New insights into LDLR regulation and trafficking: The interplay of non-lipid-related molecules with PCSK9

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

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To my family

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Abstract

PCSK9, the last member of the proprotein convertase family, is a serine protease that has been linked to familial hypercholesterolemia *via* targeting LDLR in hepatocytes. The degradation of LDLR occurs by two distinct pathways: an intracellular pathway that sends the complex of PCSK9 and LDLR to lysosomes directly from the trans-Golgi network (TGN), and an extracellular pathway that sorts this cell-surface complex to endosomes and then lysosomes for degradation. PCSK9 has a C-terminal Cysteine-His-Rich-Domain (CHRD) composed of 3 repeat domains M1, M2 and M3. The M2 domain does not bind the LDLR directly, but it is necessary for PCSK9's extracellular function on LDLR. The M2 domain was suggested to interact with an RxE motif in some MHC-class I proteins, sending some of them (e.g., HLA-C) to lysosomal degradation. Here, our first two objectives were to evaluate the implication of MHC-I-like proteins, such as HFE (objective 1; involved in iron homeostasis) and HLA-C (objective 2; implicated in the immune system), in the regulation of the lysosomal sorting of the LDLR/PCSK9 complex. Apart from MHC-I-like molecules, another convertase PACE4 was shown to bind PCSK9. Although PACE4 is highly expressed in hepatocytes and has an emerging role in cardiovascular diseases, its function in the liver is not yet understood. Hence, our third objective raised the question of whether this interaction could affect the activities and properties of PCSK9 in liver.

In this work, we found that although the M2 domain of PCSK9 is needed to sort the complex to lysosomes, it is not necessary for the internalization of the complex. Furthermore, our cell-based assays revealed that HFE inhibits the activity of PCSK9 toward LDLR degradation, whereas HLA-C enhances this function. We showed for the first time that HFE interacts with PCSK9 via the same RxE motif that is critical for HLA-C binding to PCSK9 and both receptors are degraded by extracellular PCSK9. Although, the degradation of HLA-C is LDLR-independent, the presence of LDLR is necessary for HFE degradation. Indeed, the PCSK9-induced degradation of HFE depends on the presence of both caveolin and clathrin heavy chains, whereas that of HLA-C is likely dependent only on clathrin heavy chains. Interestingly, physiological conditions such as iron levels can favor one of two pathways that affect PCSK9's activity. Thus, we propose that HFE and HLA-C might compete to interact with PCSK9 in different physiological conditions to either inhibit or activate PCSK9's activity to balance the levels of LDLR in the human body.

Moreover, our data reveal that the distribution of PACE4 mRNA in different tissues is very similar to LDLR and PCSK9. We show that PACE4 can regulate LDLR levels either in PCSK9-dependent (intracellular function) or -independent pathways. In the PCSK9-dependent pathway, PACE4 directly interacts with PCSK9 and inhibits its intracellular activity. Paradoxically, extracellular PACE4 enzymatic activity positively regulates the levels of LDLR and enhances diI-LDL uptake in a PCSK9-independent fashion. This suggests that PACE4 can cleave and inactivate an endogenous protein that negatively regulates LDLR levels. One such possibility is CAP1, whose absence (siRNA) leads to higher LDLR levels. These data suggest that PACE4 is a new regulator of PCSK9 and LDLR and emphasize the need to study this protein in the liver more thoroughly, both in vivo and in vitro. In general, our work unravels the new regulatory pathways/proteins involved in PCSK9 and LDLR trafficking along with new possible functions of PCSK9 in non-lipid cellular metabolism such as iron homeostasis.

Résumé

PCSK9 est une sérine protéase associée à l'hypercholestérolémie familiale grâce à son ciblage du récepteur LDLR au niveau des hépatocytes. La dégradation du LDLR se produit via deux voies distinctes : une voie intracellulaire qui dirige directement le complexe PCSK9-LDLR vers les lysosomes depuis le réseau trans Golgien (TGN), et une voie extracellulaire qui envoie ce complexe de la surface cellulaire aux lysosomes via les endosomes, pour être dégradé. La région C-terminale de la PCSK9, connue sous le nom de CHRD, comprend 3 domaines M1, M2 et M3. •Bien que le domaine M2 de PCSK9 ne se lie pas directement au LDLR, il est nécessaire à l'activité extracellulaire de la PCSK9 sur le LDLR. Il a été démontré que le domaine M2 interagit avec le motif RxE d'une protéine CMH de classe I, l'envoyant à la dégradation lysosomale. Ici, notre question principale est de déterminer si des protéines de type CMH-I, telles que HFE (impliquée dans l'homéostasie du fer) et HLA-C (impliquée dans le système immunitaire), peuvent réguler le trafic du LDLR/PCSK9. Ainsi, les deux premiers objectifs de ce projet de mon projet de doctorat sont d'étudier ces deux protéines et de définir leurs implications dans la régulation de la fonction de la PCSK9. •Le troisième objectif consiste à analyser la régulation de la fonction de la PCSK9 par la PACE4, le 6ieme membre de la famille des proprotéines convertases qui lie directement la PCSK9. Bien que PACE4 soit fortement exprimée dans les hépatocytes et qu'elle joue un rôle émergent dans les maladies cardiovasculaires, sa fonction dans le foie reste encore méconnue. Notre troisième objectif soulève donc la question de savoir si cette interaction peut affecter les activités et les propriétés de la PCSK9.

Dans cette étude, nous avons constaté que bien que le domaine M2 de PCSK9 soit nécessaire pour diriger le complexe vers les lysosomes, il n'est pas nécessaire à son internalisation. De plus, nos expériences cellulaires ont révélé que l'HFE inhibe l'activité extracellulaire de la PCSK9, tandis que HLA-C augmente son effet sur la dégradation du LDLR. Nous avons montré pour la première fois que HFE est également dégradée par la PCSK9 extracellulaire, tout comme HLA-C. HFE interagit avec PCSK9 via le même motif RxE qui est essentiel pour la liaison de HLA-C à PCSK9. Bien que la dégradation de HLA-C soit indépendante du LDLR, sa présence est nécessaire pour la dégradation de HFE. En effet, la dégradation de HFE induite par PCSK9 dépend de la présence de vésicules à la fois de cavéoline et de clathrine à chaînes lourdes, tandis que celle de

HLA-C dépend probablement des endosomes à chaînes lourdes de clathrine. Certaines conditions physiologiques telles que le taux de fer peuvent favoriser l'une des deux voies qui affectent l'activité de PCSK9. Ainsi, notre hypothèse est que HFE et HLA-C sont en compétition pour interagir avec PCSK9 dans différentes conditions physiologiques pour l'inhiber ou l'activer, afin d'équilibrer les niveaux de LDLR dans le corps humain.

De plus, nos données révèlent que la distribution de l'ARNm de PACE4 dans différents tissus est très similaire à celle du LDLR et de PCSK9. Nous montrons que PACE4 peut réguler les niveaux de LDLR, soit dépendamment de PCSK9 (fonction intracellulaire), soit de manière indépendante. Dans la voie dépendante de PCSK9, PACE4 interagit directement avec la PCSK9 et inhibe sa fonction intracellulaire. Le possible effet direct de PACE4 sur le LDLR a ensuite été étudié dans les hépatocytes n'exprimant pas la PCSK9. Nos données montrent que non seulement l'activité enzymatique de PACE4 augmente les niveaux de LDLR, mais également qu'elle améliore l'internalisation des diI-LDL. Il est donc possible que PACE4 clive et inactive une protéine qui régulerait négativement les niveaux du LDLR, telle que CAP1 (basé sur des études de siRNA de CAP1). Ces résultats suggèrent que PACE4 est un nouveau régulateur de la PCSK9 et du LDLR, et soulèvent la nécessité d'étudier plus en détails cette protéine dans le foie, à la fois in vivo et in vitro.

De façon générale, notre travail dévoile de nouvelles voies/protéines de régulation impliquées dans le trafic de PCSK9 et du LDLR, ainsi que de nouvelles fonctions possibles de PCSK9 dans les métabolismes cellulaires non lipidiques, tels que l'homéostasie du fer.

