential interplay between PREP, excess free fatty acids, and lipotoxicity in the progression of non-alcoholic fatty liver disease could also offer valuable insights into the broader implications of PREP modulation in liver inflammation. To do this we can integrate a multi-omics approach, using lipidomics, proteomics and metabolomics, and other large-scale screens to get a more comprehensive view of the intricate regulatory network involving miRNA-122 and PREP. It would also be interesting to look at the diverse roles of PREP across various tissues and under different physiological conditions to see if its activity and

regulation vary in different organs. Previously disregarded, studying PREP's non-enzymatic functions could help uncover some of the mysteries which have impeded its success as a therapeutic agent in the past (Svarcbahs et al., 2019). Eventually, extending our research to in vivo models, like a mouse model, would provide a more accurate understanding of how miRNA-122 and PREP interact within a whole organism, accounting for systemic effects. By addressing these limitations and pursuing these future directions, this research has the potential to significantly advance our understanding of the interplay between miRNA-122, PREP, and liver metabolism.

4.2 Activation of MGLL and ABHD12 by SARS-CoV-2 membrane protein

Chapter 3 of this thesis applies ABPP to determine the effects of SARS-CoV-2 on the regulation of serine hydrolases. We identified 2 serine hydrolases involved in endocannabinoid metabolism, MGLL and ABHD12 as being differentially activated following transfection with the SARS-CoV-2 membrane (M) protein and HCoV-229E infection. Further experiments involving siRNA knockdown of MGLL and ABHD12 demonstrated their necessity in viral replication. Lastly, the MGLL inhibitor, MJN110, exhibited a dose-dependent reduction in viral glycoprotein expression, indicating a potential therapeutic avenue. Overall, these preliminary results open further questions into the role of endocannabinoid metabolism in SARS-CoV-2 infection. Our study focused on the SARS-CoV-2 M protein, but it would be interesting to create a library of activity profiles, including all other SARS-CoV-2 structural and non-structural proteins. This would allow comparisons to be made between the different profiles, potentially bringing structure-specific interactions to light. Next, extending the research to animal models, such as mice, would provide a more comprehensive understanding of how MGLL and ABHD12 interact

with the virus in a whole organism. Also, the use of a surrogate virus model, HCoV-229E, introduces extrapolation biases since our goal is studying SARS-CoV-2. Collaboration with a facility equipped for working with SARS-CoV-2, or animal models, could help address these limitations. Another main goal for the future is to understand the molecular mechanisms through which MGLL and ABHD12 mediate their pro-viral effects. Some possible ways to do this could be performing co-immunoprecipitation experiments to identify interaction partners of MGLL and ABHD12. We could also carry out substrate specificity studies against a library of possible substrates to identify molecules MGLL and ABHD12 interact with. Other possible avenues of exploration include looking at specific stages in the viral life cycle such as entry, replication, or assembly to see what the implication of MGLL and ABHD12 on those processes are. In the upcoming sections, we comment on the role of the endocannabinoid system during infection and speculate if its overstimulation could contribute to the prolonged inflammation observed post-SARS-CoV-2 in some individuals.

4.2.1 ABPP as a tool for studying the endocannabinoid system

As mentioned previously, ABPP is a valuable tool for studying the functions of enzymes within complex biological systems. Here are a few examples of how ABPP has been useful in studying the endocannabinoid system (ECS). The FP-Biotin probe has been applied to label multiple serine hydrolases of the ECS, including FAAH, MGLL, DAGLα, DAGLβ, ABHD6 and ABHD12. It was ABPP combined with targeted lipidomics that allowed for MGLL to be discovered as the predominant enzyme in 2-AG hydrolysis followed by ABHD12 and ABHD6 (Blankman et al., 2007). ABPP has also been applied to studying fatty acid modulation of the ECS and the effects it may have on food intake and metabolism (Naughton et al., 2013). In this study researchers discovered that certain dietary fats, including oleic acid, might indirectly

influence the ECS. For example, oleyl trifluoromethyl ketone which is an analogue of oleic acid, was revealed to react with multiple brain membrane serine hydrolases by ABPP with FP-biotin, suggesting that dietary fats may influence endocannabinoid signaling in the brain, potentially affecting appetite and energy balance (Kidd et al., 2001). In our work, we use ABPP to study SARS-CoV-2 and found upregulation of three serine hydrolase involved in endocannabinoid metabolism, MGLL, ABHD12 and ABHD6. Once again illustrating the wide-range of applications ABPP has to offer.

