Quantitative Subcellular Analysis of the Effects of the Enigmatic Protein PCSK9

By

Nicholas Denis

Department of Biochemistry, Microbiology, and Immunology

Submitted in partial fulfillment

Of the requirements for the degree of

Masters of Science

Faculty of Graduate Studies

The University of Ottawa

Ottawa, ON

December 2010

© Nicholas Denis, Ottawa, Canada, 2011

ABSTRACT

PCSK9 is the third gene implicated in autosomal dominant hypercholesterolemia, due to its role in promoting the degradation of the low density lipoprotein receptor (LDLR). Little is known regarding the mechanism by which it promotes the degradation of LDLR, nor the effects PCSK9 has on other cellular proteins. I report here the first quantitative subcellular proteomic study of proteins affected by the expression of a variant of PCSK9. I show that the expression levels of 293 proteins were affected by the expression of the PCSK9-ACE2-V5 construct. Of particular interest, is a protein involved in receptor recycling, EHBP1, which shows reduced protein levels by both PCSK9-ACE2-V5 and the PCSK9-D374Y mutant. I show that an EHBP1 binding protein, EHD4, binds with PCSK9 and LDLR. These results establish novel effects of PCSK9 on liver cell protein levels, of which some relating to endosomal sorting are shown to bind to PCSK9 and LDLR in complex, providing insight into the mechanism of PCSK9 mediated LDLR degradation.

Acknowledgements

My time in the lab started with a nervous phone call to a potential supervisor, unsure of how to pronounce his name. Dr. Daniel Figeys has been a great supervisor to me and has always been open, honest and helpful with many discussions about science, but more so about important life matters outside of the lab. My path through graduate school has not been that of a straight line, but one with lots of turns and changes. Throughout this, Daniel was both critical and understanding, a great combination to keep me focused, but also allow me to pursue my passion outside of science. For this I am very grateful, thank you Daniel! I would also like to thank the members of my thesis advisory committee, Dr. Mbikay and Dr. Bennett, for their support, criticisms and suggestions over the years.

Over the years I have had the great pleasure of meeting many students and post-docs in Daniels lab, and have been lucky enough to become friends with most of them. Weimin Hou, the first person to train me on the reactor and since then became great friends. Dr. Julian Vasilescu, my ubiquitination colleague and friend. Dr. Jeffrey C. Smith and Dr. Theodore Glenn Wright, two great post-docs. Dr. Hou Zhou, Dr. Riujun Tian, and of course Dr. Houjiang Zhou, you guys have all helped me continuously in the lab and had fun both doing it. Dr. Maroun Bou-Khalil, Mohamed Abu-Farha and Jean-Phillippe Lambert, thanks for always taking the time to help me when I needed it. To everyone else, thanks!

Finally I would like to thank my parents John and Linda Denis, and my brother Joel Denis, for their constant support, interest and encouragement over the years. In addition to my supervisor, I have them to thank for always listening and talking to me about my crazy mind-changing decisions, thank you.

Table of contents

List of Abbreviations

PCSK9	Proprotein convertase subtilisin kexin type 9
LDLR	Low-density lipoprotein receptor
MS/MS	Tandem mass spectrometry
PC	Proprotein convertase
ER	Endoplasmic reticulum
ACE	Angiotensin converting enzyme
EV	Empty vector
NARC-1	Neural apoptosis-regulated convertase 1
ADH	Autosomal dominant hypercholesterolemia
LDL-C	Low-density lipoprotein cholesterol
SREBP	Sterol regulatory element-binding protein
EGF	Epidermal growth factor
BACE1	β-site amyloid precursor protein (APP)-cleaving enzyme 1
VLDLR	Very low-density lipoprotein receptor
DMEM	Dulbecco's Modified Eagle Medium
SILAC	Stable isotope labelling of amino acids in cell culture
FBS	Fetal bovine serum
PBS	Phosphate buffered saline
EDTA	Ehylenediamine tetraacetic acid
PNS	Post-nuclear supernatant
PBS-Tw	PBS-Tween
TGN	Trans Golgi network
HRP	Horseradish peroxidise
HPLC	High performance liquid chromatography
ESI	Electrospray ionization
LTQ	Linear trap quadrupole
SDS-Page	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
IP	Immunoaffinity purification
IPP	Insilicos proteomic pipeline

List of Figures

List of Tables

Chapter 1. INTRODUCTION

The Proprotein convertase subtilisin kexin type 9 (PCSK9) has been identified in humans to be the third genetic *loci* linked to autosomal dominant hypercholesterolemia. This finding was due to the mutations found throughout this protein that affect its ability to regulate the low-density lipoprotein receptor (LDLR) [25]. Higher levels of PCSK9 or its gain-of-function mutants lead to increased levels of LDL, whereas lower levels of PCSK9 or it loss-of-function mutants lead to lower levels of LDL [27,28]. PCSK9 is a member of the family of proteins called the proprotein convertases. The primary role of proprotein convertases is to cleave a wide variety of proteins leading to the activation or inactivation of the proteins by cleaving a key fragment or the generation of smaller biologically active peptides/proteins. PCSK9 is a unique convertase in many ways; despite having a functional proteolytic active site that leads to autocatalysis, no known substrates have been identified to date. As well, PCSK9 moves through the secretory pathway and is secreted into the plasma. Circulating PCSK9 then binds cell surface LDLR in the liver, is internalized with the LDLR and disrupts the normal recycling of the LDLR to instead promote the degradation of the LDLR in acidic lysosomal vesicles. To date, the mechanism of how PCSK9 mediates the degradation of the LDLR remains unclear, as are the other potential functions and roles that PCSK9 has in liver cells.

In this study I have employed various proteomic technologies, including qualitative and quantitative approaches to gain insight into novel roles PCSK9 plays in liver cells. Through the quantitative analysis of subcellular fractionated HuH7 liver proteins, it was possible to identify proteins whose relative abundance levels were significantly affected by

PCSK9 expression. Through the use of immunoaffinity purifications of PCSK9 containing protein complexes coupled with liquid chromatography tandem mass spectrometry (LC-MS/MS), it was possible to identify novel interaction partners of PCSK9. These proteins have potential links to the PCSK9 mediated degradation of the LDLR, such as the endocytic machinery proteins EHBP1 and RME-8, as well as many other interesting functions unrelated to LDLR regulation such as vesicle trafficking, chaperone activity, post-translational modifications, cell adhesion and cell signalling. The results found in this study, and future studies stemming from them, provides a better understanding of PCSK9 mediated degradation of the LDLR, as well as other novel functions PCSK9 plays. Overall, this study of PCSK9 demonstrates the advantages of taking global, large-scale and quantitative approaches to studying a protein of limited understanding, in gaining novel information regarding the proteins functions, both known and unknown.

1.1 Literature Review

I.Proprotein Convertases

i. General Background

Biologically active polypeptides and proteins are often the product of post-translational processes and regulations. Often, such polypeptides and proteins are activated following intracellular proteolytic cleavages of inactive precursor proteins. Such precursor proteins regulated by proteolytic activation include hormones, cell surface receptors, proteases, growth factors, cell signalling proteins, as well as pathogenic factors such as viral and bacterial proteins.

The proteases responsible for such proteolytic activation of precursor proteins are called proprotein convertases (PCs), and are secretory proteins containing serine protease activity. The first proprotein protease to be discovered was the kexin protease (KEX2 gene) from *S. cereveisiae,* which was later characterized as a Ca^{2+} -dependant serine protease[1,2]. Soon after the discovery of Kex2 protease, homologues in humans were discovered[3,4].

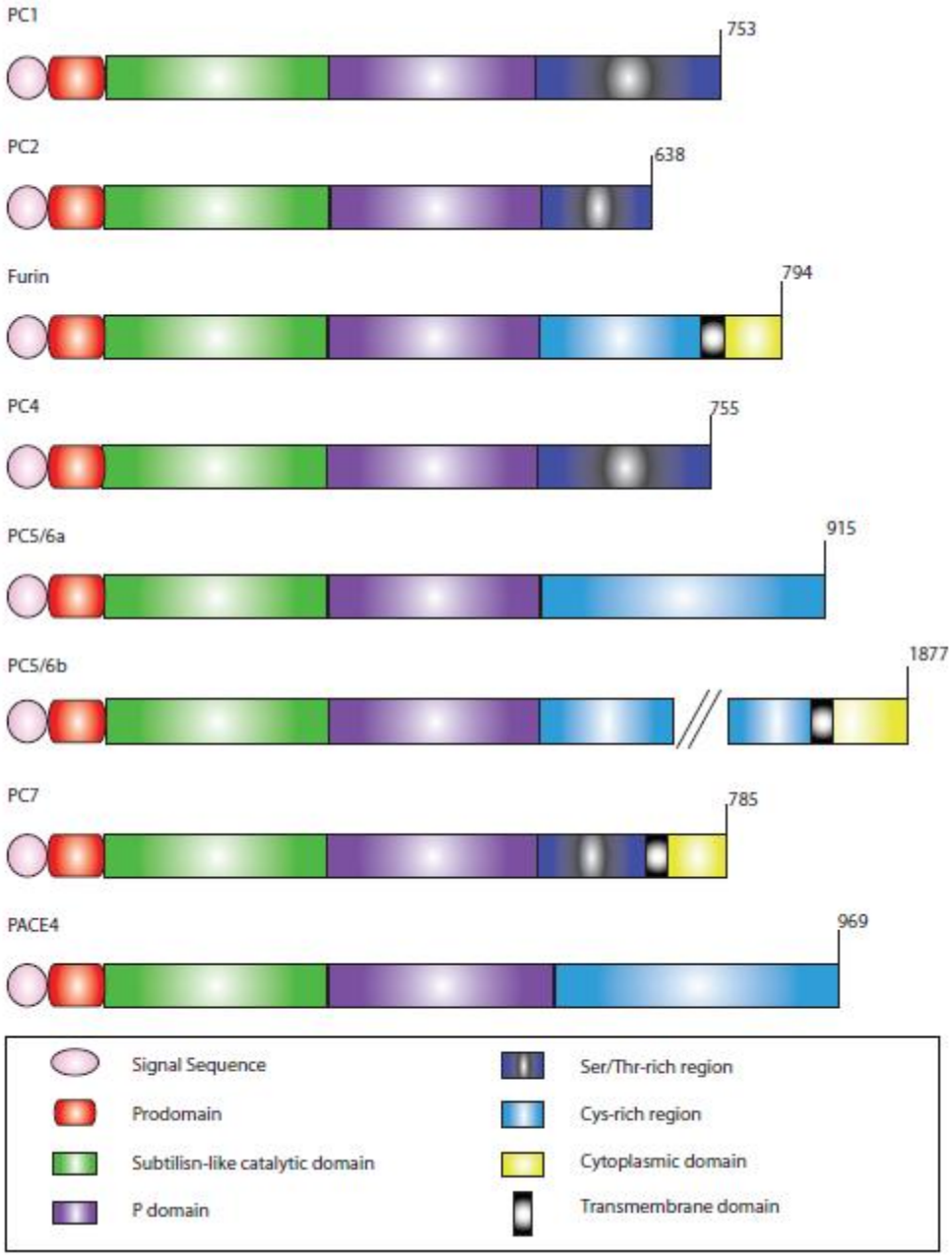
To date nine mammalian PCs have been characterized, and all belong to a conserved family of subtilisin-like proteinases, due to the structural homology between their catalytic domains and that of bacterial subtilisins. These proprotein convertases include seven enzymes that cleave protein precursors containing the multi-basic (K/R)-$(X)_n$-(K/R)↓ motif, where ↓ represents the cleavage site, X represents any amino acid besides cysteine, and n= 0,2,4 or 6. These PCs include furin, PC1/3, PC2, PC4, PACE4, PC5/6 and

PC7. The two remaining PCs include Subtilisin/kexin-like isozyme-1 (SKI-1) which cleaves at (R/K)-X-(L,I,V)-Z↓ motifs, where Z represents any amino acid except cysteine, glutamic acid, valine, or proline. The newest member of the proprotein convertase family is proprotein convertase subtilisin/ kexin type 9 (PCSK9). To date no known substrates exist for PCSK9, however it does undergo autocatalytic cleavage of its prodomain at the VFAQ↓SIP motif[5-11].

ii. Proprotein Convertase Structure and Function

Each currently known PC contains a signal peptide at its N-terminus, a pro-segment (also known as a pro-domain), a catalytic domain, followed by a C-terminal domain which is unique to each convertase (Figure 1). Most PCs are soluble proteins, however furin, PC5-B, PC7 and SKI-1 each contain a type-I single pass transmembrane domain near their C-terminus. The signal peptide functions as a signal to target the newly synthesized protein to the secretory pathway, beginning at the endoplasmic reticulum (ER). The pro-domain has multiple functions. This domain serves both as an intramolecular chaperone, aiding in the correct folding of the polypeptide during its translation, as well as auto-inhibition of the catalytic domain. The pro-domain of mammalian PCs have high sequence similarity (30-67%)[12]. Importantly, the pro-domain is always N-terminal to the catalytic domain, ensuring the protease is synthesized as an inactive zymogen immediately upon synthesis. By inhibiting the catalytic domain of the PC, the pro-domain allows for proper temporal and

Figure 1. Structure of human proprotein convertases. Schematic representation of the protein structure of human proprotein convertases. Each PC contains a N-terminal signal sequence, a self-inhibitory prodomain, a subtilisin-like catalytic domain, as well as a P domain responsible for calcium binding and pH dependence. The PCs contain either serine and threonine rich regions or cysteine rich regions, and sometimes contain transmembrane domains followed by C-terminal cytoplasmic domains.


PC1
753
PC2
638
Furin
794
PC4
755
PC5/6a
915
PC5/6b
1877
PC7
785
PACE4
969
Signal Sequence
Prodomain
Subtilisn-like catalytic domain
P domain
Ser/Thr-rich region
Cys-rich region
Cytoplasmic domain
Transmembrane domain

spatial activation of the catalytic activity. This in turn regulates the convertase which ensures that proteolysis occurs only in the presence of its intended target substrates. Following autocatalytic cleavage of the pro-domain, the pro-domain remains tightly bound through non-covalent interactions with the catalytic domain and functions as a competitive inhibitor. As the PC moves throughout the secretory pathway the complex remains tightly associated until it reaches its final destination where the organelle environmental milieu becomes optimal for a secondary cleavage to occur and/or sufficiently low pH and increasingly high calcium concentrations favour the dissociation [13-20].

The catalytic domains of PCs are related to bacterial subtilisins as well as enzymes found in the trypsin superfamily [22]. With the conserved active site catalytic triad of Asp, His and Ser, PCs are able to catalyze the acyl transfer reactions [22]. Additionally, the oxyanion hole stabilizing Asn residue (PC2 uses Asp oxyanion hole) is also conserved among the PCs, thereby acting as a hydrogen bond donor, which acts as a hydrogen bond donor during the transition state which undergoes an accumulation of negative charge on the scissile carbonyl group. Following the conserved catalytic domain, PCSK1-8 contain a β-barrel P-domain that functions in stabilizing the catalytic domain and pocket of the convertase. Finally the C-terminal domains of PCs are unique from one another, and play a role in regulating their trafficking and cellular localization [23]. The structure of these PC's can be seen in Figure1.

II. PCSK9, a Novel Proprotein Convertase that Regulates the Low-Density Lipoprotein Receptor

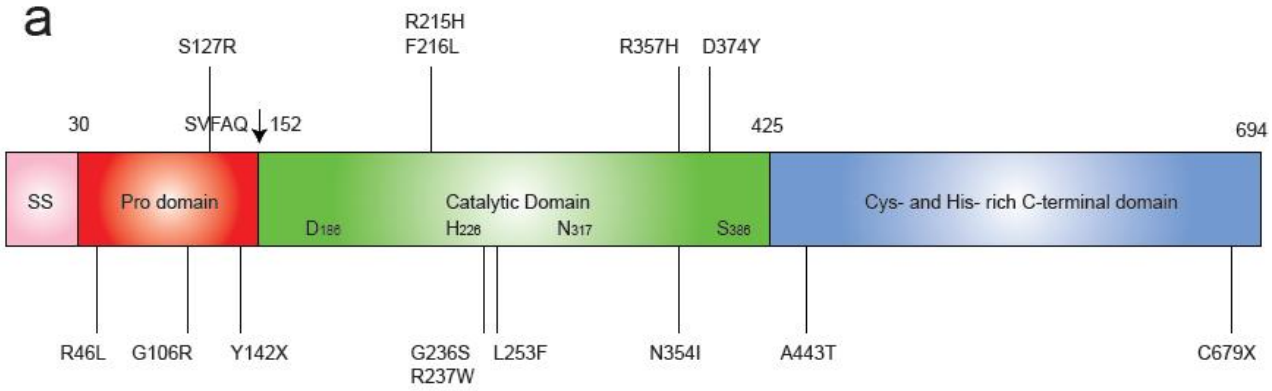
i. PCSK9 Discovery

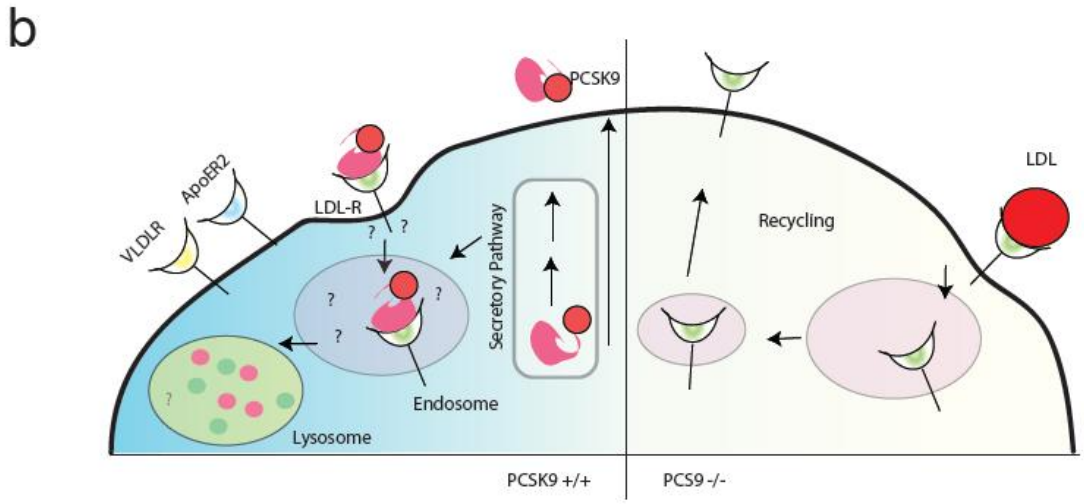
Proprotein convertase subtilisin kexin type 9 (PCSK9) was initially discovered and named neural apoptosis-regulated convertase 1 (NARC-1) for the discovery of its expression in the cerebellum and brain telencephalon cells [24]. It was found to promote cortical neurogenesis most likely via promoting the recruitment of undifferentiated neural progenitor cells towards a neuronal lineage, when NARC-1 was overexpressed in mice [24]. Soon after, human mutations in PCSK9 lead to the implication of PCSK9 as the third genetic locus that can cause autosomal dominant hypercholesterolemia (ADH) [25]. It was shown that wild type and mutant forms of PCSK9 responsible for ADH, when overexpressed in mice and in primary hepatocytes leads to a decrease in low density lipoprotein receptor (LDLR) levels post-transcriptionally, both in the presence and absence of LDLR adaptor protein ARH [26]. This study provided a link between the identification of PCSK9 as a protein involved in hypercholesterolemia in humans and its function. The link between PCSK9 and hypercholesterolemia was found as PCSK9 leads to decreased LDLR levels post-transcriptionally, less LDL-cholesterol (LDL-C) can be internalized from circulation into the liver, thus leading to hypercholesterolemia. Conversely, in mice lacking PCSK9 a decrease in plasma cholesterol was observed [27], leading to the finding that serum LDL-cholesterol levels correlate directly with PCSK9 in serum [28].

ii. PCSK9 Structure, Activation and Post-translational modification

PCSK9 is produced as a 694-amino acid (72kDa) zymogen in the endoplasmic reticulum(ER). It is comprised of an ER signal sequence (Figure 2), and pro-domain, which like other PCs, acts as an intramolecular inhibitor of PCSK9. Following the pro-domain is the catalytic domain which contains a classical catalytic triad (D186, H226, S386), an oxyanion hole (N317) and a histidine rich C-terminal domain. Pro-PCSK9 undergoes autocatalytic cleavage to produce a 63kDa mature PCSK9 at the ^{152}FAQ↓SIP^{155} site, removing the signal sequence and pro-domain. This type of cleavage is unique for PC's, in that most cleave either after basic amino acids or after hydrophobic amino acids, as is the case for site 1 protease (S1P). In 2007, the crystal structure of PCSK9 was solved and showed a high structural similarity to proteinase K for the catalytic domain and catalytic triad [29]. It was also shown that a β sheet from the pro-domain interacts quite strongly to the catalytic domain, via hydrophobic and electrostatic interactions, thereby shielding the catalytic domain and inhibiting it [30]. Despite no known proteolytic substrates of PCSK9 (aside from itself), and that proteolytic activity is not required for LDLR internalization and degradation, proteolytic activity is important as the proper intramolecular cleavage of the pro-domain is necessary for proper trafficking and secretion of PCSK9 outside of the cell [31,32]. In addition to PCSK9s autocatalytic cleavage, PCSK9 is also regulated by other proteolytic cleavages by other separate proprotein convertases. PCSK9 is cleaved by convertases furin and/or PCSK5/6A at the conserved R-X-X-R arginine cleavage site ^{215}RFHR^{218}↓, leading to its inability to degrade the LDLR [33].