Acknowledgment

I am deeply grateful to all those who have contributed to the completion of this thesis. First and foremost, I am especially grateful for my supervisor, Professor Nabil G. Seidah, who supported me in this journey. In moments of uncertainty and challenge, Dr. Seidah stood by me patiently and provided me with valuable insights that propelled me forward. Without him, it would not have been possible to overcome the challenges of this project. I am also grateful for all my current and former colleagues and friends at Dr. Seidah's lab who helped me through this journey: Dr. Annik Prat, Dr. Delia Susan-resign, Dr. Rachid Essalmani, Dr. Ali Ben Djoudi Ouadda, Alexandra Evagelidis, Anna Roubtsova Stepanova, Mailys Ledevehat, Vatsal Sachan, Priyanka Prabhala, Chloe Porcheron, Kalista Lam, Jadwiga Marcinkiewicz, and Jisca Borgela. During my doctoral studies, I also benefited from various institutions and services both within and outside McGill University and IRCM institution.

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I was also lucky to benefit from exemplary collaborators during this research. Their assistance in providing access to resources and facilities necessary for conducting experiments and data collection have been crucial in overcoming various challenges and ensuring the quality of the research. Thanks to all collaborators of different projects of this thesis: Dr. Carole Fruchart, Dr. Kostas Pantopoulos, Dr. May Faraj, Dr. Robert Day, and Oscar Henrique Pereira Ramos.

I want to express my heartfelt appreciation to my family and friends for their consistent support. Their presence during both the ups and downs of this journey has been my foundation, providing me with the strength and encouragement to overcome challenges and celebrate

successes. Their firm belief in me has been a driving force, and I am truly grateful for their role in my life.

Contribution to original knowledge

This PhD thesis was written in accordance with McGill University's traditional thesis guidelines. It is presented as four chapters including a comprehensive literature review, experimental procedures, results, and a concluding thesis discussion. The data presented is the original work of Sepideh Mikaeeli.

The research presented here contributes significantly to the advancement of our knowledge of the complex interplay between PCSK9, LDLR, and various regulatory proteins in the context of cholesterol metabolism. The complex nature of these interactions has been a challenging area of study. One of the main contributions of this study lies in its elucidation of the role of various proteins, including HFE, HLA-C, and PACE4, in modulating PCSK9 activity and LDLR degradation. The characterization and identification of protein X as an essential factor in facilitating the lysosomal degradation of LDLR by PCSK9, provides a crucial missing piece in understanding the intricate trafficking mechanisms involved. Furthermore, the revelation of HFE's dual role in positively regulating intracellular PCSK9 and negatively impacting extracellular PCSK9's activity through competitive interactions with HLA-C illustrates the complexity of PCSK9 regulation. The research also sheds light on the possible impact of PCSK9 in iron metabolism by targeting HFE protein. Furthermore, the investigation about the PACE4's involvement in regulating both PCSK9 and LDLR adds an additional layer of complexity to the network of interactions. These findings suggest potential novel therapeutic avenues, particularly in cases of PCSK9 resistance and in addressing lipid metabolism disorders. Overall, the findings presented in this study significantly advance our knowledge of the molecular mechanisms governing cholesterol metabolism and iron homeostasis.

Contribution of authors

Compilation of the work presented in this study is attributed to Sepideh Mikaeeli. The conception of the project and design of experiments were contributions from both Dr. Nabil G. Seidah and Sepideh Mikaeeli. The procurement and development of materials, along with the execution of experimental work, data collection, and subsequent analysis were primarily carried out by Sepideh Mikaeeli, unless otherwise specified below.

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Chapter III.II

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List of abbreviations

ACE2: Angiotensin-converting enzyme 2

ACLY: ATP citrate synthase

ADH: Autosomal dominant hypercholesterolemia

ANGPLT: Angiopoietin-related protein

APLP2: Amyloid precursor protein-like protein-2

ApoB: Apolipoprotein B

ApoE: Apolipoprotein E

ApoER2: Apolipoprotein E receptor 2

ARH: Autosomal recessive hypercholesterolemia

ART : Antiretroviral therapy

ASGR1: Asialoglycoprotein receptor 1

ASGR1: Asialoglycoprotein receptor 1

ASO: Antisense oligonucleotide

ATF6: ER transducers activating transcription factor 6

β2M: Beta-2 microglobulin

CAP1: Cyclase associated protein 1

CCC complex: COMMD/CCDC22/CCDC93

CD36: Cluster of differentiation 36

cDNAs: Complementary DNAs

CETP: Cholesteryl ester transfer protein

CHCs: Clathrin-heavy chains

CHD: Coronary heart disease

CHO: Chinese hamster ovary

CHRD: Cys-His-rich domain

CHX: Cycloheximide

CLCs: Clathrin-light chains

CM: Chylomicrons

CMr: Chylomicron remnants

Co-IP: Co-immunoprecipitation

COPII: Coat protein complex II

CRCLM: Colorectal cancer liver metastatic

CRD : Cys-rich domain

CTLs: Cytotoxic T cells

CVDs: Cardiovascular diseases

Dab2: Disabled-2

DENV: Dengue virus

DFA: Deferoxamine

DMEM: Modified Eagle Medium

DMEM: Modified Eagle Medium supplemented

Dyn B: Dynorphin B

E2:17β-estradiol

EDL: Endothelial lipase

EMEM: Eagle's Minimum Essential Medium

ERK/MAPK: Extracellular signal-regulated kinase/mitogen-activated protein kinase

ERSD: ER storage disease

FA: Fatty acids

FAC: Ferric ammonium citrate

Fam20C: Family with sequence similarity 20 member C

FBS: Fetal bovine serum

FFA: Free fatty acid

FH: Familial hypercholesterolemia

FXR: Foresaid X receptor

GDF15: Growth differentiation factor 15

GH: Growth hormone

GL: Glycolipids

GOF: Gain of function

GP: Glycerophospholipids

GRP78: Glucose-regulated protein 78

GWAS: Genome-wide association studies

GX sPLA2: Group X secretory phospholipase A2

HAND: HIV-associated neurocognitive disorders

HCV: Hepatitis C virus

HDL: High-density lipoprotein

HeFH: Heterozygous FH

HEK293: Human embryonic kidney-derived epithelial cells

HepG2: Human hepatocellular carcinoma

HFE: Human hemochromatosis

HH: Hereditary hemochromatosis

HINFP: Histone nuclear factor P

HIV: Human immunodeficiency virus

HLA: Human leukocyte antigen

HLA-C: Human leukocyte antigen C

HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A

HNF1-α: Hepatocyte nuclear factor-1 alpha

HNSCC: Head and neck squamous cell carcinoma

HoFH: Homozygous FH

HSPG: Heparan sulfate proteoglycans

Huh7: Human hepatocellular carcinoma cells

IDL: Intermediate density lipoprotein

IDOL: Inducible degrader of LDLR

IF: Immunofluorescence assay

IGFs: Insulin-like growth factors

IHH: Immortalized human hepatocytes

IRB: Insulin receptor isoform B

IRE1α: Glucose-regulated protein 1

ISH: In situ hybridization

KO: knockout

LCAT: Lecithin–cholesterol acyltransferase

LCFA: Long-chain saturated FA

LDL: Low-density lipoprotein

LDLc: LDL cholesterol

LDLR: Low-density lipoprotein receptor

LOF: Loss of function

LOXL2: Lysyl oxidase-like 2

Lp(a): Lipoprotein(a)