4.2.2 Endocannabinoid metabolism and SARS-CoV-2

The endocannabinoid system (ECS) is a widely distributed regulatory network consisting of cannabinoid receptors, natural signaling molecules known as endocannabinoids, and the enzymes responsible for producing and breaking down these signaling molecules. As touched on in Chapter 3 introduction, the ECS plays a pivotal role in a range of physiological and pathological functions, including neural communication, neural growth, adaptability of neural connections, emotional state, appetite regulation, and the immune response. The enzymes involved in the production and breakdown of endocannabinoids have garnered significant attention from both academic and industrial sectors due to their potential therapeutic applications. A surprising result from our investigation was that transfection of the SARS-CoV-2 membrane protein into lung cells caused increased levels of MGLL, ABHD12 and ABHD6, all of which metabolize the 2-AG endocannabinoid. Furthermore, pharmacological inhibition and siRNA knock-down of MGLL showed a dose-dependent antiviral effect against HCoV-229E. While MJN110 has shown promise as an MGLL inhibitor, further investigations are needed to assess its specificity and potential toxicity. Conducting additional experiments, including cell viability assays, will be crucial in the future. Researchers are only now starting to uncover some links between

endocannabinoid metabolism and SARS-CoV-2 and the mechanism through which upregulation of MGLL is pro-viral is not yet known. Elevated levels of MGLL during infection could increase the conversion of 2-arachidonoylglycerol to arachidonic acid and glycerol, potentially decreasing activation of the cannabinoid receptor type 2 (CB2). The role of the CB2 receptor in modulating inflammation and immune responses is well documented (Hernández-Cervantes et al., 2018). It is also known that the stimulation of CB2 receptors limits the release of pro-inflammatory cytokines, shift the macrophage phenotype towards the anti-inflammatory M2 type and enhance the immune-modulating properties of mesenchymal stromal cells (Rossi et al., 2020). This would agree with our findings that SARS-CoV-2 M protein activates MGLL and offers a possible explanation to how this could be beneficial to the virus. Further studies should investigate if MGLL is in fact leading to increased CB2 signaling and if this has any biological effect relevant to the SARS-CoV-2 life cycle. Lastly, some researchers have identified prolonged inflammation in the nervous system post-SARS-CoV-2 infection (Maamar et al., 2022). It would be interesting to investigate the potential long-term effects of chronic endocannabinoid signaling modulation on the immune response. It may offer some light on mechanisms underlying post-SARS-CoV-2 inflammation.

4.3 Concluding Remarks

The work presented in this thesis explores miRNA-122 interactions, HCV infection, endocannabinoid metabolism, and SARS-CoV-2 infection. Our findings not only complement current knowledge on these topics, but also paves the way for further investigations into host-virus interactions. Additionally, in both experimental Chapters, 2 and 3, we emphasize the significance of a powerful tool in chemical biology, namely, activity-based protein profiling. Collectively, the findings from all three projects showcased in this work set the stage for further exciting new avenues of research in the fields of chemical biology, host-virus interactions, and immunometabolism.