Figure 2. Structure, mutations and trafficking of PCSK9 as it relates to LDLR processing and degradation. a) Schematic showing PCSK9 structure including domains, various gain of function (above) and loss of function (below) mutations found in humans responsible for hyper- and hypocholesterolemia, as well as specific amino acids relating to the catalytic triad and oxyanion hole. b) Diagram showing the general trafficking of the LDLR in the absence (right) and presence (left) of PCSK9. In the absence of PCSK9 LDLR is internalized into endosome vesicles and eventually recycled back to the cell surface, whereas in the presence of PCSK9 it co-internalizes with PCSK9 into endosomes then trafficked to the lysosome for degradation.


a
S127R
R215H
F216L
R357H
D374Y
30
SVFAQ
152
425
694
SS
Pro domain
Catalytic Domain
Cys- and His- rich C-terminal domain
D186
H226
N317
S386
R46L
G106R
Y142X
G236S
R237W
L253F
N354I
A443T
C679X


b
PCSK9
LDL
ApoER2
VLDLR
LDL-R
Recycling
Secretory Pathway
Endosome
Lysosome
PCSK9 +/+
PCS9 -/-

Besides proteolytic cleavages, PCSK9 also undergoes various other forms of post-translational modification. These modifications include N-linked glycosylation in the cysteine and histidine rich C-terminal domain, which occurs throughout the endoplasmic reticulum, sulfation of the prodomain at Tyr38, and phosphorylation of both the prodomain at Ser47 and in the cysteine and histidine rich C-terminal domain at Ser688 [24,34]. Glycosylation of PCSK9 can be expected as it is quite common for secreted proteins and those found in the secretory pathway [34,35]. To date, sulfation of PCSK9 has no known functional significance and appears to have no effect on PCSK9 mediated LDLR degradation. While PCSK9 phosphorylation is not quite understood at the moment, it appears to be dependent on cell type, and thought to play a role in shielding the prodomain from proteolytic cleavage.

iii. PCSK9 Expression and Localization

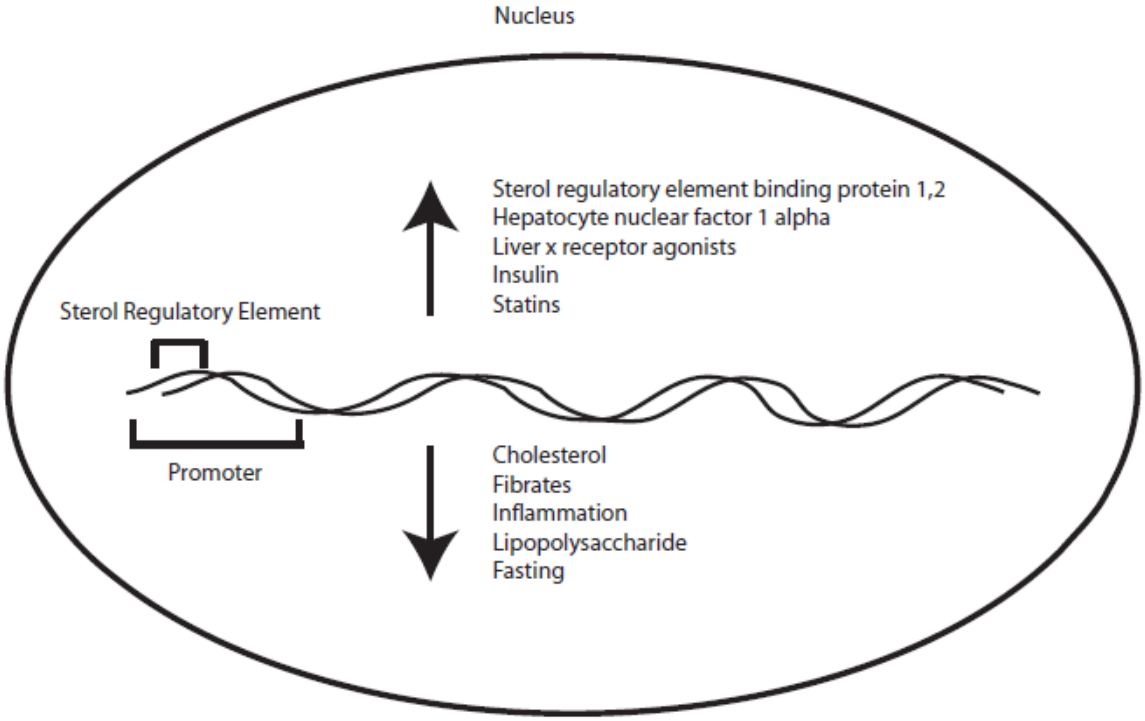
PCSK9 was initially found to be expressed in neuroepithelioma, hepatic and colon carcinoma cell lines, as well as in hepatocytes, kidney, pancreatic, spleen, testes, thymus and intestinal tissues [24,46]. As a proprotein convertase, PCSK9 is synthesized in the endoplasmic reticulum, and following proper folding, maturation and processing, moves throughout the secretory pathway towards the cis- and trans- Golgi apparatus en route to becoming secreted into the extracellular milieu and circulating plasma. Throughout the circulating plasma, PCSK9 can be internalized and act intracellularly in hepatic, lung, kidney and adipose cells [31, 32]. Interestingly, it has been shown that PCSK9 trafficking and

localization is affected by the presence of LDLR [36]. In the absence of LDLR, PCSK9 remains in the endoplasmic reticulum, however in the presence of LDLR, PCSK9 is found in post-ER compartments throughout the secretory pathway (including Golgi and endosomal compartments), as well can co-localize with LDLR at the cell surface.

PCSK9 gene expression can be highly modulated depending on the presence of many proteins and nutritional factors (Figure 3). PCSK9 expression is regulated by sterol regulatory element-binding proteins 1 and 2 (SREBPs), as the promoter for PCSK9 carries a sterol regulatory element [37], as well by the co-factor hepatocyte nuclear factor 1 α [38]. Because of this, liver x receptor agonists (which activate the liver x receptor and thus SREBP1c) can increase PCSK9 gene expression, as does insulin, while cholesterol, fibrates, inflammation and lipopolysaccharide [39] and fasting cause a decrease in PCSK9 expression [40,41].

Interestingly, despite not having been found to be a nuclear protein, studies have been carried out to see the effects PCSK9 plays on global gene expression. To this end, it appears that PCSK9 gene expression in turn affects the gene transcript levels of not only genes belonging to cholesterol and steroid biosynthesis, but also affects genes related to immune and viral response, sterol metabolism, the ubiquitin proteasome pathway, cell cycle regulation, inflammation and stress response, suggesting that PCSK9 plays, *in vivo*, other functions that have yet to be discovered [42,43].

Figure 3. PCSK9 gene expression. PCSK9 gene expression is known to be affected by multiple stimulus, dietary factors and proteins. Gene expression is controlled by a sterol regulatory element in the promoter of PCSK9. This leads to an increase in gene expression in the presence of sterol regulatory element binding protein 1 and 2, HNF-1α, liver x receptor agonists, insulin and statins, while factors that decrease gene expression include cholesterol, fibrates, inflammation, LPS and fasting.


Nucleus
Sterol regulatory element binding protein 1,2
Hepatocyte nuclear factor 1 alpha
Liver x receptor agonists
Insulin
Statins
Sterol Regulatory Element
Promoter
Cholesterol
Fibrates
Inflammation
Lipopolysaccharide
Fasting

iv. PCSK9 and LDLR Degradation

Upon discovery that PCSK9 was implicated in hypercholesterolemia, and that PCSK9 overexpression causes a decrease in LDLR levels and an increase in circulating plasma cholesterol, the relationship between PCSK9 and LDLR degradation has been studied extensively. PCSK9 overexpression causes a decrease in hepatic LDLR protein levels while mRNA levels remain unchanged, suggesting an effect at the protein level, while circulating PCSK9 can decrease adipose, lung and kidney tissue LDLR, in addition to hepatic LDLR [44]. Oppositely, inhibition of PCSK9 expression leads to a decrease in circulating LDL levels [45,46]. Additionally, it was found that PCSK9 mediated LDLR degradation is unaffected by proteasome inhibition, but must occur in some post-endoplasmic reticulum compartment, as treatment with brefeldin A inhibited the degradation of LDLR. PCSK9 was then shown to work extracellularly. This was demonstrated by adding purified PCSK9 to hepatic cell cultures which reduced cell surface LDLR levels, implying that LDLR mediated endocytosis and interaction is necessary for PCSK9 internalization [48,49]. However PCSK9 mediated degradation of the LDLR does involve an internal pathway, the importance of both pathways remains unclear [50]. The internalization and degradation of LDLR by PCSK9 requires the transfer from a late endosomal acidic compartment to the lysosome [51,52]. Interestingly, *in vitro* binding assays show about an 150 fold increase in affinity of PCSK9 to LDLR at an acidic pH of 5.3 as compared to a neutral pH of 7.4, strengthening the results that implicate PCSK9 mediated degradation of the LDLR occur in late endosome/lysosome compartments [53]. Following this study, it was shown that the interaction was mapped to occur at one of LDLRs many extracellular epidermal growth factor (EGF) like-repeat

domains, specifically the EGF-A domain, which required calcium as a co-factor for binding. The binding of PCSK9 to the EGF-A domain of the LDLR was also stronger (150x) at a pH of 5.2 as compared to a neutral pH of 7 [54]. This interaction occurs at the surface of the catalytic domain of PCSK9, specifically between Asp-374 of PCSK9 and the His-306 of the LDLR EGF-A repeat domain [55]. Interestingly, this site is known for as the strongest gain-of-function mutation of PCSK9. In humans the D374Y mutation leads to a stronger association with LDLR (25x) and accelerated degradation of the LDLR [56]. Most recently it was postulated that the C-terminal cytoplasmic domain of LDLR, which interacts with endocytic machinery, does not play a role in PCSK9 mediated internalization and degradation of the LDLR [53b]. This was approached through studies that showed that replacement of the cytoplasmic domain of the LDLR with that of the transferrin receptor still lead to PCSK9 mediated degradation of the chimeric LDLR in CHO cell lines [53b].

v. PCSK9 Mutations in Humans

Many variants of PCSK9 naturally occur throughout various human populations, leading to a wide array of gain-of-function or loss-of-function PCSK9 variants, in terms of ability to degrade LDLR(Figure 2). The study of familial hypercholesterolemia pedigrees have led to the discovery of gain-of-function PCSK9 mutants caused from single point mutations, such as D374Y [57], S127R, F216L [25], and N157K, among others [58]. These mutants mechanism of action are not all fully understood, however they do have in common the drastic effect on promoting LDLR degradation and thus increase in circulating serum LDL and cholesterol levels.

PCSK9 loss-of-function mutants naturally occurring in humans are also observed, and range from missense mutations that reduce the autocatalytic cleavage of the prodomain, such as G106R [59]and N354I [60], to mutations that lead PCSK9 unable to exit the endoplasmic reticulum, such as G236S, to nonsense mutations that lead to the production of truncated forms of PCSK9 that are not processed or trafficked and secreted properly, such as Y142X and C679X [61].

vi. PCSK9 Outside of LDLR Degradation

PCSK9 is able to bind the EGF-A domains of LDLR and other EGF-A domain containing proteins. These proteins include cell surface receptors ApoER2 and very low density lipoprotein receptor (VLDLR) [62], which are also subject to PCSK9 mediated degradation, possibly through a similar mechanism, as the natural gain-of-function D374Y mutant also increases the rate of degradation of these proteins [63].

PCSK9 was discovered to play a novel role in the endoplasmic reticulum when it was shown to promote the degradation of non-acetylated β-site amyloid precursor protein (APP)-cleaving enzyme 1 (BACE1) intermediates [64]. The acetylation of BACE1 in the endoplasmic reticulum was shown to be compulsory for its trafficking from the ER into the Golgi, however those intermediates that are not acetylated are retained in the ER and signaled for degradation in a post-ER compartment by PCSK9, separate from the proteasome. It was also shown that secreted PCSK9, when added to cell culture media, becomes internalized and promotes the degradation of BACE1, just as it does for LDLR [64].

This was the first finding that PCSK9 has functional roles outside of LDLR degradation, as well as in the endoplasmic reticulum.

PCSK9 has been shown to degrade the cell surface receptor CD81, a membrane protein that plays a role in Hepatitis C viral (HCV) entry [65]. In HuH7 cells HCV infection was reduced when PCSK9 was able to degrade CD81 protein levels, thereby providing a potential role for PCSK9 in protecting humans from HCV infection. Despite these identified functions of PCSK9, not much else is known regarding the mechanisms in which PCSK9 affects these proteins, as well as the other functional roles PCSK9 plays.

vii. PCSK9 Interaction Partners

The first known interaction partners of PCSK9 were the EGF-A domain containing cell surface receptors, including LDLR, VLDLR and ApoER2. In 2008 a non-cell surface receptor interaction partner was discovered in the 33kDa protein, annexin A2. Annexin A2 is a soluble protein found to interact with PCSK9 in its C-terminal cysteine and histidine rich domain. This interaction occurs near the cell surface of HepG2, CHO and HuH7 cell lines, and when alone or in complex with p11, could lead to the inhibition of PCSK9 mediated degradation of the LDLR [66].

viii. PCSK9 as a Therapeutic Target

As the third identified gene responsible for autosomal dominant hypercholesterolemia (the first two being LDLR and ApoB), its physiological role in promoting the degradation of the LDLR and increase in circulating plasma cholesterol levels make inhibiting PCSK9 an attractive target to treat hypercholesterolemia. To this end, using antibodies raised against specific regions of PCSK9 responsible for interacting with LDLR is considered a valuable means for disrupting this interaction between these two proteins and potentially inhibiting the degradation of the LDLR [67]. Using this strategy, a monoclonal antibody (mAb1) of PCSK9 has been generated that binds nearby the site that mediates PCSK9-LDLR interaction. Co-incubation of mAb1 with PCSK9 inhibited this interaction, leading to higher LDLR protein levels and LDL uptake, decreasing serum LDL-cholesterol levels by up to 80% in cynomologus monkeys [68].

III. Low-Density Lipoprotein Receptor

i. LDLR Structure and Function

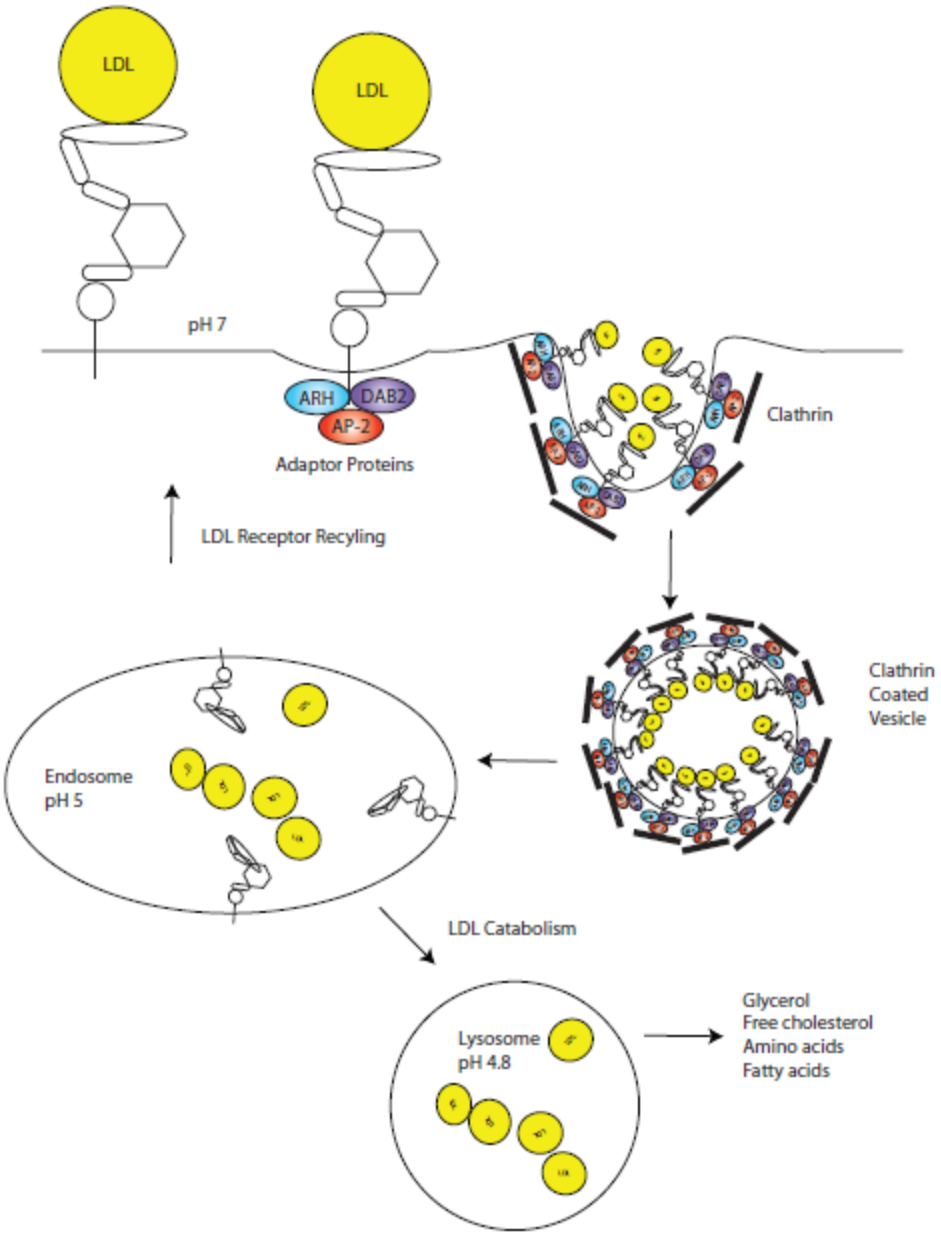
The LDLR carries an important function *in vivo* as it is the cell surface receptor responsible for internalizing and removing circulating LDL and LDL-C from the blood serum. LDLR has a very complex structure with many different subunits. The majority of the receptor is extracellular, where the ligand-binding domain is found, it is here that circulating LDL particles are able to interact and bind cell surface LDLR. This domain is comprised of seven LDLR class A folds (LA1-7), followed by the EGF precursor homology

domain which contains EGF repeats as well as a β-propeller domain. Immediately preceding the transmembrane domain is a domain responsible for receiving O-linked sugar modifications [69]. The intracellular domain of the LDLR is quite short in relation to its size, and contains the cytoplasmic tail responsible for internalization of the LDLR via protein interactions that occur through the NPXY motif, a common receptor motif responsible for internalization [70].

ii. LDLR Internalization

Upon binding of LDL to the LDLR, the protein complex is internalized into the cell via endocytosis (Figure 4). This internalization process is carried out by clathrin coated pits, and involves adaptor proteins such as autosomal recessive hypercholesterolemia (ARH). These adaptor proteins form a complex between the cytoplasmic tail of the LDLR, to clathrin machinery as well as other endosomal machinery [71,72]. Following internalization, the LDL-LDLR complex is trafficked to acidic endosomal compartments. It is under these conditions that the LDLR undergoes a conformational change in which the β-propeller moves towards the ligand binding domain, which in turn causes the release of the LDL for future metabolism, while the LDLR is recycled back to the cell surface to repeat this process to allow for more LDL and LDL-C to be internalized [73,74].

Figure 4. Life cycle of LDLR. Following the binding of cell surface LDLR and circulating LDL particles at a neutral pH, cytoplasmic adaptor proteins autosomal recessive hypercholesterolemia (ARH), disabled 2 (Dab2) and adaptor protein complex 2 (AP-2) participate in binding the cytoplasmic tail of LDLR and recruit endocytic machinery proteins as well as clathrin. Invagination of the cell surface occurs leading to the internalization of LDLR into a clathrin coated vesicle (CCV). This CCV fuses with an endosome (pH 5) where the LDLR undergoes a conformation change, releasing LDL, where LDLR is then trafficked back through a recycling pathway back to the cell surface while LDL is transported to the lysosome (pH 4.8) for catabolism.


LDL
LDL
pH 7
ARH
DAB2
AP-2
Adaptor Proteins
Clathrin
LDL Receptor Recyling
Clathrin
Coated
Vesicle
Endosome
pH 5
LDL Catabolism
Lysosome
pH 4.8
Glycerol
Free cholesterol
Amino acids
Fatty acids

1.2 Hypothesis Statement

PCSK9 studies to date have helped to elucidate various factors that affect LDLR degradation, expression, post-translational regulation and the effects of mutations on human physiology. Little is known regarding the cellular functions of PCSK9 outside of its role in LDLR degradation, nor are the mechanisms or protein complexes that participate in PCSK9 mediated LDLR degradation. In order to increase our understanding of the effects of PCSK9 in the cell and PCSK9 cellular functions, both known and currently uncharacterized, I utilized quantitative subcellular fractionation, LC-MS/MS analysis and protein interaction studies to test the hypothesis that *PCSK9 has multiple effects on the liver cellular proteome*, and that these effects signify novel cellular functions and provide insight into currently known functions of PCSK9. This approach utilizes quantitative proteomics and immunoaffinity purification to study PCSK9 in a global view of its effects on the cellular proteome, as well as its interaction partners. By observing the novel effects PCSK9 plays on liver cell proteins in conjunction with purifying and identifying PCSK9 interaction partners, information can be attained regarding PCSK9 cellular function that was otherwise unknown. From this work future studies can be carried out to further explore these potential novel functions of PCSK9.

Chapter 2. MATERIALS AND METHODS

Reagents

See Appendix A for list of reagents, buffers, and solutions used and their recipes.

Cell culture and SILAC metabolic labelling

HuH7 liver cells stably expressing PCSK9-ACE2-V5 and empty vector control cells were generated as previously described [63] and were kindly donated by Dr. Nabil Seidah. HuH7 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% anti-mycotic antibiotic reagent at 37°C in a 5% CO_2 humidified incubator. For SILAC labelling, HuH7 cells stably expressing PCSK9-ACE2-V5 were grown in SILAC 'light' media containing only ^{12}C lysine amino acids, while empty vector control cells were grown in SILAC 'heavy' media containing only ^{13}C lysine amino acids [75]. Cells were grown in SILAC media for 10 doubling times. SILAC media kit was obtained from Invitrogen.

Subcellular Fractionation and histodenz gradient preparation

The subcellular fractionation was performed essentially as described [76]. At the end of the labelling period, cells were washed twice with ice-cold phosphate buffered saline (PBS) and then removed with a plastic scraper. The collected cells were centrifuged at 2,000 × *g* for 5 min at 15 °C. The pellet was re-suspended in membrane solubilization buffer and homogenized using a ball bearing homogenization apparatus. The cell

homogenate was then transferred to 15-ml glass Corex tubes and centrifuged at 10000 × g for 10 min at 5 °C, to remove unbroken cells, debris, nuclei and mitochondria. The post-nuclear supernatant (PNS) was set aside on ice for further centrifugation. Histodenz stock solution and saline buffer were used to prepare four histodenz solutions of increasing concentrations (10, 14.66, 19.33, and 24%) and 2.5 ml of each solution were loaded from the bottom of the tube (Beckman Polyallomer Centrifuge Tubes) in decreasing percentage order. The tubes were then sealed with a piece of parafilm, and a linear gradient was formed by placing the tubes horizontally for 45 min at room temperature. Gradient maker was obtained from Sigma. The tubes were then centrifuged at 37,000 × *g* for 4 h at 15 °C (Beckman L8–70 M Ultracentrifuge). The supernatant was layered on top of the created histodenz gradient and centrifuged at 37,000 × *g*, for 1.5 h at 15 °C (SW41 rotor). Following ultracentrifugation, each tube was fractionated into 15 aliquots (0.8 ml each) and used for Western blotting and liquid chromatography-mass spectrometry (LC-MS/MS) analyses.

Western blot analysis

Protein samples from aliquots and fractions were boiled for 5 min using 5x sample buffer, then separated on NuPage 4-12% Bis-Tris precast gels (Invitrogen) and then transferred onto a nitrocellulose membrane (Bio-Rad). The membrane was blocked over night at 4°C and washed four times for 5 min in PBS-Tween. Antibodies, unless otherwise stated, were added at 1:1000 (v/v) dilution in PBS-Tw + 5% skim milk for 1 hour at room

temperature. Membranes were washed four times at 5 min in PBS-Tw. Secondary antibodies were added at 1:5000 dilution and the membrane was incubated for 1 h at room temperature. Membranes were washed 4 times for 5 min in PBS-Tw. The membrane was then incubated in SuperSignal chemiluminescent substrate (Pierce) for 1 min and exposed to film.

HPLC-ESI-MS/MS

Following histodenz subcellular fractionation, each histodenz fraction was separated using custom made 15cm 4-12% Tris-glycine precast denaturing gels (Jule Inc., Milford, CT) and silver stained. Gel lanes were cut and separated into 15 separate 1cm bands. Bands were excised from each gel lane, and digested with trypsin as previously described [77]. From these experiments about 330 samples were analyzed by HPLC-ESI-MS/MS, equating to almost 500 hours of mass spectrometry time. The HPLC-ESI-MS/MS consisted of an automated Agilent 1100 micro-HPLC system (Agilent Technologies, Santa Clara, CA) coupled with an ESI LTQ mass spectrometer (Thermo Scientifics). Briefly, each peptide mixture was reconstituted in 20 µl of 5% formic acid and loaded on a 200 μm × 50 mm fritted fused silica pre-column packed in-house with 5 cm of reverse phase Magic C18AQ resins (5 µm; 200-Å pore size; Michrom Bioresources; Auburn, CA). The separation of peptides was performed on an analytical column (75 µm i.d. × 50 mm) packed with the same beads using a 90min gradient of 5-80% acetonitrile (v/v) containing 0.1% formic acid at an eluent flow rate of 200 nl/min after in-line flow splitting. The HPLC was interfaced to an ESI LTQ linear ion trap mass spectrometer (Thermo Electron, Waltham, MA) operated in positive ion mode. A

voltage of 1.8 kV was applied to generate the electrospray ionization. Data dependant analysis were performed in which a full scan MS was first performed to detect potential peptides which was then followed by 5 data-dependant MS/MS.