LPL: Lipoprotein lipase

LRP1: LDLR related protein 1

LRPAP1: Low-density lipoprotein receptor adapter protein 1

LXR: Liver X receptor

mAbs: Monoclonal antibodies

MASP-3: Mannose-binding lectin–associated serine protease 3

MHC-I: Major histocompatibility complex 1

MMPs: Matrix metalloproteinases

NAFLD: Non-alcoholic fatty liver disease

NARC1: Neural apoptosis-regulated convertase 1

NPC1L1: Niemann-Pick C1-like protein 1

NS: Non-significant

OSR1: Odd-skipped-related 1

PACE4: Paired basic amino acid cleaving enzyme 4

PAQR3: Progestin adipo Q receptor 3

PAR1: Proteinase-activated receptor 1

PCR: Polymerase chain reaction

PCs: Proprotein convertases family

PCSK9: Proprotein convertase subtilisin/kexin type 9

PD-1 antibody: Programmed cell death protein 1 antibody

PDGF-B: Platelet-derived growth factor B

PDL1: Programmed death-ligand 1

PERK: Protein kinase RNA–like endoplasmic reticulum kinase

PI (4,5) P2: Phosphatidylinositol-4,5-biphosphate

PI3K: Phosphatidylinositol 3-kinase

PK: Polyketide

PPARs: peroxisome proliferator-activated receptors

PR: Prenol Lipid

pro-GDF: Pro-growth differentiation factor

PTM: Post-translational modification

PUFA: Poly un-saturated FA

RCT: Reverse cholesterol transport

rhTIMP-1: Recombinant human tissue inhibitor of metalloproteinase-1

SARS-CoV-2: severe acute respiratory syndrome coronavirus-type 2

SD: Standard deviation

SH3BD: Src homology 3 binding domains

sHFE: Soluble HFE

SL: Saccharolipid

SNPs: Single-nucleotide polymorphisms

SORT1: Sortilin 1

SP: Sphingolipids

SREBP: Sterol regulatory element-binding protein

STAP1: Signal transducing adaptor family member 1

STARD3: Steroidogenic acute regulatory protein-related lipid transfer domain-3

SURF4: Surfeit locus protein 4

TAG: Triacylglycerol

TBP: TATA box binding protein

TBX5: T-box transcription factor 5

TCRs: T cell receptors

Tf: Transferrin

TfR1: Transferrin Receptor 1

TfR2: Transferrin Receptor 2

TGF-β: Transforming growth factor beta

TGN: Trans-Golgi network

TIBC: Total iron-binding capacity

TIMPs: Tissue inhibitors of metalloproteinases

TPH1: Tryptophan hydroxylase 1

UPR: Unfolded protein response

VLDL: Very-low-density lipoprotein

VLDLR: Very-low-density lipoprotein receptor

WASH complex: SCAR homolog

WB: Western blot

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The subsequent reference of all used figures and tables provided in the text.

Chapter I:

I.I. literature review:

I.I.I. lipid metabolism:

So far, more than 47,000 lipids have been identified on our planet (https://www.lipidmaps.org/), yet our understanding of their role and importance is very scant. The study of lipids is important since lipids are fundamental components of living creatures and are considered the main source of energy in animals, along with their massive role in many physiological processes. In this section, we are going to have a brief review of lipids, their metabolism, and their importance in the human body.

Lipids as an Essential Compound of the Human Body

Lipids are organic macromolecules, made of fatty acid monomers [$CH3(CH2)_xCOOH$], that are found both in animals and plants. Lipids are hydrophobic macromolecules including sterols, sphingolipids, and glycolipids. These water-insoluble compounds are essential for living beings because of their role as the energy reserves of animals. They also play many other vital roles, such as being precursors of different compounds like vitamins (e.g., Vitamin D), hormones and bile acids. Lipids are also a fundamental part of the plasma membrane and are involved in cellular transportation and membrane trafficking (Blanco & Blanco, 2017).

The balanced presence of various types of lipids is needed to maintain the healthy function of the body. Unbalanced levels of lipids, either in circulation or organs, can cause a different spectrum of diseases such as diabetes, kidney disease, hypothyroidism, liver disease, metabolic disorders, and a variety of atherosclerosis disorders like heart attack (Blanco & Blanco, 2017; Ratnayake & Galli, 2009).

Lipids consist of hydrocarbons and diverse groups of non-polar compounds that are soluble in organic solvents. These compounds and parts have significant structural variety, mostly based on their functional groups, different chain lengths, and their capability for ring formation.

Traditionally, lipids used to classify into two categories known as simple (including acylglycerols and waxes) and complex (containing phospholipids, glycolipids, and lipoproteins)

(Blanco & Blanco, 2017; Fahy et al., 2011). However, in 2005, the International Lipid Classification and Nomenclature Committee proposed a comprehensive classification system for lipids called the LIPID MAPS. This classification, established based on the various chemical and biochemical characterization of molecules, divides lipids into eight categories (https://www.lipidmaps.org/) (Fig. 1) (Fahy et al., 2011).

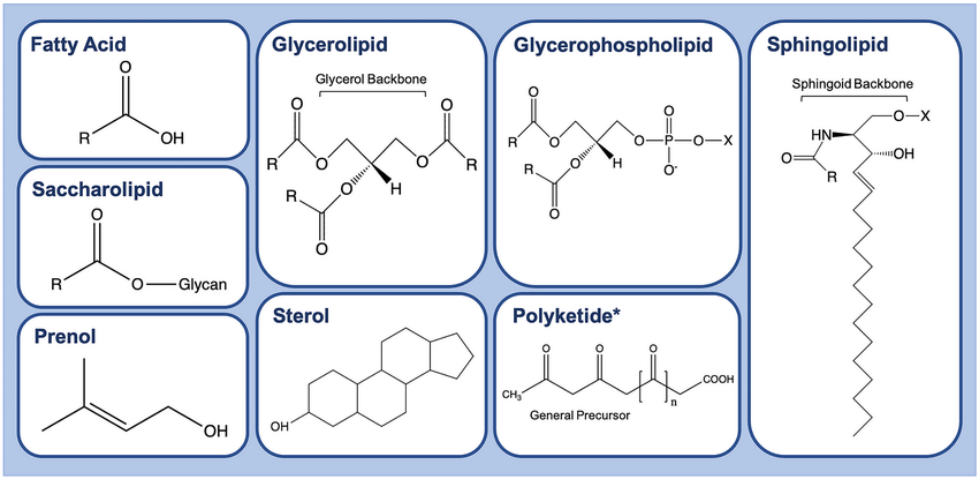


Fig. 1. The eight classes of lipids and their biochemical structures (Ward et al., 2021).

1. Fatty acids (FA): This class of lipids is the simplest form that is found primarily in plasma and is usually used as a building block of other types of lipids. Fatty acids are made of carboxylic acid along with a long aliphatic chain that can be either saturated (with no C=C double bonds) or non-saturated (with at least one C=C double bond). In non-saturated lipids, the C=C double bonds can make either *cis* or *trans* isomers (Blanco & Blanco, 2017; Fahy et al., 2011). Fatty acids can also be added to proteins by acylation process.
2. Saccharolipids (SL): In saccharolipids, that mostly found in the plasma membrane, fatty acids are directly linked to a carbohydrate backbone. These lipids are considered as an important component of the cell surface antigens (Raetz et al., 2009).
3. Prenol Lipids (PR): Prenol lipids (PR) are synthesized from the 5-carbon precursors isopentenyl diphosphate and dimethylallyl diphosphate. One of the most common types of prenols is carotenoid that is the precursor of vitamin A. It is also have shown that prenols

can have antioxidant effects on the human body (Salvatore Fanali, 2017). PRs can also be added to proteins by prenylation.

4. Plycerolipids/Glycerolipids (GL): Glycerolipids are synthesized from long-chain acyl and alkyl groups, with polar alcohols that are attached to the sn-glycerol-3-phosphate backbone. These lipids have critical roles in membrane formation, intracellular signalling, and storage of energy (Scaramozzino, 2013).
5. Sterol Lipids (ST): sterols are one of the most common types of lipids, along with triglycerides and phospholipids, that have a more complicated structure. Sterols include cholesterol and its derivatives that are biosynthesized in all animal cells. Cholesterol is an essential molecule as it serves as a critical part of the cell membrane and as a precursor for the biosynthesis of steroid hormones, bile acid and vitamin D (Blanco & Blanco, 2017).
6. Glycerophospholipids (GP): are glycerol-based phospholipids that are the main component of plasma membranes. Phospholipids generally consist of two hydrophobic fatty acids that joined the hydrophilic phosphate group by a glycerol molecule. Depending on the type of alcohol, Phospholipids split into glycerophospholipids and sphingophospholipids (Blanco & Blanco, 2017).
7. Polyketides (PK): Polyketides are a class of natural products that have yielded many antibiotics. This class of lipids is usually found in bacteria and fungi and are derivates of precursor molecules with a reduced form of ketone. The diverse structure of these lipids makes it possible for their use for drug discovery purposes (Sharbrough, 2011).
8. Sphingolipids (SP): Sphingolipids are ubiquitous components that have great involvement in plasma membranes and many biological processes, such as signal transduction (Blanco & Blanco, 2017).

Depending on the metabolic function of each lipid, they can be considered as either structural lipids or stored lipids. Each type of lipid has its distinct composition of compounds. However, some components are similar in both types, including long-chain saturated FA (LCFA), mono un-saturated FA, and poly un-saturated FA (PUFA). The structural lipids, which are normally located at the cell surface/membrane, are mostly composed of free cholesterol and phospholipids. The composition of membrane lipids can be changed depending on many factors, such as their subcellular localization and even with various diets. The main component of stored lipids, found in body fat or adipose tissue, is triacylglycerol (TAG) which can be made either by oxidation of

fatty acids from diet or by endogenous synthesis in the liver and intestine. Lipids during their transportation in circulation can be considered as an intermediate lipid and connect membrane lipids to stored lipids (Calder, 2006; Frayn, 2000).