Chapter 5: References

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Chapter 6: Appendix

6.1 Supplemental material

Table 6.1: List of primer sequences for qRT-PCR. Related to Figure 2.1

Gene ID	Forward Primer (5'-3')	Reverse Primer (5'-3')
LYPLA2	TCAAGAAGGCAGCAGAGAACATCAA	GCTCAACGCCACGATGCC
PNPLA3	TCTGGATTCTTCCCCGGAGT	ACTCAGTGCTGTAGCGAGC
ABHD2	ACAATGTGATGAGGAAGTTTCACG	CTTTCATGCACCAACGGATCG
ABHD12	ACCTTTGACTACAGAGGTTGGG	AGATGTACACGGGGTTGTCAC
ESD	ATCTTGAGCCCCGCCTTTTAC	AATGGTAGGCGATTCTACGGTG
MGLL	TGCAAACGCCAGCACATAATGG	TGGGACACAAAGATGAGGGC
LYPLAL1	GCCTCTCTGATCTTCCTGCAT	GAGTATATGATCTGGGAGGAGCTGT
PREP	GAGACCGCCGTACAGGATTAT	TGAAGTGGCAACTATACTTGGGA
LIPC	ATCAAGTGCCCTTGGACAAAG	TGACAGCCCTGATTGGTTTCT
FASN	GAAACTGCAGGAGCTGTC	CACGGAGTTGAGGCGCAT
CPVL	TGGAAGGTGATTGTTTCGCTG	GTCTCCCTTAGGTGGCATGGA
DPP4	CGTGAAGCAATGGAGGCATTC	GTGACCATGTGACCCACTGT
CTSA	TCCCAGCATGAACCTTCAGG	AGTAGGAAAGTAGACCAGGG
PAFAH1B3	ACATCCGGCCCAAGATTGTG	GGGCTGTCGCTCATTCACC
RNA18S	GCGATGCGGCGGCGTTATTC	CAATCTGTCAATCCTGTCCGTGTCC
JFH1 IRES	GTCTGCGGAACCGGTGAGTA	GCCCAAATGGCCGGGATA
HCoV-229E	TGGCCCCATTAAAAATGTGT	CCTGAACACCTGAAGCAAT

Table 6.2: Antibodies and dilutions for immunoblotting. Related to figure 2.1, 3.2, 3.3

Antibody	Dilution	Supplier
PREP	1:1000	Santa Cruz Biotechnologies
CTSA	1:5000	Santa Cruz Biotechnologies
MGLL	1:100	Santa Cruz Biotechnologies
ABHD12	1:1000	Abcam
LIPC	1:1000	Santa Cruz Biotechnologies
3xFlag	1:2000	Sigma
HCV-core	1:5000	Abcam
NS5A	1:7000	Virostat
gαm-HRP	1:20000	Jackson ImmunoResearch Laboratories Inc.
dαr-HRP	1:20000	Jackson ImmunoResearch Laboratories Inc.

Table 6.3: Predicted miRNA-122 serine hydrolase targets. miRNA-122 predicted targets were selected for from a list of AGO-CLIP and RNA-seq data (Luna et al., 2015; Moore et al., 2015; Luna et al., 2017). Serine hydrolases revealing miRNA-122 specific binding sites were compared to a list of known miR-122 targets (TargetScan) and known direct targets are indicated by (*).

Predicted miRNA-122 Targets: Serine Hydrolases		
LYPLA2	APEH	PLG
LYPLAL1	BPHL	PNPLA3
ABHD12	C1S	PNPLA6
ESD	FASN	PREP
F2	HPN	SCPEP1
CES2	IMMP2L	TMPRSS2
CPVL	LONP2	TPP1
HABP2	MASP1 *	TYSND1
CEL	MASP2	MGLL
CLPP	PAFAH1B3	LIPC
ABHD2	PCSK5	DPP4
ABHD14B	PCSK6	ABHD11 **