Database searching, quantitation and data analysis

Peak lists were generated from the raw file using Mascot Distiller 2.0.0.0 (Matrix Science, London, UK) to export *mgf files. MS/MS data were searched against the human NCBInr database Mascot 2.2.02 (Matrix Science Ltd., London, UK). The searches were performed using the following criteria, only tryptic peptides with up to two missed cleavage sites were allowed; the mass tolerance was set to 2 Da for MS and 0.8 Da for MS/MS fragment ions; carbamidomethyl was set as a static modification; oxidation on methionine and $^{13}C_6$-Lys from SILAC were specified as dynamic modifications. The false discovery rate of protein identification was limited by accepting only the results with a mascot score > 30 ($P < 0.05$). Following peptide identification using Mascot, peptide and protein quantitation was carried out using Insilicos Proteomic Pipeline (IPP v1.0 rev.91, Build 200712031206, Institute for Systems Biology, Seattle, WA). Here, dat files from Mascot search results were converted to pepXML files for downstream quantitation. Peptide prophet was used for peptide validation, accepting peptide results with $P < 0.05$. XPRESS was used for quantitation, setting SILAC 'heavy' peptides to a value of 1, varying SILAC 'light' peptides relative quantitation levels. Once quantitative data from all LC-MS runs were collected and compiled, all peptides that showed a greater than 2 fold change in relative abundance were

subjected to manual validation. Here, the MS scans for these drastically changing peptides were manually validated based on peak intensities for co-eluting light and heavy peptide pairs. Labelled peptide pairs peak intensities not matching the relative quantitation reported by IPP were removed from the dataset. The resulting quantitative peptide list was expressed according to the $\log2_{SILAC\ L,H}$ in order to produce a log2 distribution curve.

Validation Experiments

The myc-tagged EHD4 construct [78], the V5-tagged human LDLR construct [63], and the V5-tagged PCSK9 wild-type construct [24] used for expression experiments were previously described. The C-terminal Flag-tagged PCSK9 wild-type construct was made by transferring the PCSK9 gene from pIRES into the pCMV-Flag-MAT-2 expression vector. HEK293 cells were stably transfected with either human LDLR-V5 alone or in combination with Flag-tagged PCSK9 wild-type using the calcium chloride method. Each stably transfected HEK293 cell line was then transiently transfected with myc-tagged EHD4 using polyethylenimine (PEI) transfection reagent, as previously described [79]. Briefly, each stable cell line was seeded into two 150 mm tissue culture plates and was transfected when cells reached 70% plate confluency. Plates were transfected with myc-EHD4 in the presence of serum-free medium. In addition, native HepG2 cells were seeded into four 150 mm tissue culture plates and were ready for transfection when cells reached 70% plate confluency. Two 150 mm plates of HepG2 cells were transfected with myc-EHD4 alone, while another two plates were transfected with myc-EHD4 in combination with wild-type

PCSK9-V5, in serum-free medium. Following an initial 3 h incubation for the various transient transfections, serum-free medium was replaced with medium containing 10% FBS and an anti-biotic/anti-mycotic stock. Cells were grown for an additional 36 h, at which time cells were washed twice with cold PBS, followed by scraping and resuspension in cold PBS. Cells were pelleted at 2000 x g for 2 min, PBS was removed and cells were either lysed directly or flash frozen using liquid nitrogen. Frozen cell pellets were stored at -80^{o}C. Upon confirmation of proper expression of transfected proteins via Western blot analysis, cell lysates were subjected to immunoaffinity purifications. For anti-Flag IP experiments, monoclonal anti-FLAG M2 beads were used (Sigma). For anti-V5 IP experiments anti-V5 agarose affinity gel was used (Sigma). For anti-Myc IP experiments anti-C-Myc agarose affinity gel was used (Sigma). For co-IP experiments, 1mg of cell lysate and 20µL of specific agarose affinity gel was used for each separate immunoaffinity purification. Antibody-beads and cell lysates were mixed on a rotator at 4°C for 2 hours. Flow-through fractions were removed and antibody-bead-antigen complexes were washed twice in modified RIPA lysis buffer. Proteins were eluted by boiling beads at 95°C for 5 minutes in 2x sample buffer. Eluted proteins were subjected to SDS-Page fractionation and Western blotting.

PCSK9 Immunoaffinity purifications and interaction experiments

PCSK9-ACE2-V5 immunoaffinity purifications using anti-V5 pull-downs were optimized using the aforementioned HuH7 cell lines. Briefly, using a constant amount of anti-V5 agarose affinity gel (10µL of 50:50 slurry), multiple IP's were tested against

increasing amounts of PCSK9-ACE2-V5 and EV expressing HuH7 cell lysates. Following mixing, antibody-beads and lysates mixtures were mixed for 2h at 4°C. Flow through was removed, beads were washed with modified RIPA, and eluted with 2x sample buffer for Western blot analysis using polyclonal anti-PCSK9 antibody. For large scale IP experiments HuH7 cells expressing empty vector control or PCSK9-ACE2-V5 were grown in 15cm plates. Following cell lysis, 15mg of protein for each sample was prepared and subjected to anti-V5 immunoaffinity purification using 150µL of anti-V5 antibody-beads for 4 hours at 4°C. Flow-through fractions were removed and antibody-bead-antigen complexes were washed eight times in modified RIPA lysis buffer. Proteins were eluted by boiling beads at 95°C for 5 minutes in 2x sample buffer. Eluted proteins were subjected to SDS-Page fractionation, silver staining, LC-MS/MS analysis and database searching, as previously described, in order to identify novel interaction partners of PCSK9-ACE2-V5. Two lists of interaction partners were generated; those proteins identified in the empty vector control cell line anti-V5 IP, and those proteins identified in the PCSK9-ACE2-V5 expressing cell line anti-V5 IP. Proteins identified in the empty vector control IP were removed from the list of potential interaction partners found in the PCSK9-ACE2-V5 IP experiment. Peptides with a mascot score of 30 or greater were accepted. This experiment was repeated for n=2.

Chapter 3. RESULTS

Subcellular Fractionation and Western blot Analysis of Histodenz Fractions

PCSK9 studies in recent years have centred around its localization, expression, mutation studies, and effects on LDLR processing. Despite the large number of studies being presented, very little is known regarding the key players and mechanisms controlling LDLR degradation. Additionally, some studies have revealed a possible functional role for PCSK9 in the ER, unrelated to its processing of LDLR for degradation [62,64,65]. It is for these reasons that we chose to investigate the effects PCSK9 plays on a human liver subcellular proteome. For the study, human HuH7 liver cells were used. Cells were kindly donated from Dr. Nabil Seidah. One set of cells stably expressed a variant of PCSK9, containing an ACE2 transmembrane domain from the angiotensin converting enzyme 2 protein, followed by a c-terminal V5 tag. This PCSK9-ACE2-V5 construct has been previously shown to promote LDLR degradation stronger than wild type PCSK9 can [63]. This is thought to occur as the ACE2 transmembrane domain localizes the protein towards endosomal/lysosomal membranes, the sites thought to promote the degradation of the LDLR [63]. These cells, as well as an empty vector expressing control cells were grown for ten doubling times in SILAC light and heavy media, respectively. Following metabolic labelling of proteins using SILAC, light and heavy labelled cells were combined in equal amounts, homogenized and subjected to a subcellular fractionation protocol (Figure 5). 15

subcellular fractions were collected and subjected to Western blot analysis using known markers for resident proteins of specific subcellular organelles ranging from ER to lysosomes (Figure 6). The more dense fractions (fraction 15) contained the heavier subcellular organelles such as the ER, and as the density of the fractions decreased, so did the size of the organelles contained within them. Found in these histodenz fractions was that the later fractions contained mostly ER, Golgi was found in mid-level fractions, and finally the least dense fractions contained smaller organelles such as early endosomes and lysosomes. Nuclear contamination was also tested in the fractions, and was shown to be present in the most dense subcellular fraction (fraction 15), but mostly absent from the remaining fractions.

SILAC Quantitation

Following the analysis of subcellular organelle histodenz fractions were subjected to further fractionation based on size via SDS-Page gel separation. These protein gels were then stained with silver nitrate, and all proteins were trypsinized and subjected to LC-MS/MS analysis for protein identification and quantitation. A total of 330 gel bands were digested and subjected to LC-MS/MS analysis, resulting in approximately 500 hours of mass spectrometry run time. Following peptide and protein identification, Insilico Proteomic Pipeline software was used to perform quantitation on each of the 330 mass spectrometry runs, from SILAC light and heavy paired peptides identified [109]. From two completed experiments (n=2) a total of 6187 peptides were quantitated (3169 n=1, 3018 n=2). All peptide hits found to undergo a greater than 2 fold change by IPP were manually validated

Figure 5. General workflow of SILAC metabolic labelling of HuH7 cell lines, subcellular fractionation, protein processing and LC-MS/MS analysis of resulting peptides. HuH7 cells stably expressing PCSK9-ACE2-V5 were grown in 'light' SILAC media, while empty vector expressing cells were grown in 'heavy' SILAC media. Cells were labelled in their respective media for 10 doubling times, combined in a 1:1 ratio and homogenized. PNS were separated in 15 fractions by histodenz gradient subcellular fractionation. Fractions were further fractionated based on size via SDS-Page and visualized by silver nitrate. Gel bands were excised, digested with trypsin and analyzed by LC-MS/MS followed by quantitative analysis using IPP software.

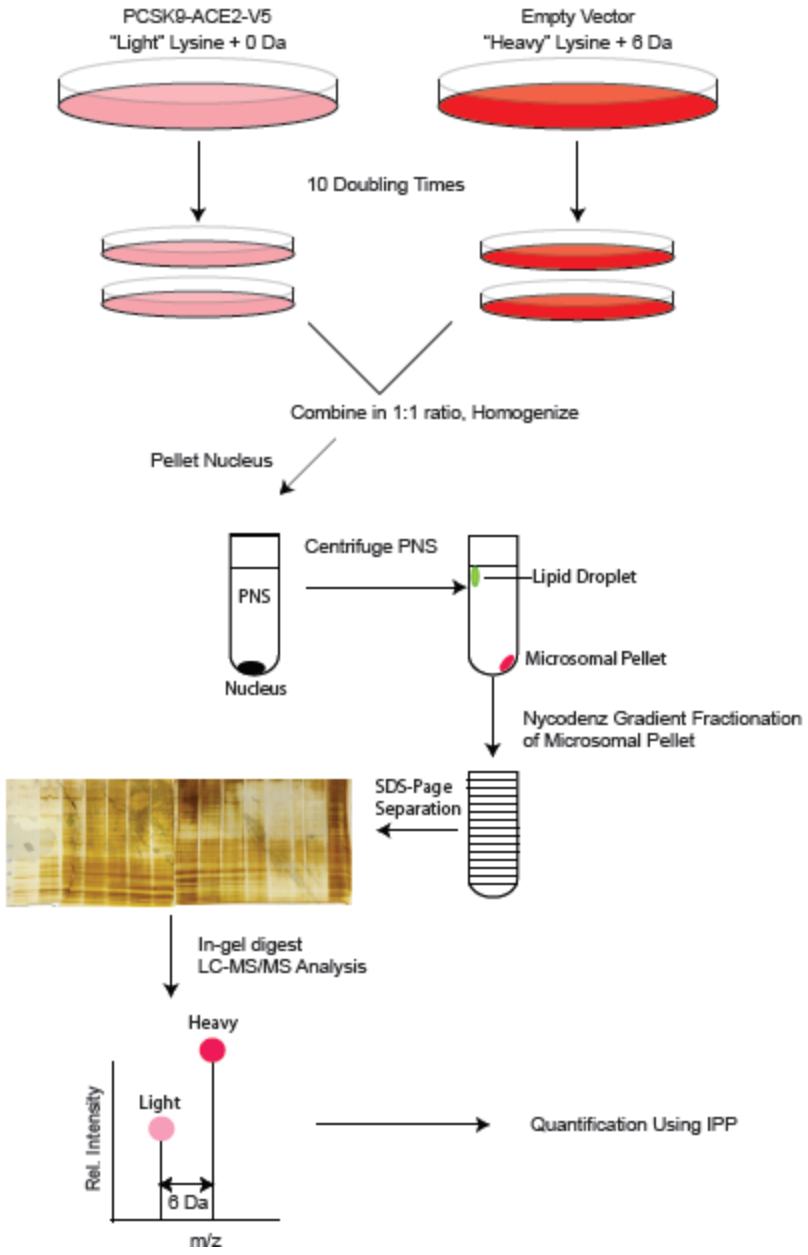
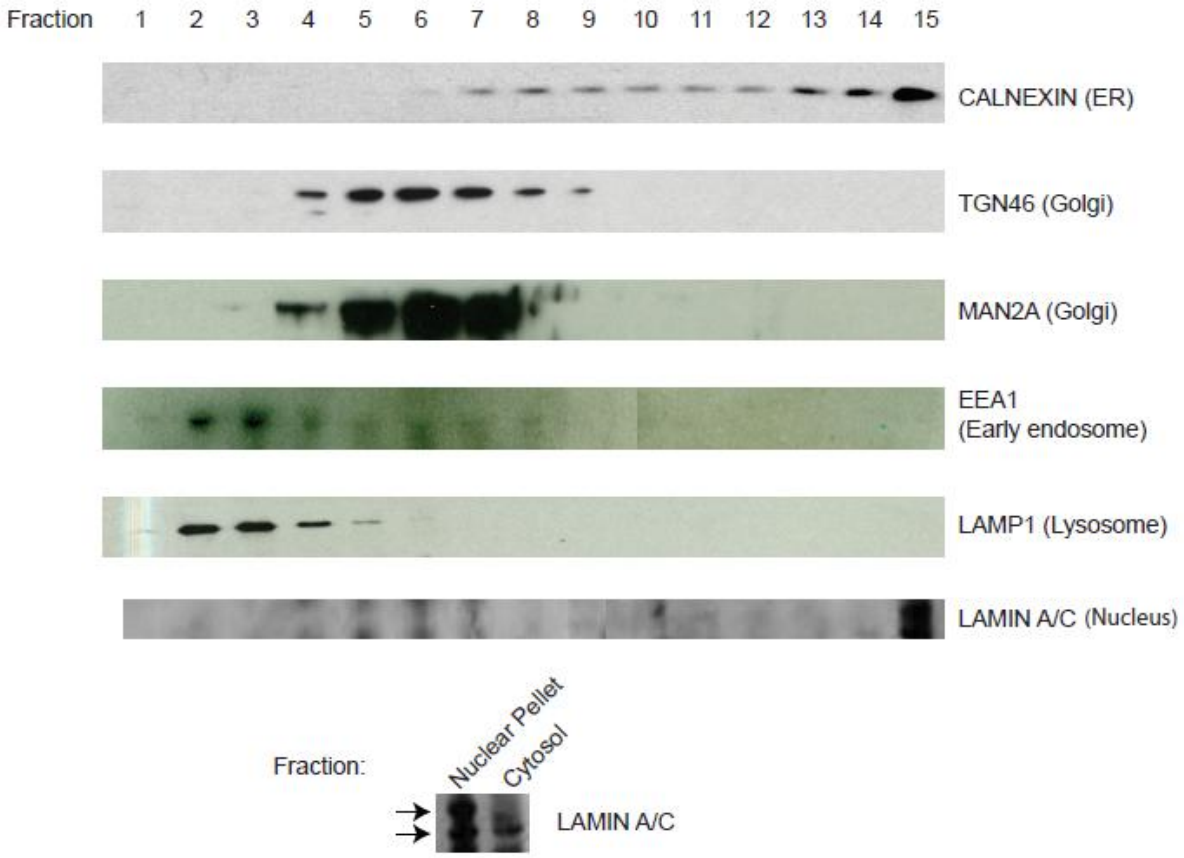

PCSK9-ACE2-V5
"Light" Lysine + 0 Da
Empty Vector
"Heavy" Lysine + 6 Da
10 Doubling Times
Combine in 1:1 ratio, Homogenize
Pellet Nucleus
PNS
Nucleus
Centrifuge PNS
Lipid Droplet
Microsomal Pellet
Nycodenz Gradient Fractionation
of Microsomal Pellet
SDS-Page
Separation
In-gel digest
LC-MS/MS Analysis
Heavy
Light
Rel. Intensity
6 Da
m/z
Quantification Using IPP

Figure 6. Western blot analysis of subcellular fractions for specific organelle markers. The 15 subcellular histodenz fractions were subjected to Western blot analysis with the following organelle markers, Calnexin (ER), TGN46 (Golgi apparatus), MAN2A (Golgi apparatus), Early endosomal antigen1 (Early endosome), Lamp1 (Lysosome), Lamin A/C (Nuclear envelope). Cytosolic and nuclear fraction were also subjected to Lamin A/C Western blot analysis for nuclear contamination.


Fraction 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
CALNEXIN (ER)
TGN46 (Golgi)
MAN2A (Golgi)
EEA1
(Early endosome)
LAMP1 (Lysosome)
LAMIN A/C (Nucleus)
Fraction:
Nuclear Pellet
Cytosol
LAMIN A/C

based on peak intensities for co-eluting light and heavy peptide pairs from the raw mass spectrum.

The SILAC light and heavy relative abundance ratios were converted to log2 values to simplify data, and all quantitated data were expressed in terms of abundance (Figure 7) in order to show the distribution of quantitated peptides. Quantitated data from both experiments followed similar distribution patterns, centred at log2 = 0, suggesting a normal distribution where the majority of proteins were unaffected by PCSK9-ACE2-V5 expression in HuH7 cells. However, a small portion of the quantitated peptides did show protein levels significantly altered as compared to the empty vector control HuH7 cells. All quantitated peptides with relative abundances of over 2 or under 0.5 were subjected to manual validation. A total of 293 proteins were found to be significantly affected by PCSK9 expression. Interesting proteins whose expression was determined to have been significantly affected by the expression of PCSK9-ACE2-V5 were listed in Table 1. Proteins that were determined to be significantly affected by the PCSK9 variant expression belonged to many different functional classes of proteins, including cytoskeletal organization, organelle transport and trafficking, lipid and cholesterol homeostasis, protease activity and other post-translational modifications, chaperone and protein folding, cell adhesion and

Figure 7. Log2 distribution of peptides quantified by IPP reveals a normal distribution of SILAC labelled peptides. Following quantitative analysis, light, heavy relative abundance ratios for peptides were converted to a log2 scale. The distribution of these proteins were plotted, with a distribution centred at log2 = 0. The first set of experiments (n=1) resulted in 3169 peptides quantitated, and the second set of experiments (n=2) resulted in 3018 peptides quantitated.

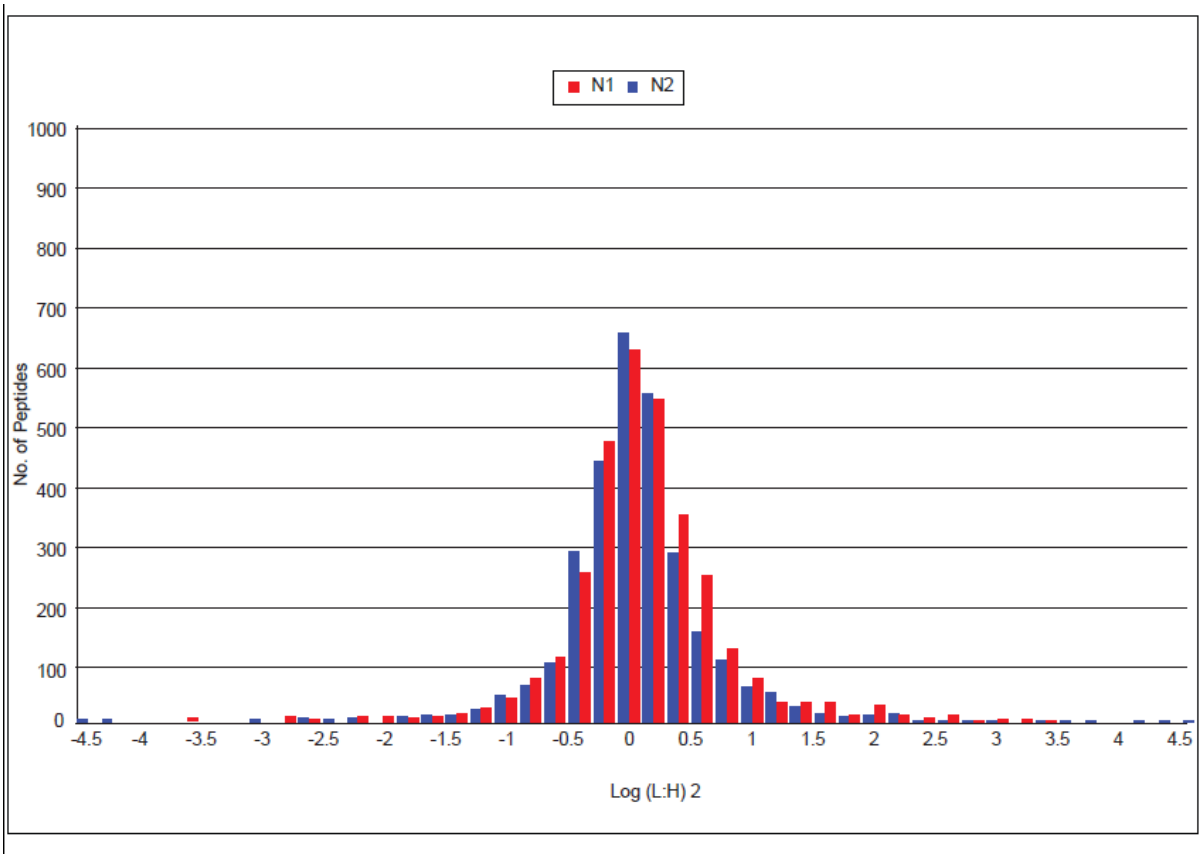

N1
N2
1000
900
800
700
600
500
400
300
200
100
0
No. of Peptides
-4.5
-4
-3.5
-3
-2.5
-2
-1.5
-1
-0.5
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
Log (L:H) 2

Table 1. Partial list of significantly affected non-nuclear proteins and their biological functions, following PCSK9-ACE2-V5 expression

Protein	gi accession	PCSK9:Ctrl	Peptide	Experiment	Fraction	Mascot Score
FUNCTIONAL CLASS, CYTOSKELETON ORGANIZATION/VESICLE AND ORGANELLE TRANSPORT						
Actin related protein 2\3 complex subunit 1b	gi|14043135	9.4	TWKPTLVILR	N1	F4	48
A-kinase anchor protein-12	gi|55959051	8.5	GSSSDEEGGPKAMGGDHQK	N1	F6	32
Rab19b	gi|51094782	8.0	IILIGDSNVGK	N2	F9	31
Actin related protein 2/3 complex subunit 1b	gi|14043135	5.5	TWKPTLVILR	N2	F5	53
phospholipase C, beta 3	gi|836665	5.2	LVAGQQQVLQQLAEEEPK	N2	F5	31
Myoneurin	gi|18448935	5.2	AGAMPQAQK	N1	F4	36
Rab35	gi|54696910	4.6	LLIIGDSGVGK	N2	F15	60
transmembrane emp24 domain-containing protein 10 precursor	gi|4885697	4.6	IPDQLVILDMK	N1	F4	38
Actin related protein 2/3 complex subunit 1b	gi|14043135	4.5	TWKPTLVILR	N2	F3	49
FERM and PDZ domain containing 4	gi|109731127	4.4	VYLENGQTK	N2	F9	32
Actin related protein 2\3 complex subunit 1b	gi|14043135	4.2	TWKPTLVILR	N1	F3	48
Rab1b	gi|89029141	3.8	IQTIELDGK	N1	F8	40
Kinesin light chain 3	gi|91825557	3.7	GEAAAGAAGMKR	N1	F3	40
Scribble	gi|32812252	3.3	SERGLGFSIAGGK	N1	F8	32
Nebulin	gi|19856971	3.3	DKYTPVPDTPILIR	N2	F14	32
Fibronectin leucine rich transmembrane protein 3	gi|7529605	3.1	KTITITVK	N2	F4	39
Actin related protein 2/3 complex subunit 1b	gi|14043135	3.1	TWKPTLVILR	N2	F6	34
FERM and PDZ domain containing 4	gi|109731127	2.9	VYLENGQTK	N1	F9	32
MAP\microtubule affinity-regulating kinase 2	gi|86990437	2.9	FLILNPSKR	N2	F4	30
Sec5	gi|15982242	0.5	KLLEELLNK	N1	F9	41
dynein light chain-A	gi|5531813	0.5	NVLLLGEDGAGK	N2	F7	35
echinoderm microtubule associated protein like 6	gi|223005862	0.5	QVTEAVVIEK	N2	F6	37
kalirin, RhoGEF kinase	gi|148839466	0.4	GSLTPGYMFKR	N2	F10	34
retinoblastoma-associated factor 600	gi|82659109	0.4	NLQGFLEQPK	N1	F3	31
Actin related protein 1	gi|119570071	0.4	VMAGALEGDIFIGPK	N2	F6	41
EH domain binding protein 1	gi|21739555	0.4	KAPAPPVLSPK	N2	F10	31
coat protein gamma2-COP	gi|51094839	0.4	SIATLAITTLLK	N1	F3	89
Rab2	gi|14286264	0.3	TASNVEEAFINTAK	N1	F5	91
Rab33a	gi|14603037	0.3	TVEIEGEK	N2	F5	31
ROCK1	gi|47605999	0.3	LLEFELAQLTK	N1	F4	72
cytoplasmic FMR1 interacting protein 1 isoform 3	gi|57545144	0.2	LADQIFAYYK	N1	F4	49
receptor mediated endocytosis 8	gi|215274246	0.3	SEETNQQEVANSLAK	N2	F10	52