Lipid metabolism

On average, in a Western diet, between 50 to 100 g of fat can be taken up, which can provide about 35% of total energy (Griffin, 2013). Lipid metabolism is a process which involves a series of events that ultimately causes the synthesis and degradation of lipids in cells. This process makes lipids available to be used for various purposes, like being used as a source of energy or synthesis of functional and structural derivates in the human body (Genest, 2003; Gordon Betts, 2013).

In general, lipid metabolism consists of the following steps:

Lipid digestion

Digestion is the first step of lipid metabolism, where lipids are broken down either for energy production or other purposes. During this pathway, free fatty acids (FFA) and glycerol are released from TAGs, cholesterol esters, and phospholipids. This process begins with chemical digestion *via* lingual lipases in the mouth that ultimately transfers lipids to the stomach. There, mechanical digestion starts while chemical digestion of lipids continues and is mediated by gastric lipases. Then, digested lipids are directed to the small intestine (where most of the absorption and digestion either mechanically or chemically happens) along with critical chemical compounds for digestion. The uptake of cholesterol and fatty acids are mediated by Niemann-Pick C1-Like 1 (NPC1L1) and CD36 receptors, respectively. After the absorption of these fatty acids, they will be hydrolyzed into FFAs and glycerol with the help of pancreatic lipases (Fig. 2). The final products of these digestive steps are individual fatty acid units that are absorbed into the small epithelial cells of the small intestine (Genest, 2003; Gordon Betts, 2013).

Lipid absorption

Absorption of fats occurs only in the small intestines, where about 90% of total dietary TAGs and 40% of cholesterol can be absorbed (Griffin, 2013). Bile acids from biliary secretion are critical components for this process as they solubilize lipids in the duodenum by forming mixed micelles that facilitate the internalization of lipids into the enterocytes (Genest, 2003; Gordon Betts, 2013). Once lipids enter the enterocytes, they are re-esterified to be further transported to the circulation and reach their destination cells (Fig. 2) (Griffin, 2013).

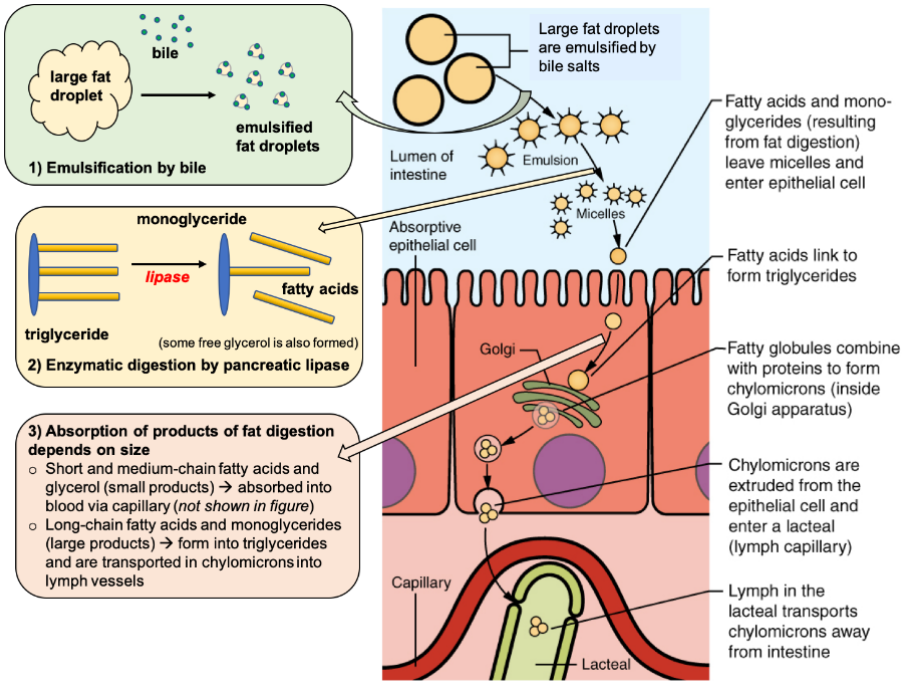


Fig. 2. Detailed illustration of lipid digestion and absorption processes in the human body (Gordon Betts, 2013).

Transporting lipids

As lipids are not soluble in water, they need some carrier proteins to help them transport during circulation. Hence, specific amphipathic proteins known as apolipoproteins help lipids to transport in circulation. Lipids with apolipoproteins and phospholipids form lipoproteins. Lipoproteins are categorized in different classes depending on their density and size, including chylomicrons (CM), chylomicron remnants (CMr), very-low-density lipoprotein (VLDL), Intermediate density lipoprotein (IDL), low-density lipoprotein (LDL), high-density lipoprotein (HDL), and lipoprotein(a) (a.k.a. Lp(a)) (Fig. 3). Each lipoprotein is responsible for carrying a certain amount and specific type(s) of lipids (Table 1) (Genest, 2003; Gordon Betts, 2013).

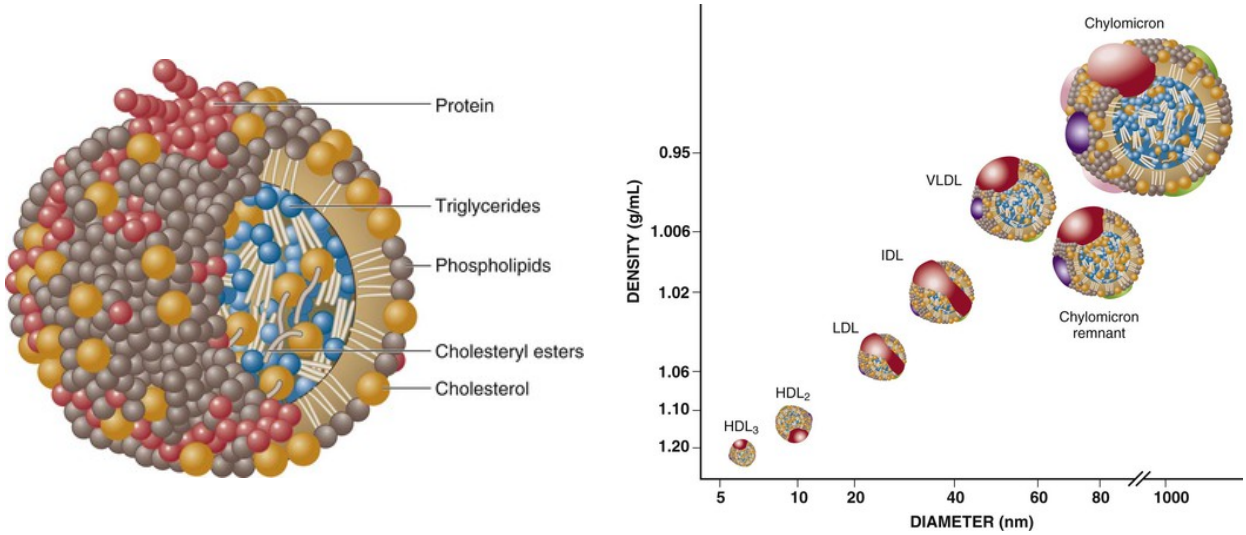


Fig. 3. A). Structure of lipoproteins and B) classification of lipoproteins based on their size and density. HDL = high-density lipoprotein; IDL = intermediate-density lipoprotein; LDL = low-density lipoprotein; VLDL = very low-density lipoprotein (Genest, 2003).

Table 1. Plasma lipoproteins Composition (Genest, 2003).

	ORIGIN	DENSITY (g/mL)	SIZE (nm)	% PROTEIN	[CHOLESTEROL] IN PLASMA (mmol/L)*	[TRIGLYCERIDE] IN FASTING PLASMA (mmol/L)†	MAJOR APO	OTHER APO
Chylomicrons‡	Intestine	<0.95	100-1000	1-2	0.0	0	B48	A-I, C's
Chylomicron remnants‡	Chylomicron metabolism	0.95-1.006	30-80	3-5	0.0	0.0	B48, E	A-I, A-IV, C's
VLDL	Liver	<1.006	40-50	10	0.1-0.4	0.2-1.2	B100	A-I, C's
IDL	VLDL	1.006-1.019	25-30	18	0.1-0.3	0.1-0.3	B100, E	
LDL	IDL	1.019-1.063	20-25	25	1.5-3.5	0.2-0.4	B100	
HDL	Liver, intestine	1.063-1.210	6-10	40-55	0.9-1.6	0.1-0.2	A-I, A-II	A-IV
Lp(a)	Liver	1.051-1.082	25	30-50			B100, (a)	

* In mmol/L; for mg/dL, multiply by 38.67.
† In mmol/L; for mg/dL, multiply by 88.5.
‡ In the fasted state, serum (or plasma) should not contain chylomicrons or their remnants.
APO = apolipoprotein; HDL = high-density lipoprotein; IDL = intermediate-density lipoprotein; VLDL = very low-density lipoprotein.