***** 62-69, 1455-1461 of MASP1 3'UTR

** 380-387 of ABHD11 3'UTR

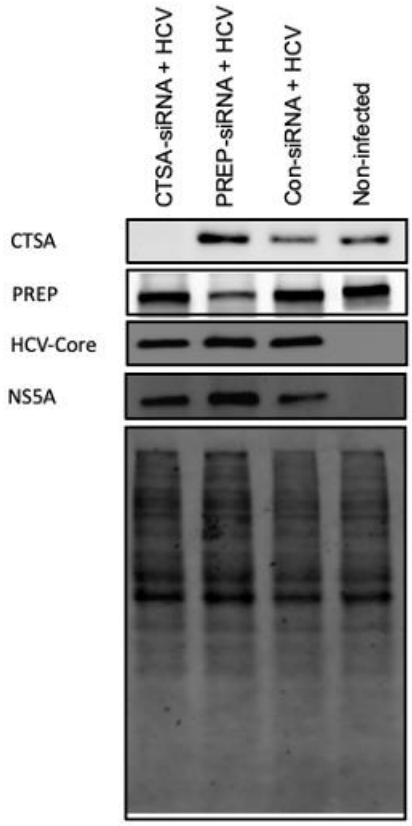

Figure 6.1: Validation of PREP and CTSA knock-down by siRNA. Huh7.5 cells were infected with HCV JFH1T strain (MOI = 0.1) and treated with either a control siRNA, CTSA siRNA, or PREP siRNA. 48 hours post-infection, intracellular RNA was harvested for analysis. Complete knock-down of CTSA was visualized by western blotting using the designated primary and secondary antibodies. Partial knock-down of PREP was also observed. Viral proteins HCV-core and NS5A served as an infection control. Blot is representative of two biological replicates (n=2).

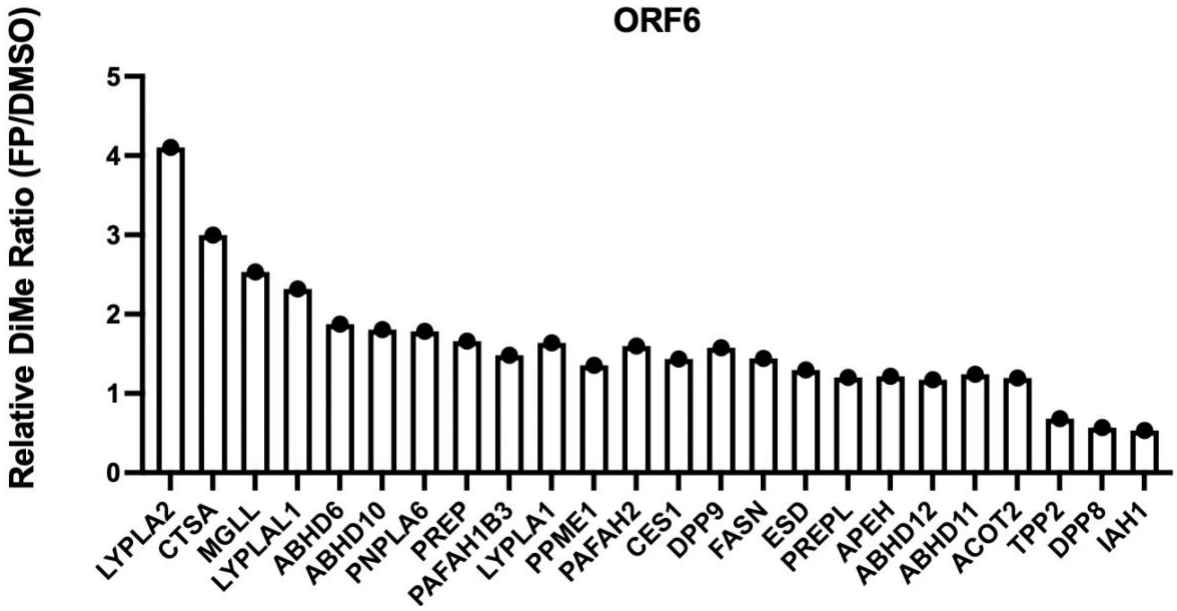

Figure 6.2: SARS-CoV-2 ORF6 dysregulates activity of serine hydrolases (by ABPP). A549 cells were transfected with the SARS-CoV-2 accessory protein ORF6 or an empty 3xFlag vector control before being labelled with the FP-Biotin probe (2μM) and isolated by streptavidin pulldown. Samples were then isotope dimethyl labelled for quantitative proteomics. Dimethyl ratio of FP labeled relative to DMSO negative control is shown (Relative DiMe Ratio). Data are represented of at least 1 biological replicates (n=1).