FUNCTIONAL CLASS, LIPID/CHOLESTEROL HOMEOSTASIS AND METABOLIC PROTEINS						
GAPDH	gi|603211	5.1	IISNASCTTNCLAPLAK	N1	F3	116
Cyclophilin A	gi|51702775	5.1	XSELFADK	N1	F15	50
gamma-butyrobetaine hydroxylase	gi|3746805	4.9	MKTGNMACTIQK	N2	F4	33
Cyclophilin A	gi|51702775	4.6	XSFELFADK	N1	F9	54
Cyclophilin A	gi|51702775	4.0	XSFELFADK	N2	F5	49
Carbamyl phosphatesynthetase I	gi|62822412	3.8	ETLMDLSTK	N1	F15	36
Hydroxysteroid (17-beta) dehydrogenase 12	gi|7705855	3.4	GVFVQSVLPYFVATK	N1	F14	37
ATP citrate lyase isoform 2	gi|38569423	3.4	KAKPAMPQGK	N2	F14	32
Cyclophilin A	gi|51702775	3.2	XSFELFADK	N1	F3	54
Protein disulfide isomerase family A, member 4	gi|37674412	3.2	RSPPIPLAK	N1	F15	40
Protein disulfide isomerase family A, member 4	gi|37674412	3.1	RSPPIPLAK	N1	F9	40
Malate deydrogenase 2	gi|6648067	3.0	IQEAGTEVVKAK	N1	F4	37
7-dehydrocholesterol reductase	gi|4581759	3.0	FLPGYVGGIQEGAVTPAGVVNK	N2	F15	141

Alpha enolase	gi|12804749	2.9	DATNVGDEGGFAPNILENKEGLELLK	N1	F4	48
Lactate dehydrogenase B	gi|12803117	2.8	MVVESAYEVIK	N1	F3	35
Calreticulin	gi|12803363	0.4	FYALSASFEPFSNK	N1	F14	96
Methylenetetrahydrofolate dehydrogenase 1	gi|14602585	0.4	YVVVTGITPTPLGEGK	N2	F7	38
Triosephosphate isomerase	gi|14043688	0.3	VVLAYEPVWAIGTGK	N1	F5	34
Cyclophilin A	gi|51702775	0.2	KITIADCGQLE	N2	F3	51
FUNCTIONAL CLASS, PROTEASE/PROTEASOME						
Plasminogen	gi|387026	6.1	LSSPADITDK	N1	F4	38
Proteasome 26S non-ATPase subunit 13 isoform 1	gi|12654533	3.7	YYQTIGNHASYYK	N2	F4	60
ADAMTS5	gi|6049180	3.6	LMSSILTSIDASKPWSK	N1	F7	33
Proteasome subunit, alpha type, 8	gi|20379541	3.3	LYQTDPSGTYHAWK	N1	F4	47
Proteasome subunit, alpha type, 2	gi|50881968	3.2	GYSFSLTTFSPSGK	N1	F3	89
Proteasome alpha 6 subunit	gi|82571777	3.2	LYQVEYAFK	N1	F3	64
Transmembrane protease, serine 11F	gi|34527807	3.1	ALYQSLKTK	N1	F4	34
Headpin	gi|12643252	3.1	DLFPDGSISSSTK	N2	F3	37
Proteasome 26S subunit, non-ATPase, 3	gi|13436065	0.4	LQLDSPEDAEFIVAK	N1	F3	104
Proteasome beta 7 subunit proprotein	gi|1531533	0.3	LDFLRPYTVPNK	N2	F5	35
Proteasome 26S non-ATPase subunit 7	gi|15214948	0.3	VVGVLLGSWQK	N2	F6	65
PAPP-A	gi|38045915	0.2	CKVLMLGGSALNHNYR	N2	F15	34
FUNCTIONAL CLASS, POST-TRANSLATIONAL MODIFICATION						
Protein tyrosine phosphatase	gi|16876892	5.3	MEKGDDINIK	N2	F4	45
Heparan sulfate 3-O-sulfotransferase 6	gi|14336772	5.1	KAQGGSRPR	N2	F9	34
Cullin 7	gi|2833262	4.8	NLLNCLIVRILK	N2	F4	34
Retinoblastoma binding protein 6 isoform 1	gi|74762440	4.8	ISKLEVTEIVKPSPK	N2	F9	32
Tssk4	gi|83405295	4.7	RATILDIIK	N2	F15	37
E1A binding protein p400	gi|56549696	4.3	AIQPQAAQGPAAVQQKITAQQITTPGAQQK	N1	F7	31
Tssk4	gi|83405295	4.2	RATILDIIK	N1	F5	40
Cullin 7	gi|2833262	3.7	NLLNCLIVRILK	N2	F5	33
Ubiquitin specific protease 4 isoform b	gi|40795667	3.3	ADTIATIEK	N2	F3	31
Protein tyrosine kinase	gi|515871	3.1	TGAFEDLKENLIR	N2	F4	41
TIP120 protein	gi|76661742	0.5	EGPAVVGQFIQDVK	N1	F5	34
N-myristoyltransferase 1	gi|10835073	0.5	GSETDSAQDQPVKMNSLPAER	N2	F6	37
Putative methyltransferase WBMT	gi|23831505	0.4	AGFSGGMVVDYPNSAK	N1	F7	54
Janus kinase 1	gi|62087694	0.3	MQLPELPKDISYK	N2	F4	31
Ring finger protein 20	gi|83405148	0.3	LQELTDLLQEK	N1	F5	52
Polypeptide GalNAc transferase 13	gi|27530993	0.3	NQEGPGEMGK	N2	F10	39

NOL1\NOP2\Sun domain family, member 5	gi|37674386	0.2	VLVYELLLGK	N2	F6	35
FUNCTIONAL CLASS, PROTEIN FOLDING/CHAPERONE						
Reticulon 4	gi|20070662	6.1	LFLVDDLVDSLK	N1	F15	38
Heat shock protein 90Ad	gi|61104905	4.4	EPHISLIPNK	N1	F7	32
Histocompatibility (minor) 13	gi|14286316	4.0	LVFPQDLLEK	N2	F15	38
Peptidylprolyl isomerase domain and WD repeat containing 1	gi|24308049	0.3	DVITHVVCTK	N1	F3	33
Chaperonin	gi|41399285	0.2	TVIIEQSWGSPK	N1	F3	71
Chaperonin containing TCP1, subunit 4 (delta)	gi|76827901	0.1	DALSDLALHFLNK	N2	F15	37
Signal sequence receptor, alpha	gi|119575608	3.0	FLVGFTNK	N1	F14	42
FUNCTIONAL CLASS, CELL ADHESION AND MEMBRANE PROTEINS						
BAI1-associated protein 3	gi|14336746	4.5	ILNDKSPR	N2	F5	31
Aggrecan core protein	gi|129886	4.6	GSVILXVKP	N1	F4	39
Solute carrier family 25, member 3	gi|15079648	4.4	FGFYEVFK	N1	F15	38

Translocase of inner mitochondrial membrane 44 homolog	gi|62897685	4.4	ILDIDNVDLAXGK	N1	F9	32
Potassium voltage-gated channel, subfamily H, member 6 isoform 1	gi|11878259	3.6	VVERTQNVTEK	N2	F11	36
Transient receptor potential cation channel, subfamily M, member 8	gi|72537227	3.5	GLLKEIANK	N1	F15	39
Semaphorin receptor	gi|6010211	3.1	FRYLVPGSNGQLTFDSGFEK	N1	F9	33
GPAD9366	gi|73619948	0.5	MVTFYCTTK	N1	F7	34
Cadherin 8, type 2	gi|6483315	0.5	HKNEPLIIK	N1	F4	33
FUNCTIONAL CLASS, SIGNALLING/CELL CYCLE						
Fibroblast growth factor 11	gi|20160215	5.6	SLCQKQLLILLSK	N1	F7	36
cell division cycle 2-like 6 (CDK8-like)	gi|57209410	3.7	DLKPANILVMGEGPER	N2	F3	34
Rotatin	gi|145046269	3.4	KSAAEQLAVIMQDIK	N2	F14	31
Cytokine induced protein 29 kDa	gi|109097133	3.3	FGIVTSSAGTGTTEDTEAK	N2	F5	91
KH domain containing, RNA binding, signal transduction associated 1	gi|12653853	0.4	ILGPQGNTIK	N2	F6	43
Cyclin-dependent kinase 9	gi|12805029	0.3	EIKILQLLK	N2	F4	33
FUNCTIONAL CLASS, OTHER						
Retinol dehydrogenase 14 (all-trans\9-cis\11-cis)	gi|10190746	5.9	GGDPGLMHGK	N1	F14	35
GTP-binding protein NGB	gi|4191616	4.1	SSFINKVTR	N2	F9	32
HLA-B associated transcript 1	gi|56207984	3.2	GLAITFVSDENDAK	N1	F3	80
Carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase	gi|50403731	3.1	HPQPGAVELAAK	N1	F6	32
Ribosomal protein L29	gi|60656425	3.0	AQAAAPASVPAQAPK	N1	F5	88
Alpha-fetoprotein	gi|31351	0.5	GYQELLEK	N1	F15	31
A kinase (PRKA) anchor protein 8-like	gi|6688138	0.3	QTADFLQEYVTNK	N1	F3	42
J-type co-chaperone HSC20	gi|41388876	0.2	QFLIEIMEINEK	N1	F3	44

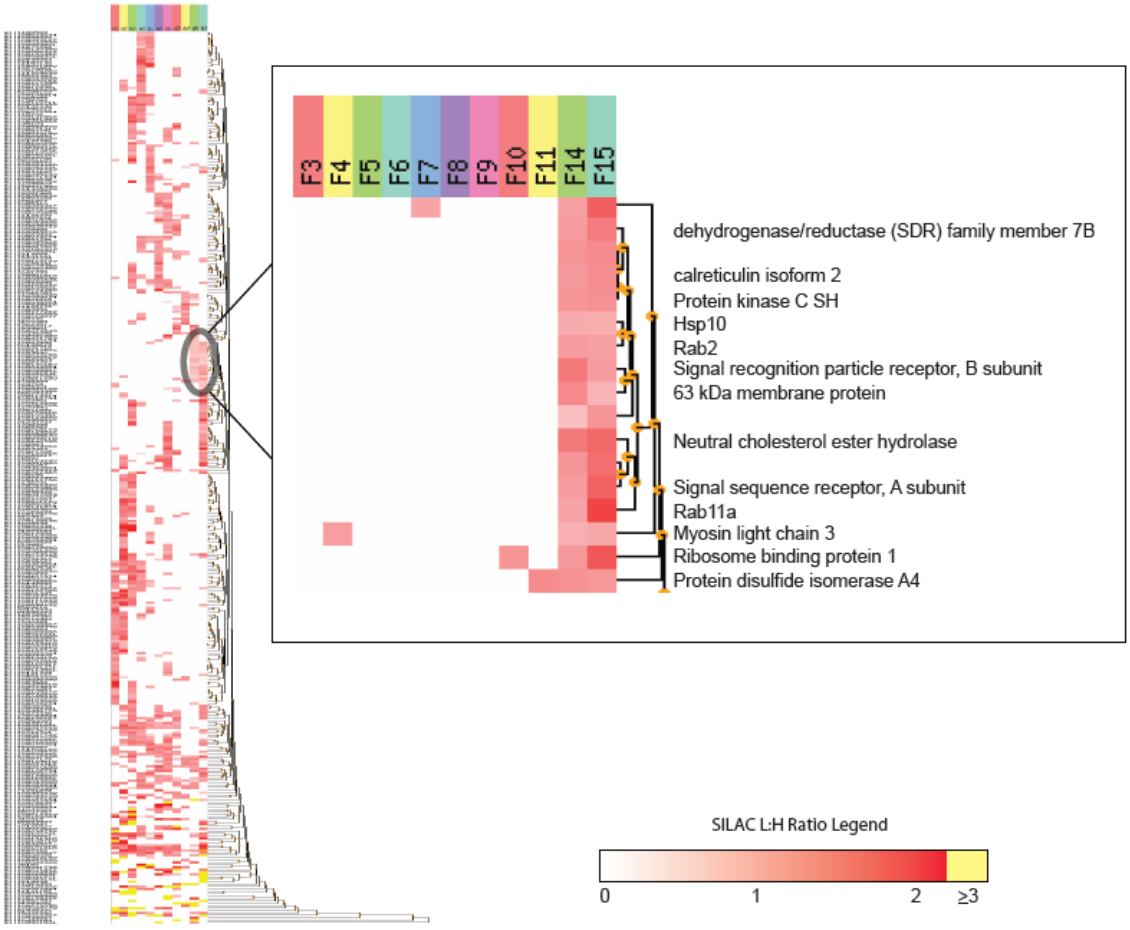
membrane proteins as well as other types of functionalities. This finding suggests that PCSK9 either directly or indirectly has an effect on many types of proteins with a wide range of functions.

All quantitated proteins that were identified among two or more fractions were then subjected to hierarchical clustering in order to observe any possible trends in the SILAC data, as well as assess the data quality (Figure 8). As shown, most proteins were identified only in similar or contiguous histodenz fractions, rather than interspersed amongst the histodenz subcellular fractions. This suggests the proteins are not present in all but instead are found in specific regions among the subcellular fractions. An example of this is found in Figure 8 (see insert) where the proteins clustered together among the more dense histodenz fractions (15 and 14) were highly enriched for ER resident proteins. This is as expected, as it was found that these fractions were comprised of ER marker proteins and lacked Golgi, endosome and lysosome marker proteins (Figure 6).

PCSK9 promotes a decrease in cellular EH domain binding protein 1 and an increase in actin related protein 2\3 at the protein level

Beyond manual validation of quantitation results at the raw MS level, two different proteins were chosen for further biochemical validation studies. One protein, actin related protein 2\3 (Arp 2\3), was chosen since its protein levels were significantly higher in response to PCSK9-ACE2-V5 expression, as well as it being seemingly unrelated to PCSK9 mediated degradation of the LDLR. SILAC quantitation results showed Arp 2\3 to have

Figure 8. Heat map clustering of quantitated fractionation data reveals non-random distribution of proteins throughout subcellular fractions. Proteins identified in two or more histodenz fractions were subjected to hierarchical clustering across the subcellular histodenz fractions. Inset shows proteins clustering together in high density fractions (F14 F15) relating to the endoplasmic reticulum, (See Figure 6) were found to be highly enriched with endopasmic reticulum resident proteins.


F3
F4
F5
F6
F7
F8
F9
F10
F11
F14
F15
dehydrogenase/reductase (SDR) family member 7B
calreticulin isoform 2
Protein kinase C SH
Hsp10
Rab2
Signal recognition particle receptor, B subunit
63 kDa membrane protein
Neutral cholesterol ester hydrolase
Signal sequence receptor, A subunit
Rab11a
Myosin light chain 3
Ribosome binding protein 1
Protein disulfide isomerase A4
SILAC L:H Ratio Legend
0
1
2
≥3

protein levels increased between 9.4 to 3.1 fold in subcellular fractions 3 to 6, in PCSK9 expressing cells. Cell lysates from empty vector control and those expressing PCSK9-ACE2-V5 were subjected to Western blot analysis for Arp 2\3. Protein levels were corrected to transferring receptor levels, a protein known to be unaffected by PCSK9 levels. Western blot analysis revealed increased protein levels of over 2-fold of Arp 2\3 in PCSK9-ACE2-V5 expressing HuH7 cells (Figure 9). Another protein, EH domain binding protein 1 (EHBP1), was chosen for its significantly decreased protein levels in response to PCSK9-ACE2-V5 expression, as well as for its potential link to PCSK9 mediated degradation of LDLR. SILAC quantitation results showed EHBP1 protein levels were 2.5 fold lower in PCSK9-ACE2-V5 expressing cells. Cell lysates from empty vector control and PCSK9-ACE2-V5 expressing HuH7 cells were subjected to Western blot analysis for EHBP1 (Figure 10). Protein levels were corrected to transferrin receptor levels, and revealed a decreased protein levels of roughly 2-fold of EHBP1 in PCSK9-ACE2-V5 expressing cell lysates, and a decrease in protein levels as low as 4-fold in subcellular fractions. These results are in accordance with, and validate the quantitation results obtained through SILAC labelling experiments.

PCSK9-ACE2-V5 and PCSK9-D374Y variants decrease both cellular LDLR and EHBP1 protein levels

PCSK9-ACE2-V5 has previously been shown to accelerate LDLR degradation greater than wild type PCSK9. To date, however, the strongest gain of function variant, in terms of

Figure 9. Western blot validation of Arp2/3 levels increased in PCSK9-ACE2-V5 expressing cells. HuH7 cells expressing empty vector and PCSK9-ACE2-V5 were grown in DMEM supplemented with 10% FBS and 1% anti-mycotic. Whole cell lysates of empty vector and PCSK9-ACE2-V5 expressing HuH7 cells were subjected to Western blot analysis for endogenous Arp2/3. Arp2/3 levels in PCSK9-ACE2-V5 expressing cells were listed relative to those found in empty vector cells. Sample load was corrected to transferrin receptor levels. SILAC quantitation found Arp2/3 levels in PCSK9-ACE2-V5 expressing cells were increased between 9.4 and 3.1 fold as compared to empty vector expressing cells, depending on the subcellular fraction it was identified in.

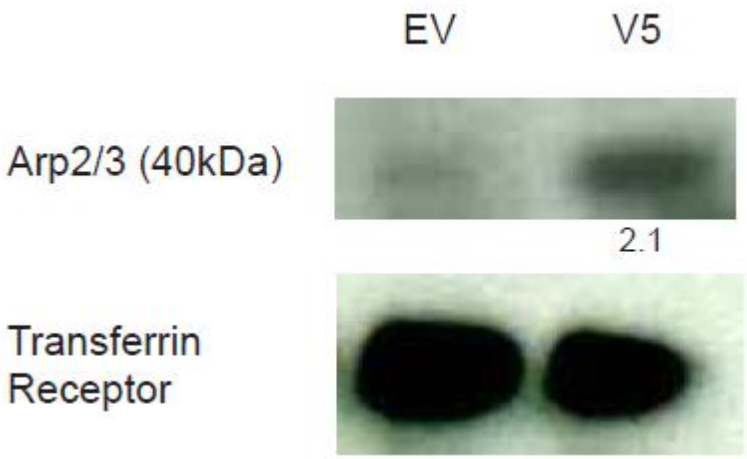
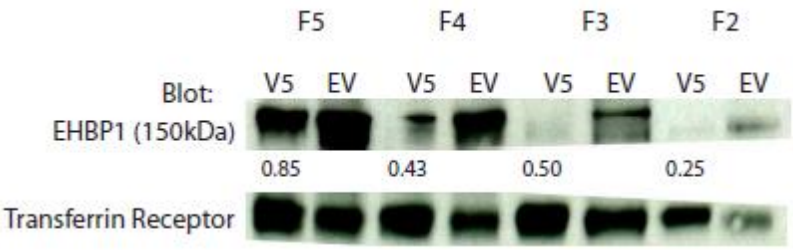
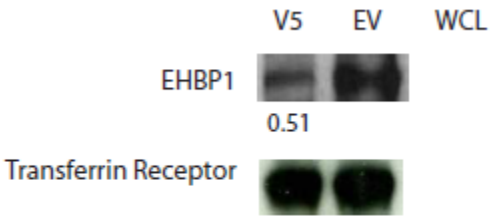

EV
V5
Arp2/3 (40kDa)
2.1
Transferrin
Receptor

Figure 10. Western blot validation of EHBP1 levels decreased in PCSK9-ACE2-V5 expressing cells. HuH7 cells expressing empty vector and PCSK9-ACE2-V5 were grown in DMEM supplemented with 10% FBS and 1% anti-mycotic. Subcellular fractions between empty vector and PCSK9-ACE2-V5 expressing HuH7 cells for fractions F2-F5 (top) and whole cell lysates of empty vector and PCSK9-ACE2-V5 expressing HuH7 cells (bottom) were subjected to Western blot analysis for endogenous EHBP1. EHBP1 levels in PCSK9-ACE2-V5 expressing cells were listed relative to those found in empty vector cells, and corrected for sample loading according to transferrin receptor levels. SILAC quantitation found EHBP1 levels in PCSK9-ACE2-V5 expressing cells were decreased 2.5 fold as compared to empty vector expressing cells.


F5
F4
F3
F2
Blot:
V5
EV
V5
EV
V5
EV
V5
EV
EHBP1 (150kDa)
0.85
0.43
0.50
0.25
Transferrin Receptor


V5
EV
WCL
EHBP1
0.51
Transferrin Receptor

specifically promoting the degradation of LDLR, of PCSK9 is the PCSK9-D374Y variant. With the belief that EHBP1 may play a role in PCSK9 mediated degradation of the LDLR, EHBP1

and LDLR protein levels were analyzed from HuH7 empty vector, PCSK9-ACE2-V5, and PCSK9-D374Y stably expressing cells (Figure 11). Compared to empty vector control cells, both EHBP1 and LDLR levels were decreased in PCSK9-ACE2-V5 and PCSK9-D374Y variant expressing cell lines, suggesting a possible link between EHBP1 and PCSK9 mediated LDLR degradation.

PCSK9-ACE2-V5 Immunoaffinity purification optimization

Despite extensive biological and physiological studies of PCSK9, at the time of this study, the only known protein interaction partners of PCSK9 were the LDLR and potentially VLDLR, and ApoER2. Determining protein interaction partners can provide a great deal of information regarding a proteins function, and in the case of PCSK9, potentially reveal novel cellular functions and/or proteins it may work in concert with to promote the degradation of the LDLR. In order to optimize the anti-PCSK9-ACE2-V5 IP conditions, increasing amounts of PCSK9-ACE2-V5 expressing HuH7 cell lysates (from 0 to 500µg) were incubated with a constant amount (10µL, 50% slurry) of anti-V5 agarose affinity gel, for 2h at 4°C on a rotator. Eluted proteins were subjected to Western blot analysis for endogenous PCSK9 (Figure 12). From the conditions tested, it was evident that from the amounts of whole cell lysates tested, using 500µg of whole cell lysates for 10µL of beads (50% slurry) was most optimal. This was determined since the recovery of PCSK9 was

Figure 11. PCSK9-ACE2-V5 and the natural gain of function variant PCSK9-D374Y decreases EHBP1 and LDLR protein levels in whole cell lysates. Cell lysates of empty vector control, PCSK9-ACE2-V5 and PCSK9-D374Y expressing HuH7 cells were subjected to Western blot analysis for endogenous EHBP1 and mature LDLR levels revealing PCSK9-ACE2-V5 and PCSK9-D374Y promote the degradation of both the LDLR and EHBP1. EHBP1 can appear as a single band or doublet depending on WB conditions.