Each lipoprotein can interact with specific receptors at the cell surface to be able to internalize in different cells. This helps various lipids reach their specific cells after their digestion and secretion in the intestine and circulation, respectively. Lipid transport can be described as either endogenous (for intracellular synthesis of lipids) or exogenous (for dietary lipids) pathways.

Exogenous lipid transport

In the exogenous pathway in enterocytes, the re-esterified TAGs and cholesterol esters interact with their specific apolipoprotein and form chylomicrons (CMs) that can be discharged into the lymph (Griffin, 2013). When CMs enter circulation, they interact with lipoprotein lipase (LPL) enzymes that catalyze the hydrolysis of TAGs and result in the release of FAs and glycerol to be taken by underlying cells (FAs; up taken by adipose tissue and skeletal muscle, glycerol; up taken by the liver). Following up on these modifications, chylomicron's size and composition changes and form chylomicron remnants which re-circulate to the liver, where they get degraded and release FAs, cholesterol, and amino acids. HDL is an essential lipoprotein in this pathway as it provides necessary apolipoproteins (ApoE and ApoCs) for chylomicrons to initiate their transformation (Fig. 4) (Blanco & Blanco, 2017; Byrnes & Griffin, 1998).

Endogenous lipid transport

In the endogenous pathway, newly synthesized TAGs and cholesterol esters are packed into VLDL particles and then secreted to the circulation. In capillaries, VLDLs undergo similar changes as chylomicrons with the help of HDL particles. This change, along with other modifications, transform VLDL into IDL and LDL particles. LDL particles can be captured in different cells *via* low-density lipoprotein receptors (LDLRs) (Fig. 4)

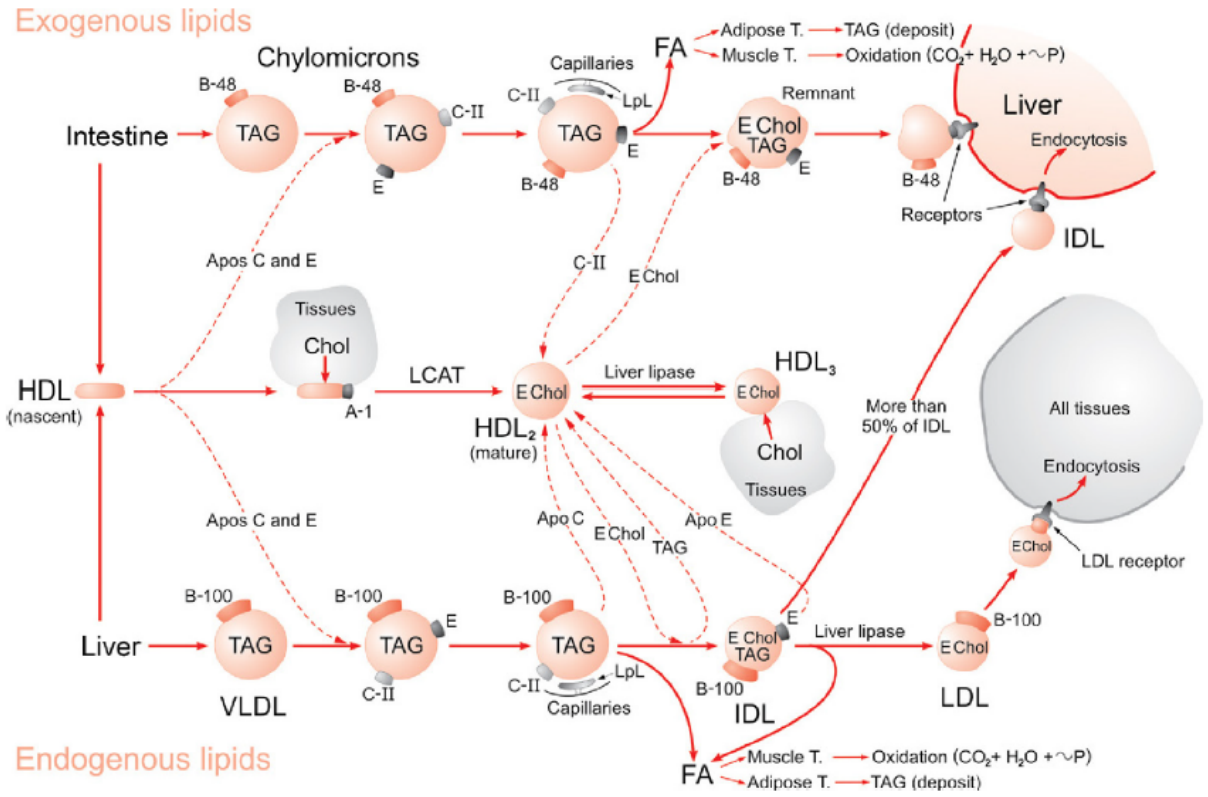


Fig. 4. Endogenous and exogenous lipid transports. Chol, Free cholesterol; EChol, esterified cholesterol; HDL, high-density lipoproteins; IDL, intermediate-density lipoproteins; LDL, low-density lipoproteins; LCAT, lecithin-cholesterol acyltransferase; LpL, lipoprotein lipase; TAG, triacylglycerol; VLDL, very-low-density. (Blanco & Blanco, 2017).

Apart from endogenous and exogenous pathways, there is another pathway that is called reverse cholesterol transport (RCT) and is mediated by HDL particles. In RCT, cholesterol is transported from peripheral tissues to the liver by HDLs to eliminate excessive cholesterol from the body.

Lipid catabolism

When the digested lipids enter the cells, they are further catabolized to produce energy. Each lipid has its own specific catabolism pathway. For example, the breakdown of fatty acids starts in the cytoplasm and is mediated by β-oxidation pathway that happens in mitochondria. The main product of the β-oxidation is acetyl-CoA which is used in the citric acid cycle to produce energy (Blanco & Blanco, 2017).

Lipid homeostasis and regulation

As it was mentioned earlier, it is important to maintain the balanced levels of each lipid to have its normal function in a healthy human. Lipid homeostasis refers to the series of mechanisms that help the body to keep balanced level of lipids either in circulation or tissues. Liver, adipose tissue, and intestine are the main parts regulating lipid homeostasis. Correct function and normal physiology of several factors can regulate lipid homeostasis. These factors include insulin, sterol regulatory element-binding protein (SREBP), FAs, leptin, and glucagon just to name a few (Blanco & Blanco, 2017).

Lipids and diseases

There is an extensive list of diseases that are either partially or fully related to imbalanced levels of lipids or their dysfunction. It has been shown that more than 80 human diseases are related to defects in complex lipid metabolism (Xiao et al., 2021). Higher levels of lipids (hyperlipidemia) seem to cause more disorders compared to lower levels of lipids (hypolipidemia). Hyperlipidemia can be classified either based on the lipid type or causing factor (Fredrickson & Lees, 1965; Verma, 2017).

Lipid diseases based on the lipid type

This classification of lipid disorders can divide diseases into the two further classes of hypercholesterolemia that is due to elevated levels of serum cholesterol, and hypertriglyceridemia which is caused by increasing levels of triglycerides (Fredrickson & Lees, 1965; Verma, 2017).

Lipid diseases based on the causing factor

This classification also subsumes hyperlipidemia into two distinct categories called familial (primary) hyperlipidemia and acquired (secondary) hyperlipidemia.

Familial hyperlipidemia is further classified into five types according to Frederickson's categorization (Table 2). Acquired hyperlipidemia is caused by underlying disorders that ultimately alter plasma lipid and lipoprotein metabolism. This class can result from conditions such as diabetes mellitus, alcohol consumption, specific endocrine and metabolic disorders, renal failure, and use of specific drugs such as estrogens (Fredrickson & Lees, 1965; Verma, 2017).

Table 2. Fredrickson classification (Verma, 2017).