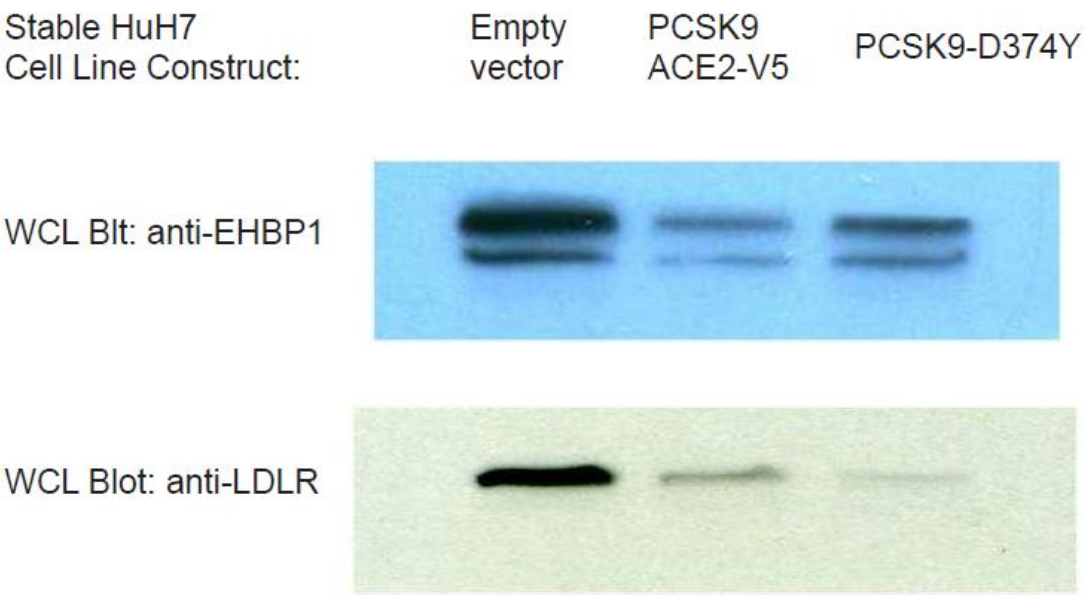
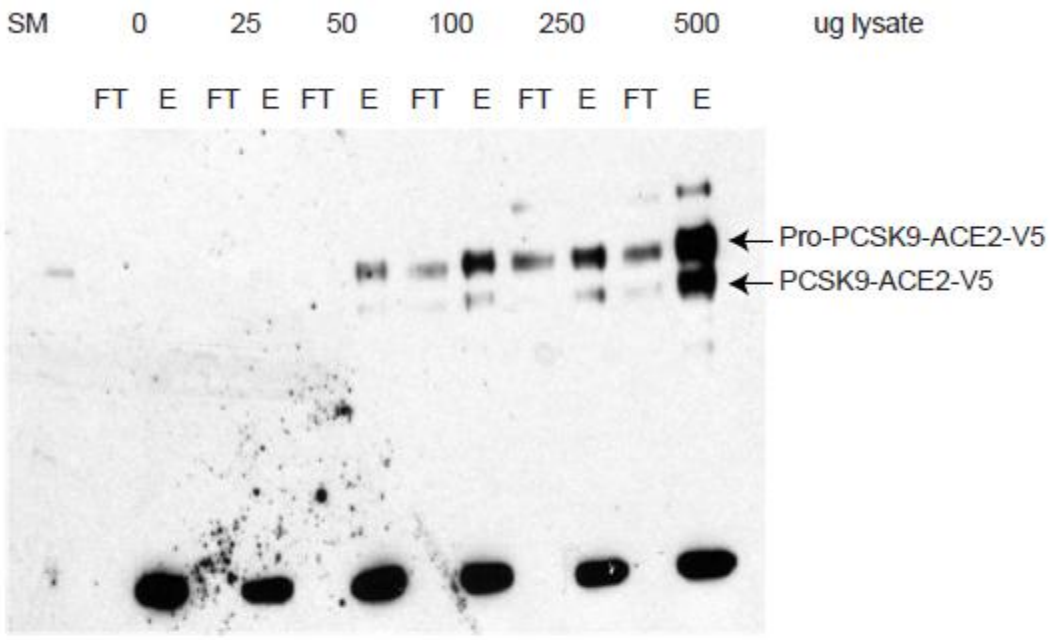

Stable HuH7
Cell Line Construct:
Empty vector
PCSK9 ACE2-V5
PCSK9-D374Y
WCL Blt: anti-EHBP1
WCL Blot: anti-LDLR

Figure 12. Optimization of anti-V5 Immunoaffinity purifications for PCSK9-ACE2-V5 pulldowns. HuH7 cells expressing PCSK9-ACE2-V5 were lysed, and purifications using 10µL of anti-V5 agarose affinity gel (50% slurry) were used against solutions containing 0, 25, 50, 100, 250 and 500 µg of cell lysates in modified RIPA buffer. Size marker (SM), and flow through (FT) and eluted (E) proteins for each IP were subjected to Western blot analysis for endogenous PCSK9. Intense lower band found in all elute lanes is IgG from anti-V5 antibody beads.



IP: Anti-V5

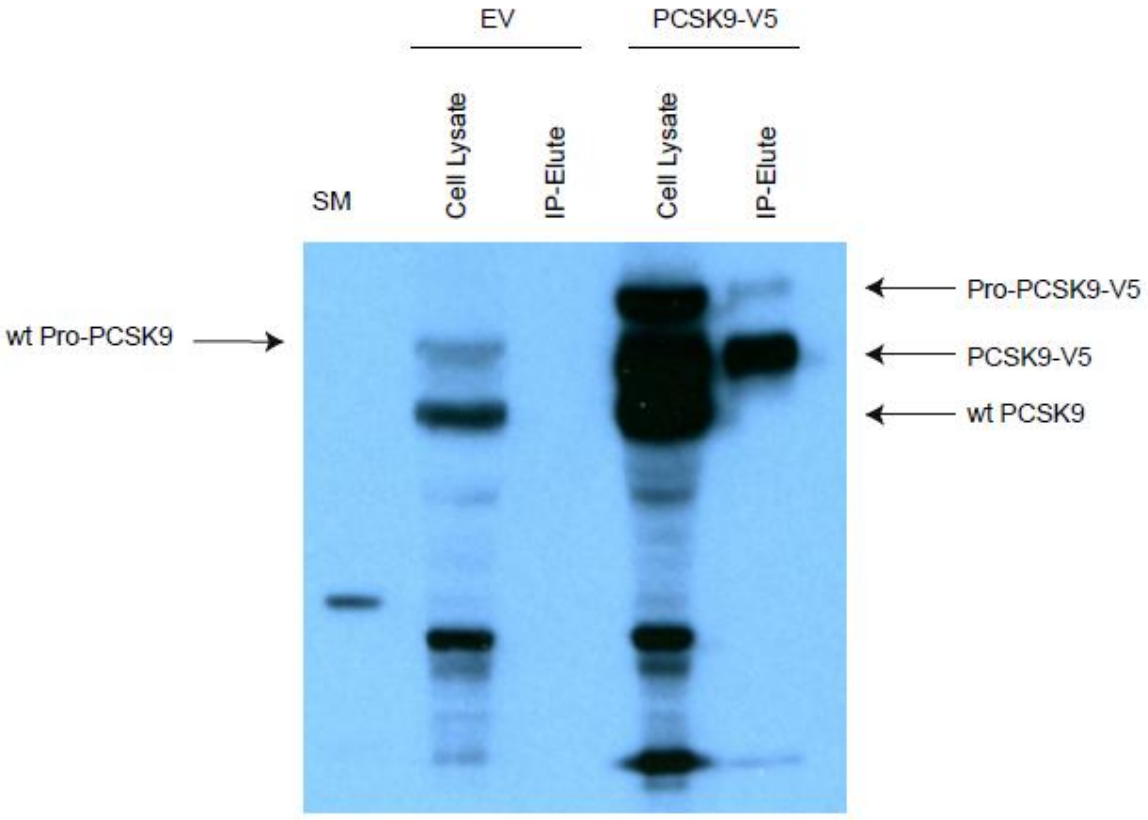
WB: Anti-PCSK9

strongest, and there was little to no increase in the amount of non-binding PCSK9, as shown by the amount of PCSK9 found in the flow through fraction.

PCSK9-ACE2-V5 IP-LC-MS/MS analysis of interaction partners

A large-scale anti-V5 IP was performed in order to co-purify interaction partners of PCSK9. 10% of starting material and eluted protein complexes were subjected to Western blot analysis to show the efficiency and specificity of the PCSK9-ACE2-V5 purification (Figure 13). In an empty vector expressing HuH7 cell lysates, endogenous pro-PCSK9 and cleaved PCSK9 are present, however following an anti-V5 IP from the empty vector HuH7 cell lysate, no forms of PCSK9 can be detected in the eluted protein fraction. Conversely, in PCSK9-ACE2-V5 expressing HuH7 cell lysates, both endogenous pro-PCSK9 and cleaved PCSK9 are present, in addition to pro-PCSK9-ACE2-V5 and cleaved PCSK9-ACE2-V5 protein variants. Following an anti-V5 IP, protein eluate contains solely the pro- and cleaved PCSK9-ACE2-V5 protein variants, and endogenous PCSK9 isoforms are not purified, suggesting a specific purification of V5 tagged PCSK9-ACE2 and its protein complexes. The remaining 90% of eluted protein complexes from both EV and PCSK9-ACE2-V5 IPs were subjected to protein separation, tryptic digestion, protein processing and LC-MS/MS analysis in order to identify potential interaction partners. In both IP experiments (n=2) PCSK9 was confidently identified in PCSK9-ACE2-V5 expressing cell IPs (Mascot score 567 and 546), but not in the EV control cell IPs, reflecting the results obtained by Western blot analysis. A complete list of potential interaction partners was obtained and following data filtering, a list of potential

Figure 13. PCSK9-ACE2-V5 Immunoaffinity Purification. 15mg of HuH7 empty vector and PCSK9-ACE2-V5 cell lysates were subjected to anti-V5 IP using 300 µL (50% slurry) of anti-V5 affinity agarose gel for 4h at 4°C. Flow through was collected, antibody-bead-antigen complexes were washed 8x with ice chilled modified RIPA buffer. Protein complexes were eluted by boiling 2x sample buffer for 10 minutes. 5% of eluted and flow through material were subjected to Western blot analysis against endogenous PCSK9.



IP: Anti-V5
WB: Anti-PCSK9

PCSK9 interaction partners of higher confidence was obtained (Figure 14 and Table 2). Interestingly, a potential interaction partner of PCSK9 that was identified was EH domain-containing protein 4 (EHD4), a member of a family of proteins that bind to EH domain binding protein 1 (EHBP1), which was shown to have decreased protein levels in PCSK9-ACE2-V5 expressing cells, as shown previously through SILAC quantitation and Western blot analysis. These results provide a link between PCSK9 interaction partners and its effect on regulating protein levels in HuH7 cells, and suggest a link to the EH domain proteins and EH domain binding protein endosomal machinery.

PCSK9 and mature LDLR both interact with EHD4

In order to test and validate the interaction between wild type PCSK9 lacking the ACE2 domain and EHD4, various cell systems were used. HepG2 cells expressing EHD4-Myc tag were co-expressed with PCSK9-V5 or an empty vector. HuH7 cells expressing EHD4-Myc tag were co-expressed with PCSK9-Flag or an empty vector. Anti-V5 and anti-Flag IP experiments were performed in both cell systems and protein eluate was subjected to Western blot analysis against anti-Myc (Figure 15). EHD4-myc was present specifically in the anti-V5 protein eluate from PCSK9-V5 expressing cells, while absent in the empty vector expressing cells. The reverse IP experiments were also performed, purifying EHD4-Myc using anti-Myc agarose affinity gel (Figure 15). Western blot analysis against anti-V5

Figure 14. PCSK9 Interaction map. Proteins identified in anti-V5 immunopurifications to co-purify with PCSK9-ACE2-V5.

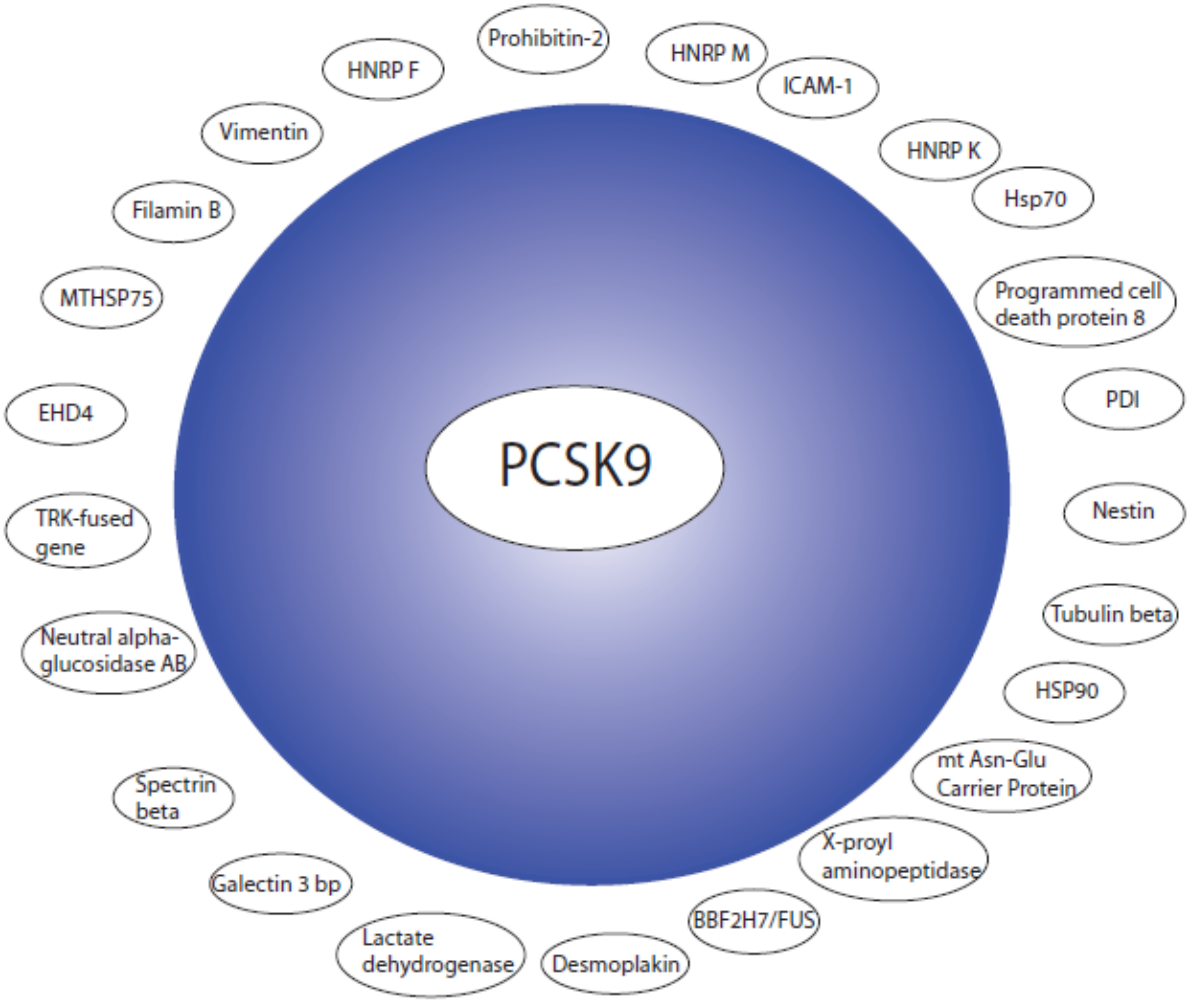
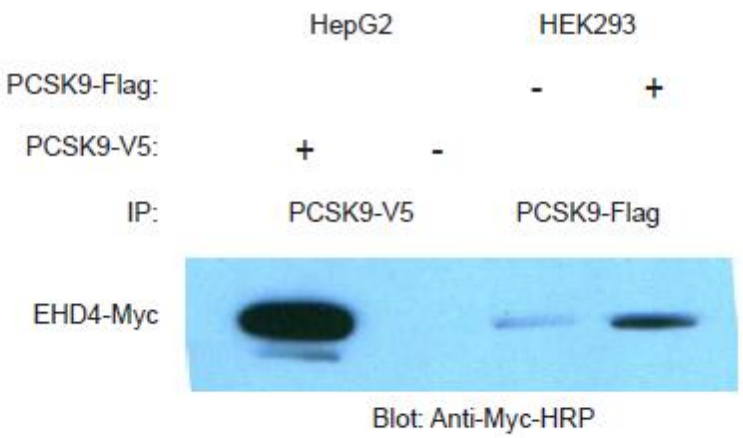

PCSK9
Prohibitin-2
HNRP M
ICAM-1
HNRP K
Hsp70
Programmed cell death protein 8
PDI
Nestin
Tubulin beta
HSP90
mt Asn-Glu Carrier Protein
X-proyl aminopeptidase
BBF2H7/FUS
Desmoplakin
Lactate dehydrogenase
Galectin 3 bp
Spectrin beta
Neutral alpha-glucosidase AB
TRK-fused gene
EHD4
MTHSP75
Filamin B
Vimentin
HNRP F

Table 2. List of proteins identified to co-immunoprecipitate with PCSK9-ACE2-V5 following anti-V5 immunoaffinity purification and LC-MS/MS analysis.

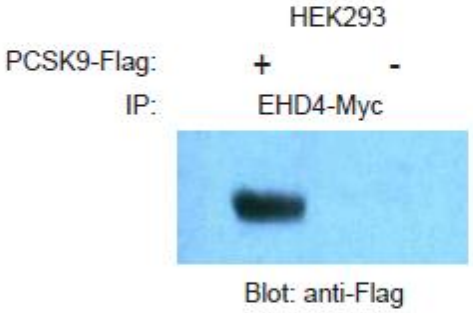
Protein Name	Mascot Score	No. Peptides	IP No.
Vimentin	1349	21	2
PCSK9	567/546	9/8	1/2
Heat shock protein 90	174/520	3/9	1/2
Nestin	569	8	2
Galectin 3 binding protein	99/180	2/3	1/2
TRK-fused gene/anaplastic large cell lymphoma kinase extra long form	360	4	1
Heat shock protein 70 protein 1	337	4	2
Spectrin, beta	327	8	2
Lactate dehydrogenase A	222	4	1
Heterogeneous nuclear ribonucleoprotein K	210	3	2
Protein disulfide-isomerase precursor	194	3	2
Heat shock protein 70 protein 8	150	2	1
Heterogeneous nuclear ribonucleoprotein F	147	3	2
MTHSP75	128	2	1
Neutral alpha-glucosidase AB precursor isoforms 2	128	3	2
X-prolyl aminopeptidease	125	2	1
Desmoplakin	121	2	1
Filamin-B	120	3	2
Mitochondrial aspartate-glutamate carrier protein	115	3	2
Programmed cell death protein 8	102	2	2
Tubulin, beta	101	2	1
EH-domain containing protein 4	91	2	2
Heterogeneous nuclear ribonucleoprotein M	88	2	2
BBF2H7/FUS protein	83	2	2
Intracellular adhesion molecular 1 precursor (ICAM-1)	74	2	2
Prohibitin-2	68	2	2

Figure 15. Co-IP Experiments reveal PCSK9 and EHD4 Interact. a) HepG2 cells were transiently transfected with EHD4-myc and either PCSK9-V5 or empty vector constructs and subjected to anti-V5 Immunopurifications. HEK293 cells were transiently transfected with EHD4-myc and either PCSK9-Flag or empty vector constructs and subjected to anti-Flag or anti-V5 immunopurifications. Eluted protein complexes were subjected to Western blot analysis against anti-myc-HRP. Reverse Co-IP experiments were performed in these cell systems involved anti-Myc-EHD4 immunopurifications, and eluted complexes were subjected to Western blot analysis against b) anti-Flag from HEK293 cells and c) anti-V5 from HepG2 cells.

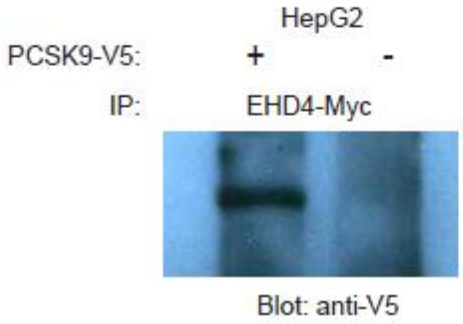
a


HepG2
HEK293
PCSK9-Flag:
-
+
PCSK9-V5:
+
-
IP:
PCSK9-V5
PCSK9-Flag
EHD4-Myc
Blot: Anti-Myc-HRP

b


HEK293
PCSK9-Flag:
+
-
IP:
EHD4-Myc
Blot: anti-Flag

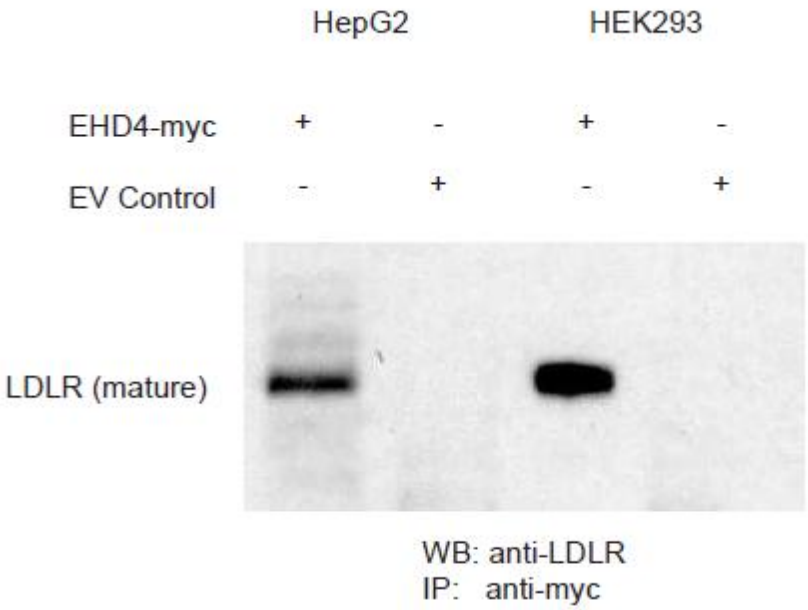
c


HepG2
PCSK9-V5:
+
-
IP:
EHD4-Myc
Blot: anti-V5

revealed an interaction between PCSK9-V5 and EHD4 when co-expressed. In HEK293 cells, EHD4-myc was co-expressed with either flag tagged wild type PCSK9 or an empty vector. When anti-Flag IP experiments were performed and eluted proteins were subjected to anti-Myc Western blot analysis, it was shown that PCSK9-Flag interacts with EHD4-Myc (Figure 15). The reverse IP experiments were also performed, purifying EHD4-myc using anti-Myc agarose affinity gel (Figure 15). Western blot analysis against anti-Flag revealed an interaction between PCSK9-Flag and EHD4-Myc in HepG2 cells. Taken together, these results validate the previous IP-LC-MS/MS interaction results between PCSK9 and EHD4, and suggest that this novel interaction is specific in nature and not due to the ACE2 domain present in the PCSK9-ACE2-V5 variant.

With previous studies showing that the EH domain containing and binding proteins are known to play a role in LDLR trafficking and recycling, the link between EHD4, PCSK9 and LDLR became a valid possibility, and therefore the interaction between EHD4 and endogenous LDLR was investigated. In both HepG2 and HEK293 cells either Myc-tagged EHD4 or an empty vector was expressed. In both cell systems, anti-Myc IP experiments were performed, and eluted protein complexes were subjected to Western blot using an antibody for endogenous cellular LDLR, in the mature, glycosylated form (Figure 16). The resulting blot showed that mature endogenous LDLR co-immunoprecipitated specifically in EHD4-Myc expressing cells. These results suggest a novel and specific interaction between LDLR and the endosomal machinery and trafficking protein EHD4.