Hyperlipo-proteinemia	Synonyms	Defect	Increased lipoprotein	Symptoms	Treatment
Type I	Familial hyperchylomicronemia Familial apoprotein CII deficiency	Decreased lipoprotein lipase (LPL) Altered ApoC2 LPL inhibitor in blood	Chylomicrons	Acute pancreatitis, lipemia retinalis, xanthomas, hepatosplenomegaly	Diet control
Type II	Familial hypercholesterolemia	LDL receptor deficiency	LDL	Xanthelasma, arcus senilis, tendon xanthomas	Bile acid sequestrants, statins, niacin
	Familial combined hyperlipidemia	Decreased LDL receptor and increased Apo B	LDL and VLDL		Statins, niacin, fibrate
Type III	Familial dysbetalipoproteinemia	Defect in Apo E2 synthesis	IDL	Tuboruptive xanthomas and palmar xanthomas	Fibrate, statins
Type IV	Familial hypertriglyceridemia	Increased VLDL production and decreased elimination	VLDL	Can cause pancreatitis at high triglyceride levels	Fibrate, niacin, statins
Type V		Increased VLDL production and decreased LPL	VLDL and chylomicrons		Niacin, fibrate

However, other researchers proposed different classifications for lipid disorders. For instance, in 2021, it was suggested to use other descriptive classification methods, in which the diseases is categorized based on the alterations in concentration of various type of lipids (Table 3) (Natesan & Kim, 2021). Our focus here is on specific lipid disease that is known as familial hypercholesterolemia (FH) that is associated with elevated levels of LDL cholesterol in the circulations.

Table 3. Descriptive classification of the dyslipoproteinemias (Natesan & Kim, 2021).

	LDL hypercholesterolemia	Hypertriglyceridemia	Mixed hyperlipoproteinemia	Low HDL
Cholesterol	Increased	Increased	Increased	Unchanged
Triglycerides	Unchanged	Increased	Increased	Unchanged
LDL cholesterol	Increased	Unchanged	Increased	Unchanged
HDL cholesterol	Unchanged	Decreased	Decreased	Decreased

Familial hypercholesterolemia (FH)

Familial hypercholesterolemia (FH) is one of the most common inherited diseases that is characterized by high levels of LDL cholesterol in circulation. Long-term exposure to high cholesterol results in tendon and skin xanthomas, arcus cornea, and vascular deposits that ultimately lead to atherosclerosis and coronary heart disease (CHD). Severe symptoms of FH can dramatically increase morbidity and mortality rates in affected patients. The heterozygous FH (HeFH) has a higher prevalence (1:200) compared to homozygous FH (HoFH) (1:160,000-300,000) (Cuchel et al., 2014; Goldstein & Brown, 1978; Nordestgaard et al., 2013).

The monogenic FH is divided into two distinct forms, namely autosomal recessive hypercholesterolemia (ARH) and autosomal dominant hypercholesterolemia (ADH). While ARH is caused by mutations in the low-density lipoprotein receptor adapter protein 1 (*ARH/LDLRAP1*) gene, ADH results from pathogenic variants in *LDLR*, apolipoprotein B (*ApoB*), and Proprotein convertase subtilisin/kexin type 9 (*PCSK9*). These three causative genes are the main pathogenic factors involved in ADH, and most patients carry diseases causing variation in the *LDLR* gene. Based on the type of defect in LDLR, FH can be classified into eight different groups (Table 4). Apart from mentioned factors, recent studies suggest that other novel genes also can implicate in ADH, such as apolipoprotein E (*ApoE*) and probably signal transducing adaptor family member 1 (*STAP1*). Indeed, many clinical and genetic reports show the implication of several heterozygous variations (Compound heterozygosity) in different genes in patients with FH (Abifadel & Boileau, 2023; Awan et al., 2013; Brown & Goldstein, 1986; Fokkema et al., 2011; Fouchier et al., 2014; Goldstein et al., 2001; Susan-Resiga et al., 2017).

In recent decades, numerous genome-wide association studies (GWAS) deciphered the involvement of many frequent and rare variants that associated with LDLc levels in circulation. This can explain the missing pathogenic variations (i.e., having no diseases causing variations in aforementioned genes) in ~20 % of FH patients and propose the presence of polygenic form of high LDLc levels in circulations (Abifadel & Boileau, 2023; Cupido et al., 2021; Talmud et al., 2013).

Table 4. Eight functional classes of FH diseases (Susan-Resiga et al., 2017).

Class	Phenotype	Example of mutant
1	No detectable LDLR protein	Stop 167
2	Either complete (2a) or partial (2b) block of transport of the LDLR from the ER	G565V
3	Defective LDL binding	D227E
4	Defective clustering in clathrin coated pits	Y828C
5	Recycling defective receptors	T454N
6	Sorting defect in polarized epithelial cells	G844D
7	Highly degraded by extracellular PCSK9	H327Y
8	Defective LDL delivery to lysosomes and resistance to extracellular PCSK9	R410S

I.I. II. Low-density lipoprotein receptors (LDLRs):

As it has been mentioned earlier, high levels of LDL cholesterol (LDLc) in blood circulation are a major hallmark for hypercholesterolemia and associated with high risk of cardiovascular diseases (CVDs). In this section we will see how LDL cholesterol levels can be regulated by their interaction with their specific receptor called low-density lipoprotein receptor (LDLR). This protein is related to the superfamily that consists of six more members including very-low-density lipoprotein receptor (VLDLR), LDLR related protein 1 (LRP1), 1b (LRP1b), 2 (LRP2; megalin), 4, (LRP4; MEGF7), and 8 (LRP8; ApoER2) (Chappell et al., 1992). While each receptor has its specific ligand, they have several structural motifs in common with other family members.

Introduction, structure, and function

It was mentioned previously that cholesterol can be obtained either by endogenous synthesis or diet. The primary pathway that removes circulating cholesterol is *via* low-density lipoprotein receptor (LDLR) (Slater et al., 1984). LDLR was discovered in 1974 as part of a study of the molecular process behind familial hypercholesterolemia (FH) when Goldstein and Brown demonstrated the defect in LDLR in the fibroblast of patients with homozygous FH (Brown & Goldstein, 1974). Furthermore, they performed a vast investigation on LDLR regulation and realized that LDLR levels are regulated by a feedback mechanism that is controlled by intracellular levels of cholesterol. Hence, high levels of intracellular cholesterol cause lower expression of LDLR, and *vice versa* (Brown & Goldstein, 1975). These preliminary studies led to additional research by other scientists throughout the world. Since then, LDLR proteins were isolated in 1982, and many structural and functional analyses have been done to characterize the functional importance of natural mutations in the LDLR (Brown et al., 1997; Schneider et al., 1982). So far, an ample number of missense mutations have been found on LDLR, and now it is clear that loss of function mutations on this protein led to FH.

Human *LDLR* (NG_009060.1; gene ID: 3949) is located on chromosome 19 (19p13.1–13.3) with a length of ~45 kb encoding 18 exons (https://www.ncbi.nlm.nih.gov/gene/3949). LDLR is a type I transmembrane protein including 839 amino acids. This protein consists of five domains containing LDLR repeat domain, epidermal growth factor (EGF) repeat domain, O-linked glycosylation domain, transmembrane domain, and cytosolic domain (Fig. 5).