Figure 16. Co-IP Experiments Reveal EHD4 and LDLR interact. HepG2 cells were transiently transfected with EHD4-myc or an empty vector construct. Cells were lysed and subjected to anti-myc immunoaffinity purifications. The resulting eluted protein complexes were subjected to Western blot analysis against endogenous mature LDLR.


HepG2
HEK293
EHD4-myc + - + -
EV Control - + - +
LDLR (mature)
WB: anti-LDLR
IP: anti-myc

Chapter 4. DISCUSSION

In these studies I have utilized large-scale proteomic techniques including subcellular fractionation, relative quantitation using SILAC metabolic labelling, and immunoaffinity purification of protein complexes in order to explore cellular roles PCSK9 plays in the liver. The aim of my work was to determine whether PCSK9 affected cellular proteins and cellular activities in liver cells. The work discussed thus far suggests that PCSK9 expression does affect the abundance of many liver cell proteins, many of which at significant levels. Additionally, the work here also aimed to determine if PCSK9 plays any novel cellular roles in the liver. The results obtained from the SILAC relative quantitation and interaction were not limited to the known established functional roles of PCSK9 such as lipid and cholesterol homeostasis and regulation, but suggests PCSK9 plays novel roles in liver cells outside of LDLR degradation. These novel functional roles which include cytoskeleton organization and regulation, vesicle transport and trafficking, post-translational modification and regulation of proteins including proteasomal regulation and chaperone regulation, cell membrane and adhesion protein regulation, chaperone mediated autophagy and even potential roles in Alzheimer's via amyloid precursor protein (APP) processing and regulation. Despite this, there is a lack of understanding of mechanisms in which PCSK9 plays a role in these cellular processes, the results obtained in this study provide a new insight into PCSK9 cellular function and show that PCSK9 is a multi-functional cellular protein with roles outside of LDLR regulation.

Subcellular fractionation and quantitative SILAC labelling of PCSK9-ACE2-V5 expressing HuH7 cells reveals effects on cellular proteome

In order to optimally study the effects PCSK9 expression plays on the HuH7 liver cell proteome, it was advantageous to perform subcellular fractionation on the proteome. This method would provide another layer of protein fractionation before LC-MS/MS analysis, identification and quantitation, as well provide cell locale relative protein quantitative information throughout the study. Following these experiments, quantitation and quality control, it became clear that PCSK9 expression affected much more than LDLR, lipid and cholesterol homeostasis. PCSK9 affected the expression of 341 HuH7 cellular proteins. Most protein expression levels were unaffected by PCSK9, including those that were known to be unaffected by PCSK9, such as transferrin receptor. The transferrin receptor is constantly recycled from endosomes back to the cell surface and has been previously shown to be unaffected by PCSK9 over-expression [42], as it is seen in this study to be unaffected, whose levels are found to range from a ratio of 1.0 to 1.2 (PCSK9-ACE2-V5 expressing cells to empty vector control cells).

Those proteins that were affected by the expression of PCSK9 were grouped into functional classes and found to represent diverse cellular roles, ranging from cell surface membrane and cell adhesion proteins, lipid and cholesterol metabolism, cytoskeleton organization, endocytosis and vesicle trafficking, post-translational modification, proteasome function, chaperone mediated autophagy, protein folding and chaperone activity, and amyloid precursor protein processing and regulation.

Comparison of SILAC quantitation results with previous PCSK9 gene expression studies

Previously, two studies have been published with the aim of looking at genes whose expression is affected by PCSK9 expression. The first study looked at the genes upregulated when the variant PCSK9-D374Y was expressed in comparison to an empty vector control [42]. This study revealed that most genes significantly affected by PCSK9-D374Y are related to lipid and cholesterol metabolism, as well as stress response. The second study treated cells with purified wild type or PCSK9-D374Y for various time points and looked at the genes that were affected by these treatments [80]. This study was able to expand upon novel pathways and functions that which PCSK9 affects, including cell cycle, inflammation and protein ubiquitination, as well as confirming that lipid and cholesterol metabolism and stress response related genes are affected.

Like the previous gene expression studies performed, this protein quantitation study was able to expand upon the understanding of what cellular functions and pathways are affected by PCSK9 expression. However, this study goes even further than the previously discussed gene expression studies, by documenting changes at the protein level, not simply at the gene expression level, as well as in a cell location specific level, not a global level. The results from this study mirrored those obtained from the previous gene expression studies. These studies suggest that PCSK9 expression affects proteins involved in cellular processes such as lipid and cholesterol metabolism, stress response, cell cycle, protein ubiquitination and proteasome function. However, in light of the data presented here, we suggest that PCSK9 expression also affects proteins involved in other cellular

processes not previously known to be associated with PCSK9 function, including cytoskeleton organization, organelle trafficking, chaperone activity and post-translational modification, among others.

A complete comparison of proteins/genes identified overlapping between these studies can be found in Table 3. By comparison, very few protein/genes were found to overlap between this study and those found in the two previous mRNA expression studies. Of those that do overlap, not all the results are harmonious, as is often the case when comparing gene expression results and protein expression levels. For example, a protein whose levels are almost non-existent may have high/up-regulated gene expression levels as a feed-back mechanism in order to raise the protein levels of such a gene, as is the case for the LDLR [80]. However there are some results that are congruent, as is the case for gene/protein 7-dehydrocholesterol reductase, whose gene expression when treated with purified wt PCSK9 was shown to increase in a range from 1.4 to 2.5 fold, and from 1.5 to 2.9 when treated with purified PCSK9-D374Y. Like the gene expression study, my results showed an increased level of this protein involved in lipid and cholesterol metabolism, ranging from 1.4 to 3.0. Baculoviral IAP repeat-containing 4, a gene/protein involved in ubiquitination in apoptotic cells was shown to have to a 9.6 fold increase in expression when treated with purified wt PCSK9, while I was able to show that PCSK9 expression caused this protein level to be increased by 3.1 fold. Additionally, both the second gene expression study utilizing exogenously treated purified wt PCSK9 and the study presented here showed similar decreases for the Zinc finger DAZ interacting protein. Proteins

Table 3. Literature comparison between SILAC quantitation and previous gene expression studies. Overlapping proteins/genes identified in PCSK9 overexpression studies [42,80] are compared to the SILAC protein quantitation data. For the second study [80] wild type or PCSK9-D374Y was added exogenously to cell media for cellular uptake. The gene expressions listed were of those following 48hrs of wt/PCSK9-D374Y exogenous treatment.

Protein/Gene	SILAC L:H Ratio	PCSK9-D374Y Protein overexpression study[a]	Wt and or PCSK9-D374Y media treatment study	
			Wt[b]	DY[c]
kinectin 1 (kinesin receptor)	24.3		0.7	0.6
myosin IXA	17.5		2.0	1.5
	14.2			
	13.9			
	6.3			
palladin, cytoskeletal associated protein	8.6		6.4	7.7
	5.2		9.9	10.4
			11.8	14.9
			15.0	12.0
			10.4	9.9
cell division cycle 40 homolog (S. cerevisiae)	8.0		0.3	0.6
within bgcn homolog (Drosophila)	7.4		0.2	0.2
	2.7			
small nuclear ribonucleoprotein polypeptide A	7.0		9.1	2.8
protein tyrosine phosphatase, non-receptor type 2	6.2		4.9	3.8
			10.4	6.1
reticulon 4	6.1		3.6	2.2
WW domain containing transcription regulator 1	6.1		4.9	7.0
			7.0	5.0
heterogeneous nuclear ribonucleoprotein M	5.8		0.3	0.2
	3.6		17.6	3.8
			0.2	0.4
			0.2	0.3
retinoblastoma binding protein 6	5.5		0.04	0.2
			0.2	0.4
			0.1	0.4
			0.5	0.7
transmembrane and tetratricopeptide repeat containing 3	4.7		0.3	0.2
			0.5	0.7
			0.2	0.3
transmembrane emp24-like trafficking protein 10 (yeast)	4.6		0.5	0.7
histocompatibility (minor) 13	4.3		0.6	0.5
			0.5	0.5
cell division cycle 2-like 6 (CDK8-like)	4.0		2.5	1.6
cyclin-dependent kinase 8	4.0		2.3	2.3
			1.9	1.5

			2.0	1.6
fibronectin 1	4.0		2.0	1.5
			0.2	0.4
heterogeneous nuclear ribonucleoprotein C (C1/C2)	3.9		0.3	0.2
			0.2	0.3
rotatin	3.9		2.2	1.7
ATP citrate lyase	3.8		1.7	1.7
			1.6	1.5
ubiquitin specific peptidase 4 (proto-oncogene)	3.8		2.3	2.3
			2.4	2.2
			2.8	2.5
			2.3	2.3
four and a half LIM domains 2	3.7		4.3	2.6
hairy and enhancer of split 1, (Drosophila)	3.5		2.0	1.7
fibronectin leucine rich	3.5		4.8	5.8
transmembrane protein 3	3.1		3.1	2.4
	2.8		5.8	4.7
			2.4	3.1
7-dehydrocholesterol reductase	3.0		1.5	1.4
			1.6	1.5
			1.5	1.61
			1.4	1.5
synaptotagmin binding, cytoplasmic RNA interacting protein	3.4		0.3	0.5
			0.3	0.6
SFRS protein kinase 1	3.4		0.5	0.7
proteasome (prosome, macropain) subunit, alpha type, 2	3.2		0.3	0.2
			0.2	0.3
HLA-B associated transcript 1	3.2		10.7	4.9
ribosomal protein L11	3.2		0.5	0.4
BAT2 domain containing 1	3.2		1.2	1.0
			1.0	0.8
protein disulfide isomerase	3.2		0.5	0.5
family A, member 4	3.1		0.5	0.6
high-mobility group box 1	3.1		0.4	0.5
			0.3	0.5
			0.5	0.7
MAX interactor 1	3.1		2.5	1.8
amyotrophic lateral sclerosis 2 (juvenile)	3.1		6.1	3.2

malate dehydrogenase 2, NAD (mitochondrial)	3.0		0.3	0.5
methyl-CpG binding domain protein 4	3.0		0.6	0.4
			0.4	0.6
			0.4	0.5
			0.4	0.5
			0.2	0.5
solute carrier family 23 (nucleobase transporters), member 1	3.0		0.2	0.1
			0.1	0.2
bromodomain adjacent to zinc finger domain, 1A	3.0		2.0	1.9
far upstream element (FUSE) binding protein 1	2.9		0.2	0.1
			0.1	0.2
			0.04	0.2
signal sequence receptor, alpha	2.9		0.4	0.6
			0.4	0.6
			0.4	0.4
			0.3	0.4
fusion (involved in t(12;16) in malignant liposarcoma)	2.9		0.3	0.2
	0.5		4.7	1.5
			0.1	0.1
			0.3	0.4
			0.2	0.3
F-box protein 11	2.8		0.3	0.4
			0.4	0.3
THO complex 2	2.8		2.3	2.3
			2.2	2.3
contactin associated protein-like 3	2.7		2.9	2.1
cold inducible RNA binding protein	2.7		2.1	1.3
			0.4	0.5
			0.5	0.8
FtsJ homolog 3 (E. coli)	2.7		0.5	0.6
proteasome (prosome, macropain) 26S subunit, non-ATPase, 7	2.7		0.4	0.4
			0.4	0.4
splicing factor, arginine/serine-rich 7, 35kDa	2.6		3.6	2.2
			0.2	0.4
trinucleotide repeat containing 6A	2.6		0.1	0.3
DIP2 disco-interacting protein 2 homolog C (Drosophila)	2.6		2.4	3.0
			2.5	2.5
			3.0	2.5

RNA binding motif protein 15	2.6		2.3	1.6
			2.0	1.5
ataxin 1	2.6		2.0	2.4
			2.4	2.0
			10.0	1.5
			5.2	1.2
proteasome (prosome, macropain) 26S subunit, ATPase, 2	2.5		0.2	0.1
			0.1	0.2
kelch-like 14 (Drosophila)	2.5		0.1	0.3
trafficking protein particle complex 3	2.5		0.4	0.5
enhancer of mRNA decapping 4	0.5		0.4	0.6
	0.4			
	0.3			
replication factor C (activator 1) 3, 38kDa	0.5		0.4	0.5
			0.3	0.5
SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5	0.5		0.4	0.5
cullin-associated and neddylation-dissociated 1	0.5		7.6	3.2
TAF15 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 68kDa	0.5		3.1	2.1
interleukin enhancer binding factor 3, 90kDa	0.5		0.3	0.5
	0.1			
heterogeneous nuclear ribonucleoprotein U (scaffold attachment factor A)	0.5		0.3	0.3
			0.2	0.3
			0.3	0.3
kalirin, RhoGEF kinase	0.5		2.9	3.0
			3.0	2.9
			0.1	0.2
KH domain containing, RNA binding, signal transduction associated 1	0.5		3.9	1.9
EH domain binding protein 1	0.5		3.7	2.4
			2.4	1.0
U2 small nuclear RNA auxiliary factor 1	0.4		1.9	3.9
			4.4	2.1
			3.9	1.9
U2 small nuclear RNA auxiliary factor 1-like 4	0.4		1.9	1.5
Janus kinase 1	0.4		7.4	2.3

structural maintenance of chromosomes 4	0.4 0.1		0.4	0.6
ribonucleotide reductase M1	0.4		0.4	0.3
			0.4	0.4
			0.3	0.4
			0.4	0.4
coatomer protein complex, subunit gamma 2	0.4		2	2.9
family with sequence similarity 118, member A	0.4		0.2	0.5
(GalNAc-T13); UDP-N-acetyl-alpha-D-	0.3		0.4	0.5
			0.1	0.3
galactosamine:polypeptide N-acetylgalactosaminyltransferase 1 (GalNAc-T1)			0.4	0.6
A kinase (PRKA) anchor protein 8-like	0.3		2.3	1.5
intraflagellar transport 140 homolog (Chlamydomonas)	0.3		2.2	1.6
SET binding factor 2	0.2		2.1	1.7
cytoplasmic FMR1 interacting protein 1	0.2		0.5	0.6
interleukin 1, alpha	0.2		3.2	1.3
retinoblastoma binding protein 4	0.1		0.4	0.6
			0.4	0.6
			0.1	0.2
			0.2	0.2
retinoblastoma binding protein 7	0.1		0.5	0.6
polymerase (DNA directed), epsilon	0.1		0.4	0.5
Baculoviral IAP repeat-containing 4	3.1		15.1	5.4
Zinc finger DAZ interacting protein 3	0.75		0.1	0.3
			0.2	0.3
inositol 1,4,5-triphosphate receptor, type 1	1.0		1.8	1.6
			2.1	2.1
			2.1	2.1
cytochrome P450, family 51, subfamily A, polypeptide 1	1.4 0.9 0.8	1.2		
lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)	0.9	1.5		

ATP citrate lyase	3.8 1.2 1.1 0.2	1.2	
stearoyl-CoA desaturase (delta-9-desaturase)	1.1 1.0 0.8	1.4	
cold inducible RNA binding protein	2.7 1.2	1.3	
DnaJ (Hsp40) homolog, subfamily A, member 1	1.6 1.1	0.7	
excision repair cross-complementing rodent repair deficiency, complementation group 6-like	1.1	1.2	
guanine nucleotide binding protein (G protein), beta polypeptide 1	1.4	1.3	
heat shock 70kDa protein 1A	1.22	0.3	
heat shock 70kDa protein 8	1	0.7	
heat shock 105kDa/110kDa protein 1	1.6 0.9	0.7	
polymerase (DNA directed) kappa	3.9	0.6	
ubiquitin specific peptidase 1	0.6	1.2	

[a] Taken from reference [42]. Gene expression ratio of PCSK9-D374Y over-expressing cells as compared to wild type cells.

[b] Taken from reference [80]. Gene expression ratio of wt-PCSK9 treated cells to those with no treatment at time point 48hr.

[c] Taken from reference [80]. Gene expression ratio of PCSK9-D374Y treated cells to those with no treatment at time point 48hr.

with congruent up- and down-regulations as compared to the first gene expression study that over-expressed PCSK9-D374Y, include upregulated proteins RECQ protein-like (DNA helicase like), ATP citrate-lyase, cold inducible RNA binding protein, and guanine nucleotide binding protein (G protein), and downregulated proteins signal transducer and activator of transcription I and heat shock 105/110 kDa protein I. Furthermore, the original PCSK9-D374Y gene expression study also showed that transferrin receptor gene levels were left unchanged, as was observed here in this study.

Comparison between the proteomic study presented here and the previous mRNA gene expression studies published previously suggest that there are similarities between the studies, both in the functional groups of genes/proteins affected by PCSK9 as well as the up- and down-regulation of specific gene/proteins recorded. Moreover, each subsequent study performed was able to increase and expand upon the genes/proteins affected and regulated by PCSK9, with the most expansive list presented here. It is worth noting that the gene expression studies and proteomic quantitative analysis performed here did not always produce similar trends and results. This could be attributed to differences often observed between protein levels and gene expression levels as well as small differences in experimental choices made between the three studies.

A novel unidentified role for PCSK9 in cytoskeleton organization and organelle transport

To date PCSK9 has shown no evidence of playing a role in affecting cytoskeleton organization, and involved in organelle transport solely as a secreted protein that itself is

transported throughout the secretory pathway, and internalized and trafficked from endosomes to lysosomes. The relative quantitative data obtained through SILAC experiments here show that many proteins of this nature are affected by the expression of PCSK9 in HuH7 cells. In PCSK9 expressing cells, actin related protein 2\3 complex subunit 1b (Arp2\3) was shown have increased protein levels between 9.4 to 3.1 fold, in various fractions, as compared to control cells. The increased levels of Arp2\3 was verified in Western blot analysis (Figure 9). Arp2\3 promotes the polymerization of actin tubules for vesicle transport and trafficking, and interestingly has been shown to regulate the fate of epidermal growth factor receptor (EGFR) containing early endosomes. Under certain conditions, Arp2\3 will enclose EGFR containing early endosomes with F-actin, preventing the proper trafficking and recycling of these vesicles and thereby promotes their degradation [81]. In addition, Arp2\3 requires Annexin A2, a known interaction partner of PCSK9, in order to promote actin polymerization for the transport of early endosomes towards late/mature endosomes [82]. Whether or not the increased levels of Arp2\3 plays a role in LDLR degradation or not is yet to be tested. This finding remains quite interesting as both of the above functions of Arp2\3 mirror what is happening in the PCSK9 mediated degradation of the LDLR. In the presence of PCSK9, LDLR is internalized, its trafficking is altered, unable to be recycled back to the cell surface, and is transported from an early endosomes towards late endosomes and ultimately lysosomes.

Rho associated protein kinase 1 (ROCK1) is another major protein affecting cytoskeletal organization in the cell that is affected by PCSK9 expression. In PCSK9-ACE2-V5 expressing cells ROCK1 is found have decreased protein levels by 3.3 fold. This decrease is

biologically interesting as it has been shown that in LDLR knockout mice, those that have a ROCK1 deficiency in bone-marrow derived cells are protected against atherosclerosis [83]. In addition to the results obtained from the SILAC relative quantitation, IP-LC-MS/MS results obtained interaction partner results for both Filamin-B, a protein involved in actin cytoskeleton organization, and linking actin networks to membranes and cell membrane receptors [84] and vimentin, an intermediate filament that interacts with many cell surface proteins, and also plays a role in transport and processing of LDL derived cholesterol [85,86]. However the meaning of these protein-protein interactions will require future studies in order to determine both the relationship of these proteins with PCSK9, and the biological significance of the interactions.

PCSK9 expression appears to have a diverse impact on organelle transport proteins. Proteins responsible for regulating the transport, trafficking and recycling of internalized cell surface receptors. In this study, Rab2a (0.3), receptor mediated endocytosis 8 (RME-8, 0.3), and EH-domain binding protein 1 (EHBP1, 0.4) are all decreased when PCSK9 is overexpressed. Rab2 affects membrane internalization, post-endocytic trafficking in *C. Elegans*, and regulates transport of membrane receptors and secretory proteins [87-89]. RME-8, as its name implies, plays a role in receptor mediated endocytosis, and also regulates internalized cell surface receptor trafficking. RME-8 is essential for EGF receptor recycling, and when downregulated, internalized EGFR is degraded even in the presence of Erb2 overexpression, a protein that prevents EGFR degradation. Very little is known about RME-8, however it appears it may have a role in PCSK9 mediated LDLR degradation, as it has been shown previously in Hela cells that RME-8 downregulation using siRNA leads to a

decrease in LDLR recycling and increase in LDLR degradation [90-91]. The direct regulation of internalized LDLR containing vesicles by RME-8 provides a potentially interesting link between PCSK9 and its regulation of LDLR. Similarly, EHBP1 is also a protein involved in regulating endocytic vesicles. EHBP1 interacts with EH-domain containing proteins, which are known for functioning as endocytic machinery proteins, binding cell surface receptor proteins, regulates their recycling back to the cell surface, and couples endocytosis to actin cytoskeleton organization [92,93]. Decreased protein levels of EHBP1 was validated from both subcellular fractions and whole cell lysates of PCSK9-ACE2-V5 expressing HuH7 cells by Western blot (Figure 10). In addition, this study showed that the natural gain of function variant of PCSK9, D374Y, also promotes decreased protein levels of EHBP1 (Figure 11). Furthermore, upon performing IP-LC-MS/MS interaction experiments, EH domain containing protein 4 (EHD4) was shown to interact with PCSK9. These interaction results were further verified by various Co-IP experiments (Figure 15) which showed that EHD4 was able to interact with different epitope tagged PCSK9 variants, and in different cell systems. Additionally, this study showed that EHD4 also interacts with the mature glycosylated LDLR, possibly in complex with PCSK9 (Figure 16). These findings were made even more attractive as it has been previously shown that EHD4 regulates LDL receptor trafficking, recycling and transport, as seen by the delayed recycling of LDLR endocytic vesicles, accumulation of LDL-C in early endosomes and increased degradation of LDLR in EHD4 siRNA treated cells [94].

These results suggest PCSK9 plays a biological role in regulating cytoskeletal organization and organelle transport, both through the vast effect PCSK9 plays on this

group of proteins expression, as well as through the identification of novel protein-protein interaction partners, however no clear mechanisms are known. It is unclear for some of the proteins affected by PCSK9 whether they are related to LDLR degradation or not, however some clearly have distinct links to this PCSK9 mediated function. The other novel cytoskeletal and organelle trafficking related functions of PCSK9 will require future studies.

A potentially novel unidentified role for PCSK9 in chaperone related functions

A recent study began to explore the possible role of PCSK9 in the ER, as it may function as a chaperone of acetylated intermediates throughout the chaperone mediated processing of various proteins in the ER, acting as a step of quality control for such proteins as BACE1 [64]. Since the release of this study, the field on PCSK9 as it relates to chaperone activity in the cell has been quiet and no further progress has been made. In this study, it appears a set of proteins relating to chaperone activity, as well as chaperone mediated autophagy, have had their protein levels affected by PCSK9, and been found to interact with PCSK9 through IP-LC-MS/MS experiments. Calreticulin (PCSK9-ACE2-V5 expressing cells levels increased by 1.6 fold) and Erp72/Protein disulfide isomerise A4 (increased levels in PCSK9-ACE2-V5 expressing cells ranging from 3.1 to 3.2 fold) both act as chaperone proteins together in complex in the ER in order to promote the proper folding of and processing of proteins such as apolipoprotein B, the major constituent protein of LDL [95]. In addition to ER chaperone activity, there is some evidence that suggests that PCSK9 may play a role in chaperone mediated autophagy (CMA), a process in which Hsc70 and Hsp90

act as molecular chaperones, which bind and transport proteins containing the KFERQ-like degradation motif (K/R, F/I/L/V, D/E,K/R, Q) necessary for transport into the autophagosome, often via a gated membrane protein such as the lysosomal antigen membrane protein 2 (LAMP2) [96,97]. Interestingly, both PCSK9 ($RFHRQ_{219}$) and a known binding partner Annexin A2 contain KFERQ-like motifs. As well, the IP-LC-MS/MS analysis repeatedly found Hsp90 to be a strong (via Mascot score) interaction partner of PCSK9. From the SILAC relative quantitation experiments it was found that Rab33a protein, a protein involved in the formation of autophagosomes, was decreased by 0.3 fold in PCSK9-ACE2-V5 expressing cells. Although no further studies were performed in order to find the meaning of these interactions and links of PCSK9 to chaperone/CMA activity, these results suggest a potential functional role of PCSK9 in chaperone and CMA activity and provide a basis for future studies on this potential novel role of PCSK9.

A potential link between PCSK9 and Amyloid precursor protein processing

Amyloid precursor protein (APP) is a single pass membrane protein that is known best for its ability to be sequentially cleaved by β-secretase (BACE) followed by γ-secretase to produce the neurotoxic Amyloid β (Aβ) peptide which forms aggregate plaques and leads to Alzheimer's progression [98]. The expression of PCSK9 suggests it may have an effect on APP regulation as PCSK9 affects the protein levels of many proteins that in turn affect APP. These proteins include reticulon-4 (6.1 fold in increase in protein levels in PCSK9-ACE2-V5 expressing cells) which is able to inhibit the production of Aβ peptide via interacting with

BACE [99], Rab1b, (3.8 fold increase in PCSK9-ACE2-V5 expressing cells) which has been shown to play a role in processing and trafficking beta amyloid precursor protein from the ER to the Golgi [100], cyclophilin A (increased between 3.2 and 5.1 fold in PCSK9-ACE2-V5 expressing cells) which protects PC12 cells against the cytotoxic effects of Aβ(25-35) peptide treatment by reducing the anti-apoptotic effects of the peptide [101], and transmembrane protein 21 (tmp21) which has been shown to form a complex which modulates gamma secretase activity, and affects the level of Aβ production [102,103], among other proteins.

Other evidence exists to support a possible link between PCSK9 and APP processing, or even Alzheimer's disease, as it is previously been reported that PCSK9 acts as a chaperone of BACE1 in the ER, and depending on its acetylation state, can promote the degradation of this APP processing enzyme [64]. Also of importance is the finding that the LDLR is affected in Alzheimer's, with a decrease in expression levels and decrease in localization at the cell surface, and the finding that increased levels of LDLR can protect against Alzheimer's by promoting the clearance of Aβ [104,105]. Taken together, these findings could implicate PCSK9 in APP processing and possibly even Alzheimer's disease. However, although PCSK9 was first discovered as a neuronal expressed protein (NARC-1), the vast majority of the studies involving PCSK9 have been performed in liver tissue and cells. Future studies would need to be carried out in tissues/cell cultures that are more relevant to APP processing and Alzheimer's disease in order to gain insight into this potentially new role of PCSK9. It is quite possible that proteins that interact, or are affected

by PCSK9 in liver cells may not be expressed or are regulated differently than in neuronal cells and tissues.

4.1 Conclusions

The objective of this study was to determine if the expression of PCSK9 had multiple effects in the HuH7 liver cell line, with the intention of gaining insights into the functional roles PCSK9 plays in the cell, both known and unknown. This study was successful in determining that PCSK9 expression has a great and varied effect on the liver cell proteome, causing 293 proteins relative abundance to become significantly altered. These proteins have a wide range of cellular functions, implicating PCSK9 in all of these cellular functions, most of which were previously uncharacterized for PCSK9. In addition, many novel PCSK9 interaction partners have been discovered in this study. These protein-protein interaction partners also represent protein complexes that provide insight into novel cellular functions of PCSK9, as well as those that interactions that form throughout the process of regulating the LDLR. Although the large-scale experiments performed in this study utilized PCSK9-ACE2-V5 variant, there is confidence in the results as congruent validation experiments were performed with wild type PCSK9, the natural gain-of-function D374Y PCSK9 variant, as well as PCSK9 constructs with various epitope tags.

Proteins whose relative abundance levels that significantly change due to PCSK9 expression are potential candidates for future studies relating to novel functions that PCSK9 plays in the cell. As well, the identified protein interaction partners of PCSK9 can also

provide a starting point for future studies to help elucidate the mechanisms of PCSK9 functions, or how these interactions might affect PCSK9s cellular roles.

Future Directions

The approach used in this study was highly informative, and at the time of this writing, could be improved upon in various ways. I believe future studies aimed at looking into novel PCSK9 functions could employ a similar quantitative subcellular fractionation approach, with the use of liver cell lines (HuH7 or HepG2) that have low-to-no expression of PCSK9 or using cell lines with stable knock-down of PCSK9. Additionally, the study could be repeated with expression of wild type PCSK9, rather than a gain-of-function variant of PCSK9 (in terms of its ability to degrade the LDLR), in order to focus on novel functions of PCSK9 outside of LDLR degradation. In these studies where protein quantitation is the main focus, I would employ the use of a higher resolution mass spectrometer, to yield higher quality data and to lessen the burden of having to manually verify as much raw MS results.

To determine what the meaning of a specific PCSK9 mediated protein effect, individual proteins, or groups of proteins based on functional class, can be chosen for future studies to be over-expressed, or have their expression silenced/knocked down. Depending on the protein chosen and its known function, assays could be performed to see what affects this may have on the cell.

The results obtained from this study are interesting and new for the field of PCSK9. Finding a novel interaction partner of PCSK9 that is involved in endocytic processes, such as EHBP1 and EHD4, could have great importance for both the LDLR related and unrelated functions of PCSK9. Future studies could focus on the importance of the relationship between PCSK9, EHD4 and EHBP1 in order to understand the functional relationship these proteins have. To test if these proteins play a role in PCSK9 mediated degradation of the LDLR, over-expression and knock down studies of each of these proteins can be carried out, meanwhile monitoring the ability of PCSK9 to internalize and promote the degradation of LDLR. If internalization of LDLR and/or LDLR levels are affected in these studies, depending on the system used (over-expression/knock down), a model can be formulated for the roles these proteins play in PCSK9 mediated LDLR internalization and degradation. If the results did show that they are necessary for PCSK9 mediated LDLR internalization and degradation, it would be worthwhile to extend the protein interaction network of this complex by expressing epitope tagged copies of these proteins in a liver cell system and perform Immunoaffinity purifications with the aim of identifying their interaction partners by LC-MS/MS. Chemical cross-linking might also be utilized as these endocytic machinery proteins might form many interaction partners, however such interactions may be transient in nature and hard to capture without chemical cross-linking. The results of these experiments might provide information regarding the other proteins in complex with PCSK9 and LDLR, and/or other proteins that take part in this process, and could help to elucidate the mechanism by which PCSK9 promotes the degradation of the LDLR.

Furthermore, as the C-terminal EHD proteins 1,2,3 and 4 function by forming homo- and various heterodimers with each other [78] and all are known to interact with EHBP1, a decrease of EHBP1 may in turn affect the functionality of the other EHD proteins either directly or indirectly through EHBP1. If this is the case, evaluation of all the EHD (1-4) protein levels could be compared between wild type liver cells, and those over-expressing the D374Y gain of function variant of PCSK9, in order to observe if their levels are affected. Furthermore, the functionality of these EHD proteins should be observed in the presence and absence of PCSK9. This could be carried out by looking at which receptors each play a role in internalizing and endocytosis. For example, EHD1 is a known interaction partner of insulin-like growth factor 1 receptor (IGF-1R) and plays a role in down-regulating the IGF-1 signalling pathway [106]. Alterations in EHD1 protein levels can affect the down-stream signalling pathways of the IGF-1R [106] and thus, we could evaluate whether PCSK9 may also have an effect on this pathway. Another great area to explore is whether PCSK9 may affect EHD protein functions. This could be studied by characterizing the effect PCSK9 levels (overexpression vs knock out) has on glucose transporter protein 4 (Glut4) levels and function. Studies show that EHD proteins interact with Glut4 and that siRNA mediated depletion of EHD1 leads to a dispersion of Glut4 containing vesicles throughout the cytoplasm reduction in Glut4 trafficking to the cell surface which is necessary for glucose transport following insulin response [107]. Also of interest is the finding in the same study that siRNA depletion of EHBP1 leads to a complete inhibition of Glut4 in the presence of insulin. As this work has shown that EHBP1 protein levels are decreased in the presence of PCSK9, this data would suggest that PCSK9 most likely affects Glut4 activity, and possibly

glucose homeostasis as previously suggested [108]. With this potential links between PCSK9, EHBP1, EHD proteins and Glut4 function, various assays could be carried out tolook at the localization and ability to internalize glucose in response to insulin in cell cultures expressing PCSK9.These studies could also be reproduced in animal models for PCSK9 as our collaborators have access to both transgenic mice expressing human PCSK9, and PC9 $^{-/-}$ KO mice.

In addition, of interest to me would be the study of the protein RME-8 with regards to PCSK9 mediated degradation of the LDLR. As discussed previously, it plays a direct role in regulating and degrading internalized LDLR, and therefore future studies, such as the ones described above for EHD4 would be beneficial to increasing our understanding of how RME-8, PCSK9 and the LDLR relate to one another.

References

1. Fuller, R.S., Brake, A.J., Thorner, J. (1989) Intracellular targeting and structural conservation of a prohormone-processing endoprotease. Science 246,482-486
2. Julius, D., Brake, A., Blair, L, Kunisawa, R., Thorner, J. (1984) Isolation of the putative structural gene for the lysine-arginine-cleaving endopeptidase required for processing of yeast prepro-a-factor. Cell 37,1075-1089
3. Smeekens, S.P., Steiner, D.F. (1990)Identification of a human insulinoma cDNA encoding a novel mammalian protein structurally related to the yeast dibasic processing protease Kex2. J Biol Chem 265,2997-3000
4. Seidah N.G., Gaspar, L., Mion, P., Marcinkiewicz, M., Mbikay, M., Chretien, M. (1990) cDNA sequence of two distinct pituitary proteins homologous to Kex2 and furin gene products, tissue-specific mRNAs encoding candidates for pro-hormone processing proteinases. DNA Cell Biol 9,415-424
5. Smeekens, S.P., Avruch, A.S., LaMendola, J., Chan, S.J, Steiner, D.F. (1991) Identification of a cDNA encoding a second putative prohormone convertase related to PC2 in AtT20 cells and islets of Langerhans. Proc Natl Acad Sci USA 88,340-344
6. Kiefer, M.C., Tucker, J.E., Joh, R., Landsberg, K.E., Saltman, D., Barr, P.J. (1991) Identification of a second human subtilisin-like protease gene in the fes fps region of chromosome 15. DNA Cell Biol 10,757-769
7. Lusson, J., Vieau, D., Hamelin, J., Day, R., Chretien, M., Seidah, N.G. (1993) cDNA structure of the mouse and rat subtilisin kexin-like PC5 – a candidate proprotein convertase expressed in endocrine and nonendocrine cells. Proc. Natl Acad Sci USA 90,6691-6695
8. Nakagawa, T., Hosake, M., Torii, S., Watanabe, T., Murakami, K., Nakayama, K. (1993) Identification and functional expression of a new member of the mammalian Kex2-like processing endoprotease family, its striking structural similarity to PACE4. J Biochem 113,132-135
9. Bruzzaniti, A., Goodge, K., Jay, P., Taviaux S.A., Lam, M.H.C., Berta, P., Martin, T.J., Moseley, J.M., Gillespie, M.T. (1996) C8, a new member of the convertase family. Biochem J 314,727-731
10. Nakayama, K., Kim, W.S., Torii, S., Hosaka, M., Nakagawa, T., Ikemizu, J., Baba, T., Murakami, K. (1992) Identification of the fourth member of the mammalian endoprotease family homologous to the yeast Kex2 protease. J Biol Chem 267,5897-5900
11. Seidah, N.G., Hamelin, J., Mamarbachi, M., Dong, W., Tadros, H., Mbikay, M., Chretian, M., Day, R. (1996) cDNA structure, tissue distribution, and chromosomal

12. localisation of rat PC7, a novel mammalian proprotein convertase closest to yeast kexin-like proteinases. Proc Natl Acad Sci USA 93,3388-3393
13. Seidah, N.G, Day, R., Marcinkiewicz, M., Chertien, M (198) Precursor convertases, an evolutionary ancient, cell-specific, combinatorial mechanism yielding diverse bioactive peptides and proteins. Ann. N.Y. Acad. Sci. 839,9-24
14. Vey, M., Schafer, W., Berghofer, S., Klenk, H-D., Garten, W. (1994) Maturation of the trans-Golgi Network protease furin, Compartmentalization of propeptide removal, substrate cleavage, and COOH-terminal truncation. J Cell Biol 127,1829-1842
15. Leduc, R., Molloy, S.S., Thorne, B.A., Thomas, G. (1992) Activation of human furin precursor processing endoprotease occurs by an intramolecular autoproteolytic cleavage. J Biol Chem 267,14304-14308
16. Anderson, E.D., VanSlyke, J.K., Thulin, C.D., Jean, F., Thomas, G. (1997) Activation of the furin endoprotease is a multi-step process, requirements for acidification and internal propeptide cleavage. EMBO J 16,1508-1518
17. Goodman, L.J., Gorman, C.M. (1994) Autoproteolytic activation of the mouse prohormone convertase mPC1. Biochem Biophys Res Commun 201,795-804
18. Matthews, G., Shenna, K.I., Seal, A.J., Taylor, N.A., Colman, A., Docherty, K. (1994) Autocatalytic maturation of the prohormone convertase PC2. J Biol Chem 269,588-592
19. Shennan, K.I.J., Taylor, N.A., Jermany, J.L., Matthews, G., Docherty, K. (1995) Differences in pH optima and calcium requirements for maturation of the prohormone convertases PC2 and PC3 indicates different intracellular locations for these events. J Biol Chem 270,1402-1497
20. De Bie I., Marcinkiewicz, M., Malide, D., Lazure, C., Nakayama, K., Bendayan, M., Seidah, N.G. (1996) The isoforms of proprotein convertase PC5 are sorted to different subcellular compartments. J Cell Biol 135,1261-1275
21. Nour, N., Basak, A., Chretien, M., Seidah, N.G. (2003) Structure-function analysis of the prosegment of the proprotein convertase PC5A. J Biol Chem 278,2886-2895
22. Siezen, R.J., Leunissen, J.A.M. (1997) Subtilases: the superfamily of subtilins-like serine proteases. Protein Sci. 6,501-523.
23. Barr, P.J., Mason, O.B., Landsberg, K.E., Wong, P.A., Kiefer, M.C., Brake, A.J. (1991) cDNA and gene structure for a human subtilisin-like protease with cleavage specificity for paired basic-amino-acid residues. DNA Cell Biol 10,319-3285
24. Gensberg, K., Jan, S., Matthews, G.M. (1998) Subtilisin-related serine proteases in the mammalian constitutive secretory pathway. Semin Cell Dev Biol. 9, 11-17
25. Seidah, N.G., Benjannet, S., Wickham, L., Marcinkiewicz, J., Jasmin, S.B., Stifani, S., Basak, A., Prat, A., Chretien, M. (2003) The secretory proprotein convertase neural

26. apoptosis-regulated convertase 1 (NARC-1), Liver regeneration and neuronal differentiation. Proc Natl Acad Sci USA.100,928-833
27. Abifadel, M., Varret, M., Rabes, J.P., Allard, D., Ouguerram, K., Devillers, M., Cruaud, C., Benjannet, S., Wickham, L., Erlich, D., *et al*. Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. (2003) Nat Gen 34,154-156
28. Park, S.W., Moon, Y-A., Horton, J.D. (2004) J Biol Chem 279,50630-50638
29. Rashid, S., Curtis, D.E., Garuti, R., Anderson, N.N., Bashmakov, Y., Ho, Y.K., Hammer, R.E., Moon, Y.A., Horton, J.D. (2005) Decreased plasma cholesterol and hypersensitivity to statins in mice lacking Pcsk9. Proc Natl Acad Sci USA. 102,5374-5379
30. Alborn, W.E., Cao, G., Careskey, H.E., Qian, Y.W., Subramaniam, D.R., Davies, J., Conner, E.M., Konrad, R.J. (2007) Serum proprotein convertase subtilisin kexin type 9 is correlated directly with serum LDL cholesterol. Clinical Chemistry 53,1814-1819
31. Cunningham, D., Danley, D.E., Geoghegan, K.F., Griffor, M.C., Hawkins, J.L., Subashi, T.A., Varghese, A.H., Ammirati, M.J, Culp, J.S., Hoth, L.R., *et al.* (2007) Structural and biophysical studies of PCSK9 and its mutants linked to familial hypercholesterolemia. Nat Structure and Mol Biol 14,413-419
32. Piper, D.E., Jackson, S., Liu, Q., Romanow, W.G., Shetterly, S., Thibault, S.T., Shan, B., Walker, N.P.C., *et al.* (2007) The crystal structure of PCSK9, A regulator of Plasma LDL-cholesterol. Structure 15,545-552
33. McNutt, M.C., Lagace, T.A., Horton, J.D. (2007) Catalytic activity is not required for secreted PCSK9 to reduce low density lipoprotein receptors in HepG2 cells. J Biol Chem 282,20799-20803
34. Li, J., Tumanut, C., Gavigan, J.A., Huang, W.J., Hampton, E.R., Tumanut, R., Suen, K.F., Trauger, J.W., Spraggon, G., Lesley, S.A., *et al.* (2007) Secreted PCSK9 promotes LDL receptor degradation independently of proteolytic activity. Biochem J 406,203-207
35. Benjannet, S., Rhainds, D., Hamelin, J., Nasoury, N., Seidah, N.G. (2006) The proprotein convertase (PC) PCSK9 is inactivated by furin and/or PC5/6A. J Biol Chem 281,30561-30572
36. Benjannet, S., Rhainds, D., Essalmani, R., Mayne, J., Wickham, L., Jun, W., Asselin, M.C., Hamelin, J., Varret, M., Allard, D., et al. (2004) NARC-1/PCSK9 and its natural mutants, zymogen cleavage and effects on the low density lipoprotein (LDL) receptor and LDL cholesterol. J Biol Chem 279,48865-48875
37. Dewpura, T., Raymon, A., Hamelin, J., Seidah, N.G., Mbikay, M., Chretien, M., Mayne, J. (2008) PCSK9 is phosphorylated by a golgi casein kinase-like kinase ex vivo and circulates as a phosphoprotein in humans. FEBS Journal 275,3480-3493

38. Nassory, N. (2007) The cellular trafficking of the secretory proprotein convertase PCSK9 and its dependence on the LDLR. Traffic 8,718-732
39. Jeong, H.J., Lee, H.S., Kim, K.S., Kim, Y.K., Yoon, D., Park, S.W. (2008) Sterol-dependent regulation of proprotein convertase subtilisin/kexin type 9 expression by sterol-regulatory element binding protein-2. J Lipid Res. 49,399-409
40. Dong, B., Wu, M., Li, H., Kraemer, F.B., Adeli, K., Seidah, N.G., Park, S.W., Liu, J. (2010) Strong induction of PCSK9 gene expression through HNF1α and SREBP2, mechanism for the resistance to LDL-cholesterol lowering effect of statins in dyslipidemic hamsters. J Lip Res. 51,1486-1495
41. Feingold, K.R., Moster, A.H., Shigenaga, J.K., Patzek, S.M., Grunfeld, C. (2008) Inflammation stimulates the expression of PCSK9. Biochem Biophys Res Commun 374,341-344
42. Costet, P., Carlou, B., Lambert, G., Lalanne, F., Lardeux, B., Jarnoux, A.L., Grefhorst, A., Staels, B., Krempf, M. (2006) Hepatic PCSK9 expression is regulated by nutritional status via insulin and sterol regulatory element-binding protein 1c. J Biol Chem 281,6211-6218
43. Kourimate, S., May, C.L., Langhi, C., Jarnoux, A.L., Ouguerram, K., Zair, Y., Nguyen, P., Krempf, M., Cariou, B., Costet, P. (2008) Dual mechanisms for the fibrate0mediated repression of proprotein convertase subtilisin/kexin type 9. J Biol Chem 283,9666-9673
44. Ranheim, T., Mattingsdal, M., Lindvall, J.M., Holla, O.L., Berge, K.E., Kulseth, M.A., Leren, T.P. (2008) Genome-wide expression analysis of cells expressing gain of function mutant D374Y-PCSK9. J Cell Physiol 217,459-467
45. Lan, H., Pang, L., Smith, M.M., Levitan, D., Ding, W., Liu, L., Shan, L., Shah, V.V., Laverty, M., Arreaza, G., *et al.* (2010) Proprotein convertase subtilisin/kexin type 9 (PCSK9) affects gene expression pathways beyong cholesterol metabolism in liver cells. J Cell Phsyiol 224,273-281
46. Schmidt, R.J., Beyer, T.P., Bensch, W.R., Qian, Y.W., Lin, A., Kowala, M., Alborn, W.E., Konrad, R.J., Cao, G. (2008) Secreted proprotein convertase subtilisin/kexin type 9 reduces both hepatic and extrahepatic low-density lipoprotein receptors in vivo. Biocheml Biophyl Res Commun. 370,634-640
47. Graham, M.J., Lemonidis, K.M., Whippie, C.P., Subramaniam, A., Monia, B.P., Crooke, S.T., Crooke, R.M. (2007) Antisense inhibition of proprotein convertase subtilisin/kexin type 9 reduces serum LDL in hyperlipidemic mice. J Lipid Res 48,763-767
48. Zaid, A., Roubtsova, A., Essalmani, R., Marcinkiewicz, J., Chamberland, A., Hamelin, J., Tremblay, M., Jacques, H., Jin, W., Davignon, J., Seidah, N.G., Prat, A. (2008) Proprotein convertase subtilisin/kexin type 9 (PCSK9), Hepatocyte-specific low-

49. density lipoprotein receptor degradation and critical role in mouse liver regeneration. Hepatology 48,646-654
50. Maxwell, K.N., Fisher, E.A., Breslow, J.L. (2005) Overexpression of PCSK9 acelerates the degradation of the LDLR in a post-endoplasmic reticulum compartment. Proc Natl Acad Sci USA. 102,2069-2074
51. Lagace, T.A., Curtis, D.E., Garuti, R., McNutt, M.C., Park, S.W., Prather, H.B., Anderson, N.N., Ho, Y.K., Hammer, R.E., Horton, J.D. (2006) Secreted PCSK9 decreases the number of LDL receptors in hepatocytes and in livers of parabiotic mice. J Clin Invest. 116,2995-3005
52. Qian, Y.W., Schmidt, R.J., Zhang, Y., Chu, S., Lin, A., Wang, H., Wang, X., Beyer, T.P., Bensch, W.R., Li, Q., *et al.* (2007) Secreted PCSK9 downregulates low density lipoprotein receptor through receptor-mediated endocytosis. J Lipid Res 48,1488-1498
53. Poirier, S., Mayer, G., Poupon, V., McPherson, P.S., McPherson, P.S., Desjardins, R., Ly, K., Asselin, M.C., Day, R., Duclos, F.J., Witmer, M., Parker, R., Prat, A., Seidah, N.G. (2009) Dissection of the endogenous cellular pathways of PCSK9-induced low density lipoprotein receptor degradation. J Biol Chem 284,28856-28864
54. Holla, O.L., Cameron, J., Berge, K.E., Ranheim, T., L., T.P. (2007) Degradation of the LDL receptors by PCSK9 is not mediated by a secreted protein acted upon by PCSK9 extracellularly. BMC Cell Biol. 8,1-12
55. Zhang, D.W., Lagace, T.A., Garuti, R., Zhao, Z., McDonald, M., Horton, J.D., Cohen, J.C., Hobbs, H.H. (2007) Binding of proprotein convertase subtilisin/kexin type 9 to epidermal growth factor-like repeat A of low density lipoprotein receptor decreases receprot recycling an d increases degradation. J Biol Chem 282,18602-18612
56. Fisher, T.S., Surdo, P.L., Pandit, S., Mattu, M., Santoro, J.C., Wisniewski, D., Cummings, R.T., Calzetta, A., Cubbon, R.M., Fischer, P.A., *et al.* (2007) Effects of pH and low density lipoprotein (LDL) on PCSK9-dependent LDL receptor regulation. J Biol Chem 282,20502-20512

53b. Holla, O.L., Strom, T.B., Cameron, J., Berge, K.E., Leren, T.P. (2010) A chimeric LDL receptor containing the cytoplasmic domain of the transferrin receptor is degraded by PCSK9. Mol Genet Metab. 99,149-156.