The LDLR repeat domain, which is the main interaction site with various ligands (e.g., apolipoprotein B100; ApoB100 and E; ApoE), consists of seven homologous repeats (LR1-LR7 that each has ~40 amino acids; aa). The acidic charge of this region causes the ionic interaction with basic residues on either ApoB100 or ApoE (Mahley et al., 1980; Shireman et al., 1977; Yamamoto et al., 1984). The EGF repeat domain is made of three repeated sequences, in which the third repeat (EGF-C) is attached to the first two repeats (EGF-A and EGF-B) by six-bladed β-propeller (includes six repeats of YWTD motif; tyrosine, tryptophan, threonine, and aspartate) (Jeon et al., 2001; Russell et al., 1984). This domain that includes 411 aa has an important role in lipoprotein release (*via* YWTD motif) and receptor recycling pathways (Rudenko et al., 2002). Given that many missense mutations that occur in LDLR are located on EGF repeat, this domain seems to be one of the most critical parts of LDLR. Following the EGF domain, the 58 aa O-linked glycosylation section carries GalNAc-type O-glycans enriched with serine residue. This domain acts as a spacer between the EGF repeat and transmembrane. Indeed, it has been demonstrated (primarily in Chinese hamster ovary cells; CHO) that this domain prevents the proteolytic cleavage (shedding) of the extracellular domain of LDLR and enhances its stability (Kozarsky et al., 1988; Russell et al., 1984). The transmembrane domain of LDLR has ~25 aa and is responsible for anchoring/attaching the receptor to the cell surface (Brown et al., 1997). The last module of LDLR is a cytosolic domain which is critical for the endocytosis of the LDLR and LDLc to the clathrin-coated vesicles. This section includes 56 aa with the specific sequence of residues (NP_XY; asparagine-proline-any amino acid (x)-tyrosine) that interact with adaptor proteins such as autosomal recessive hypercholesterolemia protein 1 (ARH1) to initiate the internalization of the complex (will be detailed in the following sections). Apart from the NP_XY sequence, it seems that the first 22 aa of the receptor tail is also required for the proper endocytosis. Indeed, it was suggested that the Phenylalanine residue at position 802 is also important for the internalization of the receptor (Chen et al., 1990; Davis, van Driel, et al., 1987). Interestingly, it seems that the glycine residue at position 823 could play an important role in the proper sorting of the newly synthesized receptor to the cell surface (Garcia et al., 2001; Koivisto et al., 2001). In addition to the spacer region of LDLR, the N-terminal of LDLR seems to have O-glycans that may be important for the LDLR function (Kozarsky et al., 1988). Researchers have shown the presence of potential N-glycosylation sites on the repeat domain of LDLR that can play an important role in

LDLR metabolism as a negative regulator of LDLR expression (Fig. 6) (van den Boogert et al., 2019).

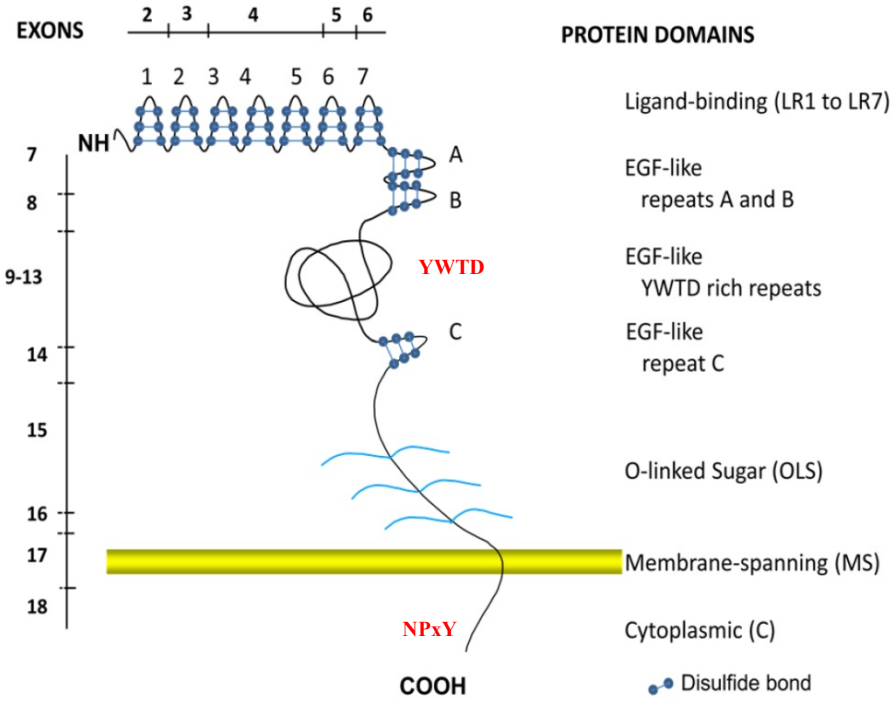


Fig. 5. Schematic of LDLR structure and its domains (Mathilde & Jean-Pierre, 2012).

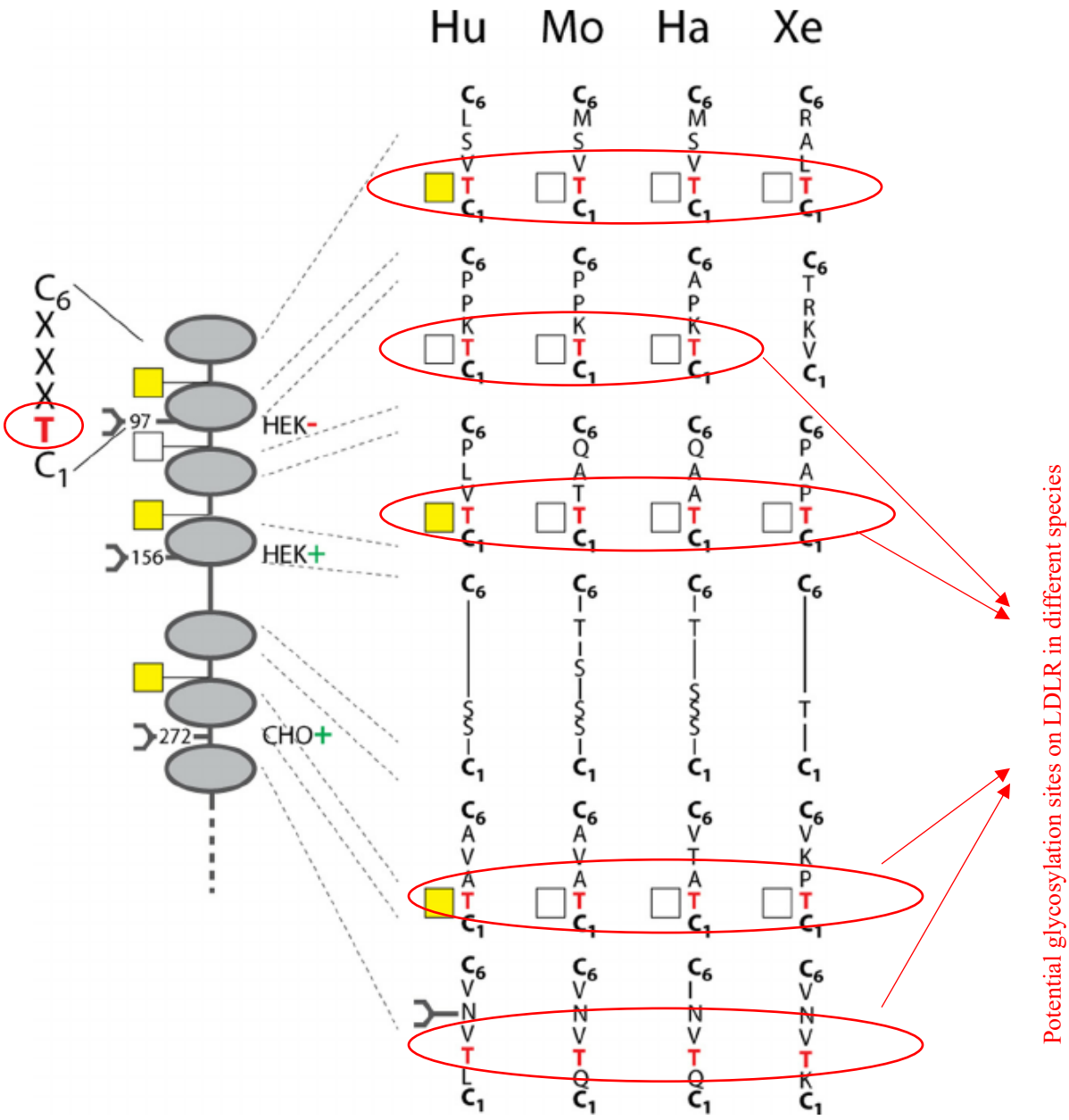


Fig. 6. Schematic diagram of glycosylation sites of LDLR. "O- and N -glycosites identified in human (Hu) LDLR are designated as described in the legend to Fig. 1 and conserved Thr residues in mouse (Mo), hamster (Ha), and X. laevis (Xe) LDLR are shown in red with a square to illustrate potential O-glycosites. Note also that the putative N -glycosylation site in repeat 2 (Asn 97, sequence not shown) and the N -glycosite in the linker between repeats 6 –7 (Asn 272) is conserved".(Pedersen et al., 2014)

LDLR regulation and trafficking

The biosynthesis of LDLR happens in the rough endoplasmic reticulum (ER), where the ~110kDa precursor proteins, also known as immature LDLR (iLDLR), are made. Over there, iLDLR partially underwent some post-transcriptional modifications (PMs) (e.g., the N-glycosylation of repeat domain). Further PMs (e.g., the O-glycosylation of spacer domain) occur in the Golgi network and result in the formation of ~150kDa mature LDLR (mLDLR) that go to the cell surface for its regulatory function. The receptor-associated RAP (LRPAP1 or α2-macroglobulin receptor-associated protein) acts as a chaperone to direct the LDLR receptor from ER to Golgi, where it dissociates from the receptor because of the lower pH (Fig. 7) (Bu, 2001).