57. Zhang, D.W., Lagace, T.A., Garuti, R., Zhao, Z., McDonald, M., Horton, J.D., Cohen, J.C., Hobbs, H.H. (2007) Binding of proprotein convertase subtilisin/kexin type 9 to epidermal growth factor-like repeat A of low density lipoprotein receptor decreases receptor recycling an d increases degradation. J Biol Chem 282,18602-18612
58. Kwon, H.J., Lagace, T.A., McNutt, M.C., Horton, J.D. (2008) Molecular basis for LDL receptor recognition by PCSK9. Proc Natl Acad Sci USA. 105,1820-1825

59. Gefhorst, A., McNutt, M.C., Lagace, T.A., Horton, J.D. (2008) Plasma PCSK9 preferentially reduces liver LDL receptors in mice. J Lipid Res 49,1303-1311
60. Timms, K.M., Wagner, S., Samuels, M.E., Forbey, K., Goldfine, H., Jammulapati, S., Skolnick, M.H., Hopkins, P.N., Hunt, S.C., Shattuck, D.M. (2004) A mutation in PCSK9 causing autosomal-dominant hypercholesterolemia in a Utah pedigree. Hum Genet 114,349-353
61. (Leren, T.P. (2004) Mutations in the PCSK9 gene in Norwegian subjects with autosomal dominant hypercholesterolemia. Clin Genet 65,419-422
62. Cameron, J., Holla, O.L., Ranheim, T., Kulseth, M.A., Berge, K.E., Leren, T.P. (2006) Effect of mutations in the PCSK9 gene on the cell surface LDL receptors. Hum Mol Genet 15,1551-1558
63. Cameron., J., Holla, O.L., Laerdahi, J.K., Kulseth, M.A., Ranheim, T., Rognes, T., Berge, K.E., Leren, T.P. (2008) Characterization of novel mutations in the catalytic domain of the PCSK9 gene. Journal of Internal Medicine. 263,420-431
64. Hallman, M., Srinivasan, S.R., Chen, W., Boerwinkle, E., Berenson, G.S. (2007) Relation of PCSK9 mutations to serum low-density lipoprotein cholesterol in childhood and adulthood (from the Bogalusa heart study). Am J Cardiol. 100,69-72
65. Shan, L., Pang, L., Zhang, R., Murgolo, N.J., Lan, H., Hedrick, J.A. (2008) PCSK9 binds to multiple receptors and can be functionally inhibited by an EGF-A peptide. Biochemical and Biophys Res Commun. 375,69-73
66. Poirier, S., Mayer, G., Benjannet, S., Bergeron, E., Marcinkiewicz, J., Nassoury, N., Mayer, H., Nimpf, J., Prat, A., Seidah, N.G. (2008) The proprotein convertase PCSK9 induces the degradation of low density lipoprotein receptor (LDLR) and its closest family members VLDLR and ApoER2. J Biol Chem 283,2363-2372
67. Jonas, M.C., Costantini, C., Puglielli, L. (2008) PCSK9 is required for the disposal of non-acetylated intermediates of the nascent membrane protein BACE1. EMBO reports 9,916-922
68. Labonte, P., Begley, S., Guevin, C., Asselin, M.C., Nassoury, N., Mayer, G., Prat, A., Seidah, N.G. (2009) PCSK9 impedes hepatitis c virus infection in vitro and modulates liver CD81 expression. Hepatology 50,17-24
69. Mayer, G., Poirier, S., Seidah, N.G. (2008) Annexin A2 is a C-terminal PCSK9-binding protein that regulates endogenous low density lipoprotein receptor levels. J Biol Chem 283,31791-31801
70. Duff, C.J., Scotty, M.J., Kirby, I.T., Hutchinson, S.E., Martin, S.L., Hooper, N.M. (2009) Antibody-mediated disruption of the interaction between PCSK9 and the low-density lipoprotein receptor. Biochem J. 419,577-584
71. Chan, J.C.Y., Piper, D.E., Cao, Q., Liu, D., King, C., Wang, W., Tang, J., Liu, Q., Higbee, J., Xia, Z., *et al.* (2009) A proprotein convertase subtilisin/kexin type 9 neutralizing

72. antibody reduces serum cholesterol in mice and nonhuman primates. ProcC Natl Acad Sci USA 106,9820-9825
73. Jeon, H., Meng, W., Takagi, J., Eck, M.J., Springer, T.A., Blacklow, S.C. (2001) Implications for familial hypercholerolemia from the structure of the LDL receptor YWTD-EGF domain pair. Nat Struct Biol 8,499-504
74. Uhlik, M.T., Temple, B., Bencharit, S., Kimple, A.J., Siderovski, D.P., Johnson, G.L. (2005) Structural and evolutionary division of phosphotyrosine binding (PTB) domains. J Mol Biol 345,1-20
75. He, G., Gupta, S., Yi, M., Michaely, P., Hobbs, H.H., Cohen, J.C. (2002) ARH is a modular adaptor protein that interacts with the LDL receptor, clathrin and AP-2. J Biol Chem 277,44044-44049
76. Mishra, S.J., Watkins, S.C., Traub, L.M. (2002) The autosomal recessive hypercholesterolemia (ARH) protein interfaces directly with the clathrin-coat machinery. Proc Natl Acad Sci USA. 99,16099-16104
77. Jeon, H., Meng, W., Takagi, J., Eck, M.J., Springer, T.A., Blacklow, S.C. (2001) Implications for familial hypercholerolemia from the structure of the LDL receptor YWTD-EGF domain pair. Nat Struct Biol 8,499-504
78. Gent, J., Braakman, I. (2004) Low-density lipoprotein receptor structure and folding. Cell Mol Life Sci. 61,2461-2470) (Rudenko, G., Henry, L., Henderson, J., Ichtchenko, K., Brown, M.S., Goldstein, J.L., Deisenhofer, J. (2002) Structure of the LDL receptor extracellular domain at endosomal pH. Science. 298,2353-2358
79. Ong, S-E., Blagoev, B., Kratchmarova, I., Kristensen, D.B., Steen, H., Pandey, A., Mann, M. (2002) Stable isotope labelling of amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol Cell Proteomics. 1,376-386
80. Tran, K., Thorne-Tjomsland, G., DeLong, C.J., Cui, Z., Shan, J., Burton, L., Jamieson, J.C., Yao, Z. (2002) Intracellular assembly of very low density lipoproteins containing apolipoprotein B100 in rat hepatoma McA-RH7777 cells. .J Biol Chem. 277, 31187-31200
81. Denis, N.J., Vasilescu, J., Lambert, J-P., Smith, J.C., Figeys, D. (2007)Tryptic digestion of ubiquitin standards reveals an improved strategy for identifying ubiquitinated proteins by mass spectrometry. *Proteomics.* 7, 868-874
82. George, M., Ying, G., Rainey, M.A., Solomon, A., Parikh, P.T., Gao, Q., Band, V., Band, H. (2007) Shared as well as distinct roles of EHD proteins revealed by biochemical and functional comparisons in mammalian cells and *C. Elegans.* BMC Cell Biol. 8,1-22

83. Goula D., Remy, J.S., Erbacher, P., Wasowicz, M., Levi G., Abdallah, B., Demeneix, B.A. (1998) Size, diffusibility and transfection performance of linear PEI/DNA complexes in the mouse central nervous system. Gene Ther 5,712-717
84. Lan, H., Pang, L., Smith, M.M., Levitan, D., Ding, W., Liu, L., Shan, L., Shah, V.V., Laverty, M., Arreaza, G. (2010) Proprotein convertase subtilisin/kexin type 9 (PCSK9) affects gene expression pathways beyond cholesterol metabolism in liver cells. J Cell Physiol. 224,273-281
85. Llado, A., Timpson, P., Vila de Muga, S., Moreto, J., Pol, A., Grewal, T., Daly, R.J., Enrich, C., Tebar, F. (2008) Protein kinase Cdelta and calmodulin regulate epidermal growth factor receptor recycling from early endosomes through Arp2\3 complex and cortactin. Mol Biol Cell. 19,17-29
86. Morel, E., Parton, R.G., Gruenberg, J. (2009) Annexin A2-dependent polymerization of actin mediates endosome biogensis. Dev Cell. 16,445-457
87. Wang, H.W., Liu, P.Y., Oyama, N., Rikitake, S., Gitlin, J., Liao, J.K., Boisvert, W.A. (2008) Deficiency of ROCK1 in bone marrow-derived cells protects against atherosclerosis in LDLR-/- mice. FASEB J. 22,3561-3570
88. van der Flier, A., Sonnenberg, A. (2001) Structural and functional aspects of filamins. Biochim Biophys Acta. 1538,99-117
89. Sarria, A.J., Panini, S.R., Evans, R.M. (1992) A functional role for vimentin intermediate filaments in the metabolism of lipoprotein-derived cholesterol in human SW-13 cells. J Biol Chem. 267,19455-19463
90. Bhattacharya, R., Gonzalez, A.M., Debiase, P.J., Trejo, H.E., Goldman, R.D., Flitney, F.W., Jones, J.C. (2009) Recruitment of vimentin to the cell surface by beta3 integrin and plectin mediates adhesion strength. J Cell Sci. 122,1390-1400
91. Dong, C., Wu, G. (2007) Regulation of anterograde transport of adrenergic and angiotensin II receptors by Rab2 and Rab6 GTPases. Cell Signal 19,2388-2399
92. Chun, D.K., McEwen, J.M., Burbea, M., Kaplan, J.M. (2008) UNC-108/Rab2 regulates postendocytic trafficking in Caenorhabditis elegans. Mol Biol Cell. 19,2682-2695
93. Tisdale, E.J., Azizi, F., Artalejo, C.R. (2009) Rab2 utilizes glyceraldehydes-3-phosphate dehydrogenase and protein kinase c{iota} to associate with microtubules and to recruit dynein. J Biol Chem. 284,5876-5884
94. Girard, M., McPherson, P.S. (2008) RME-8 regulates trafficking of the epidermal growth factor receptor. FEBS Lett. 582,961-966
95. Fujibayashi, A., Taguchi, T., Misaki, R., Ohtani, M., Dohmae, N., Takio, K., Yamada, M., Gu, J., Yamakami, M. (2008) Human RME-8 is involved in membrane trafficking through early endosomes. Cell Struct Funct. 33,35-50
96. Naslavsky, N., Caplan, S. (2005) C-terminal EH-domain-containing proteins, consensus for a role in endocytic trafficking, EH? J Cell Sci. 118,4093-4101

97. Guilherme, A., Soriano, N.A., Bose, S., Holik, J., Bose, A., Pomerleau, D.P., Furcinitti, P., Leszyk, J., Corvera, S., Czech, M.P. (2004) EHD2 and the novel EH domain binding protein EHBP1 couple endocytosis to the actin cytoskeleton. J Biol Chem. 279,10593-10605
98. Sharma, M., Naslavsky, N., Caplan, S. (2008) A role for EHD4 in the regulation of early endosomal transport. Traffic 9,995-1018
99. Linnik, K.,M., Herscovitz, H. (1998) Multiple molecular chaperones interact with apolipoprotein B during its maturation. The network of endoplasmic reticulum-resident chaperones (ERp72, GRP94, calreticulin, and BiP) interacts with apolipoprotein b regardless of its lipidation state. J Biol Chem. 273,21368-21373
100. Bejarano, E., Cuervo, A.M. (2010) Chaperone-mediated autophagy. Proc Am Thorac Soc. 7,29-39,
101. Bandyopadhyay, U., Cuervo, A.M. (2008) Entering a lysosome through a transient gate by chaperone-mediated autophagy. Autophagy. 16,1101-1103
102. Chow, V.W., Mattson, M.P., Wong, P.C., Gleichmann, M. (2010) An overview of APP processing enzymes and products. Neuromolecular Med. 12,1-12
103. Murayama, K.S., Kametani, F., Saito, S., Kume, H., Akiyama, H., Araki, W. (2006) Reticulons RTN3 and RTN4-B/C interact with BACE1 and inhibit its ability to produce amyloid beta-protein. Eur J Neurosci. 24,1237-1244
104. Dugan, J.M., deWit, C., McConlogue, L., Maltese, W.A. (1995) The Ras-related GTP-binding protein, Rab1B, regulates early steps in exocytic transport and processing of beta-amyloid precursor protein. J Biol Chem. 270,10982-10989
105. Ge, Y.S., Teng, W.Y., Zhang, C.D. (2009) Protective effect of Cyclophilin A against Alzheimer's amyloid beta-peptide (25-35)-induced oxidative stress in PC12 cells. Chin Med J. 122,716-724
106. Chen, F., Hasegawa, H., Schmitt-Ulms, G., Kawarai, T., Bohm, C., Katayama, T., Gu, Y., Sanjo, N., Glista, M., Rogaeva, E., *et al.* (2006) TMP21 is a presenilin complex component that modulates gamma-secretase but not epsilon-secretase activity. Nature. 440,1208-1212
107. Dolcini, V., Dunys, J., Sevalle, J., Chen, F., Guillot-Sestier, M.V., St George-Hyslop, P., Fraser, P.E., Checler, F. (2008) TMP21 regulates Abeta production but does not affect caspase-3, p53, and neprilysin. Biochem Biophys Res Commun. 371,69-74
108. Kim, J., Castellano, J.M., Jiang, H., Basak, J.M., Parsadanian, M., Pham, V., Mason, S.M., Paul, S.M., Holtzman, D.M. (2009) Overexpression of low-density lipoprotein receptor in the brain markedly inhibits amyloid deposition and increases extracellular A beta clearance. Neuron. 64,632-644

109. Abisambra, J.F., Fiorelli, T., Padmanabhan, J., Neame, P., Wefes, I., Potter, H. (2010) LDLR expression and localization are altered in mouse and human cell culture models of Alzheimer's disease. PLoS. 5,e8556
110. Rotem-Yehudar, R., Galperin, E., Horowitz, M. (2001) Association of insulin-like growth factor 1 receptor with EHD1 and SNAP29. J Biol Chem. 276, 33054-33060
111. Guilherme, A., Soriano, N.A., Furcinitti, P.S., Czech, M.P. (2004) Role of EHD1 and EHBP1 in perinuclear sorting and insulin-regulated GLUT4 recycling in 3T3-L1 adipocytes. J Biol Chem. 279, 40062-40076.
112. Mbikay, M., Sirois, F., Mayne, J., Wang, G.S., Chen, A., Dewpura, T., Prat, A., Seidah, N.G., Chretien, M., Scott, F.W. (2010) PCSK9-deficient mice exhibit impaired glucose tolerance and pancreatic islet abnormalities. FEBS. 584, 701-706
113. Pedrioli, P.G. (2010) Trans-proteomic pipeline:a pipeline for proteomic analysis. Methods Mol Biol. 604, 213-238.

Contributions of Collaborators

In this study, the work in its entirety was performed by myself, Nicholas J. Denis, with the exception of the following, Histodenz gradient fractionation was performed together with Dr. Theodore G. Wright for time constraint issues. Western blot analysis, as depicted in Figure 11, showing the down-regulation of LDLR and EHBP1 by both PCSK9-ACE2-V5 and PCSK9-D374Y was kindly performed by Dr. Janice Mayne. Stable and transient transfections involving HEK293 and HepG2 cell lines with either PCSK9-V5, PCSK9-Flag and/or EHD4-Myc were kindly performed by Dr. Theodore G. Wright.

Appendix A. Reagents, solutions, and buffers.

Chemicals and Reagents

- Acetonitrile (ACN) with 0.1% formic acid (J.T. Baker)
- Complete protease inhibitor cocktail (Sigma)
- NuPage 4-12% Bis-Tris precast gels (Invitrogen)
- SuperSignal chemiluminescent substrate (Pierce)
- custom made 15cm 4-12% Tris-glycine precast denaturing gels (Jule Inc.)
- monoclonal anti-FLAG M2 beads (Sigma)
- anti-V5 agarose affinity gel (Sigma)
- anti-C-Myc agarose affinity gel (Sigma)
- SILAC Media labelling kit (Invitrogen)

Enzymes

- Trypsin (Promega)

Antibodies

- Rabbit anti-calnexin antibody (Stressgen Bioreagents)
- rabbit anti-TGN46 antibody (Abcam)
- mouse anti-MAN2A antibody (Abnova)
- mouse monoclonal anti-V5 antibody (Abcam)
- polyclonal anti-PCSK9 was kindly donated from Dr. Nabil Seidah
- mouse anti-EEA1 antibody (Abcam)
- rabbit anti-Lamp1 antibody (Cell Signaling Technology)
- goat anti-Lamin A/C antibody (Cell Signalling Technology)
- mouse anti-Transferrin Receptor (Abcam)
- mouse anti-EHBP1 (American diagnostic inc.)
- anti-myc-HRP (Invitrogen)
- anti-flag-HRP (Sigma)
- LDLR (Fitzgerald)
- anti-rabbit, goat anti-mouse and donkey anti-goat horseradish peroxidise (HRP)-conjugated secondary antibodies (Cell Signalling).

Solutions

- 10x Phosphate buffered saline (PBS)
    - 80g NaCl
    - 2g KCl
    - 11.5g Na_2PO_4
    - KH_2PO_4
    - Up to 1L with distilled H_2O
- Membrane solubilisation buffer
    - 10mM Tris-HCl pH 7.4
    - 250mM sucrose
    - 5 µM ethylenediaminetetraacetic acid (EDTA)
    - complete protease inhibitor mixture
- Histodenz stock solution
    - 27.6% Histodenz
    - 10 mM Tris-HCl pH 7.4
    - 3 mM KCl
    - 1 mm EDTA
- Saline buffer
    - 0.75% NaCl
    - 10mM Tris-HCl pH 7.4
    - 3mM KCl
    - 1mM EDTA
- 5x Sample loading buffer
    - 10% SDS
    - 10mM beta-mercaptoethanol
    - 50% Glycerol
    - 250mM Tris-HCl pH 6.8
    - 0.1% Bromophenol blue
- 0.1% PBS-Tween
    - 1mL of Tween-20 in 1L of 1x PBS
- 20x MOPS buffer
    - 209.2 MOPS
    - 121.1 Tris Base
    - 20g SDS
    - 6g EDTA
    - Up to 1L with distilled water
- 20x Transer buffer

    - 81.6 Bicine
    - 104.8 Bis-Tris (free base)
    - 6g EDTA
    - Up to 1L with distilled water
- Membrane blocking solution
    - 5g skin milk powder in 100mL of 0.1% PBS-Tween
- Modified RIPA Buffer
    - 50mM Tris-HCl, pH 7.4
    - 1% NP-40
    - 0.25% Na-deoxycholate
    - 150mM NaCl
- Silver Staining
    - Fixing Solution
        - 50% Methanol
        - 5% Acetic acid
    - Solution A
        - 50% methanol
    - Solution B
        - 0.2g/L sodiumthiosulphate
    - Solution C
        - 0.2g/100mL Silver nitrate
    - Developing Solution
        - 5% sodium carbonate
    - Stopping Solution
        - 5% acetic acid

Nicholas Denis

Qualifications

- Strong analytical mind and abilities
- Driven by critical, analytical and systematic reasoning
- Experience with proteomics based technologies, including immunoaffinity purification, Western blotting, mass spectrometry, chromatography, HPLC and micro-fluidic devices
- Experience with standard molecular biology techniques, from PCR to cloning and cell culture
- Experience with Windows, Internet, Word Processing and Excel

Academic Training

2006-Present **M.Sc. Candidate, Biochemistry, University of Ottawa**

In Progress

2002-2006 **B.Sc., Honours in Biochemistry, University of Ottawa**
Diploma GPA 9.2/10

Honours Thesis, Differential analysis of protein ubiquitination using a novel gel-free approach

Supervisor, Dr. Daniel Figeys

Employment History

Winter 2007 **University of Ottawa Laboratory Course Teaching Assistant**

Experience in communicating and demonstrating various lab experiments, supervision over students and marking of their work

Summer 2006 **Laboratory Technician (Ottawa Institute of Systems Biolog**

Experience in applying proteomics based-studies towards mapping ubiquitinated proteins by mass spectrometry

Summer 2005 **FSWEP Summer student program (Agriculture Canada)**

Experience with plant molecular biology, tissue culture and molecular cloning

2001-2005 **Casual Custodian (Ottawa Carleton Catholic School Board)**
Responsible for managing multiple Ottawa high schools and the safety of those using the facilities during off-school hours

Scholarships, Awards and Achievements

2008-2011 **Natural Sciences and Engineering Research Council of Canada (NSERC) Alexander Graham Bell Canada Graduate Scholarship (CGS-D)**

Value, $105 000

2007-2008 **Natural Sciences and Engineering Research Council of Canada (NSERC) Alexander Graham Bell Canada Graduate Scholarship (CGS-M)**

Value, $17 500

Declined **Ontario Graduate Studies (OGS) Scholarship**

Value, $15 000/year

2006 **Ontario Genomics Institute Travel Award**

Value, $1 667

2006-Present **University Of Ottawa Graduate Studies Excellence Award**

Value, $4 500/year

2003-Present **Golden Key International Honour Society Member**

2002-2006 **University of Ottawa Undergraduate Entrance Award**
Value, $2 500/year

Attended Conferences and Presentations

2008 **Gordon Research Conference, Proprotein Processing, Trafficking and Secretion**

Poster Presentation, The effects of PCSK9 on the HuH7 cellular proteome using quantitative proteomics.

International conference concerned with the biological sorting and trafficking of bioactive proteins and peptides. Held in New London, NH, USA.

2006 **Progress in Systems Biology 2006**

Second annual systems biology symposium hosted by the Ottawa Institute of Systems Biology

Poster Presentation. **Probing the ubiquitome, A large-scale characterization of ubiquitination sites using a novel gel-free approach.**

2006 **Human proteome organization 5th annual world congress**

International conference concerned with the latest developments in human proteome research. Held in Long Beach, California ***Poster Presentation.*** **Probing the ubiquitome, A large-scale characterization of ubiquitination sites using a novel gel-free approach.**

2005 **Progress in Systems Biology**

First annual systems biology symposium hosted by the Ottawa Institute of Systems Biology

Publications

Gyamera-Acheampong, C., Sirois, F., **Denis, N.J.**, Mishra, P., Figeys, D., Basak, A., Mbikay, M. (2010) The precursor to the germ cell-specific PCSK94 proteinase is inefficiently activated in transfected somatic cells: evidence of interaction with the BiP chaperone. *Mol. Cell Biochem.* Manuscript number: mcbi-3625R1

Zhou, H., Elisma, F., **Denis, N.J**., Wright, T.G., Tian, R., Zhou, H., Hou, W., Zou, H., Figeys, D. (2010) Analysis of the subcellular phosphoproteome using a novel phosphoproteomic reactor. *J. Proteome Res.* 9,1279-1288.

Zhou, H., Hou, W., **Denis, N.J.,** Zhou, H., Vasilescu, J., Zou H., Figeys, D. (2009) Glycoproteomic reactor for human plasma. *J. Proteome Res.* 8,556-566.

Vasilescu, J., Smith, J.C., Zweitzig, D.R., **Denis, N.J**., Haines, D.S., Figeys, D. (2007) Systematic determination of ion score cutoffs based on calculated false positive rates, application for identifying ubiquitinated proteins by tandem mass spectrometry. *J. Mass Spectrom.* 43,296-304.

Denis, N.J., Vasilescu, J., Lambert, J.P., Smith, J.C., Figeys, D. (2007) Tryptic digestion of ubiquitin standards reveals an improved strategy for identifying ubiquitinated proteins by mass spectrometry. *Proteomics.* 7,868-74.

Vasilescu, J., Zweitzig, D.R.*, **Denis, N.J.,*** Smith, J.C., Ethier, M., Haines, D.H., Figeys, D. (2007) The proteomic reactor facilitates the analysis of affinity-purified proteins by mass spectrometry, application for identifying ubiquitinated proteins in human cells. *J Proteome Res*. 6,298-305.

*These authors contributed equally.