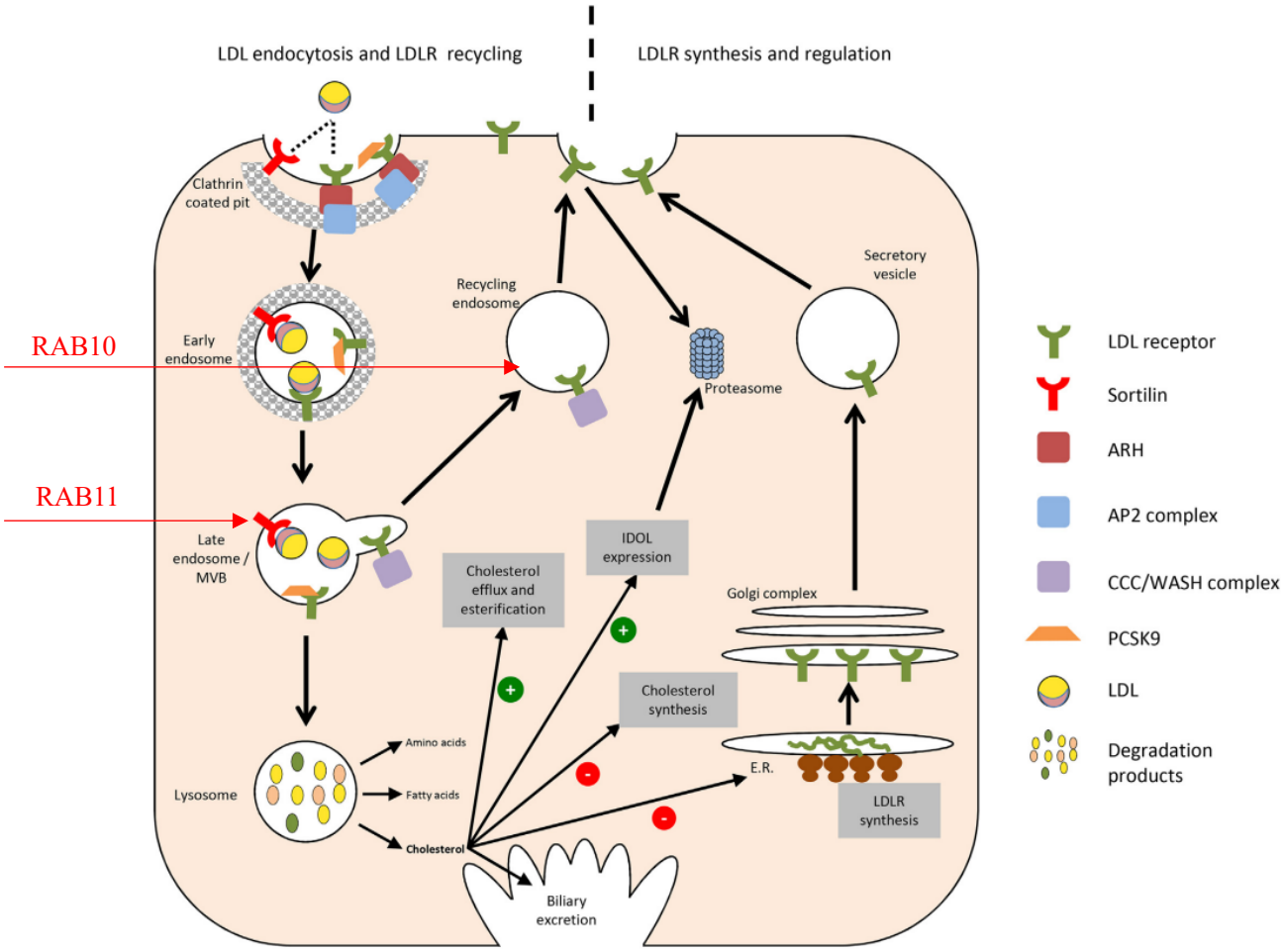


Fig. 7. LDLR and LDLc regulation and trafficking (Zanoni et al., 2018).

When LDLR reaches the cell surface, it can interact with LDL cholesterols and internalize them inside the cells *via* clathrin coated vesicles. The initiation, maturation, and growth of these vesicles are highly dependent on the proper levels of phosphatidylinositol-(4,5)-biphosphate (PI (4,5) P2) at the plasma membrane. The (PI (4,5) P2) is hydrolyzed soon after the detachment of clathrin-coated vesicles from the plasma membrane, (following with the formation of early endosomes). When there are sufficient levels of (PI (4,5) P2), adaptor proteins (i.e., ARH or Disabled-2; Dab2) interact with (PI (4,5) P2) and NP_XY motif of the cytosolic tail of LDLR and initiate endocytosis process (Maurer & Cooper, 2006; Sirinian et al., 2005). Upon internalization of the complex into the early endosomes, LDLR is dissociated from LDLc due to the acidic pH environment. The β-propeller domain of LDLR may interact with repeats 4 and 5 in the LDLR repeat domain and contributes to ligand release from the receptor following internalization once it encounters the low pH of the compartments (i.e., LDLR changes from open conformation to the closed conformation at acid pH) (Rudenko et al., 2002). Next, LDLc contours to the maturing and late endosomes and lysosomes for degradation and the majority of LDLR recycles back to the cell surface to be able to interact with more LDLc (Fig. 7). Efficient LDLR recycling needs specific proteins, including sorting nexins (SNXs) like SNX17 that interacts with the cytosolic tail of LDLR, an intact Wiskott-Aldrich syndrome protein, SCAR homolog (WASH), and COMMD/CCDC22/CCDC93 (CCC) complex (Fig. 8) (Bartuzi et al., 2016; Burden et al., 2004; Stockinger et al., 2002; Wijers et al., 2019).

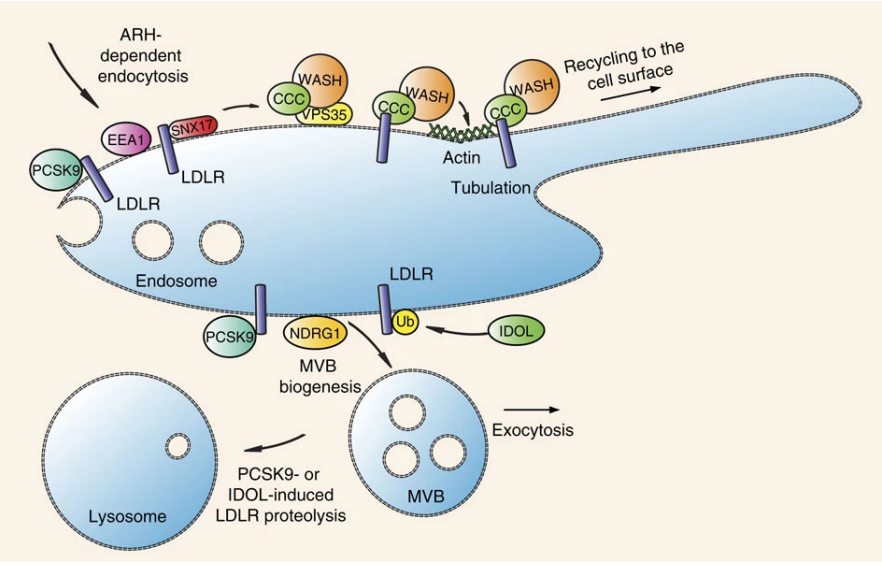


Fig. 8. Wash and CCC mediated LDLR sorting (Bartuzi et al., 2016).

Nevertheless, LDLR is not always able to get back to the cell surface and, in specific situations, will be targeted to the degradation pathway instead (Brown & Goldstein, 1979, 1986). In other words, the LDLR route changes to the degradation pathway when it cannot dissociate from LDLc in early endosomes or when targeted by specific proteins called proprotein convertase subtilisin/kexin type 9 (PCSK9), and inducible degrader of LDLR (IDOL) (Davis, Goldstein, et al., 1987; Lagace et al., 2006; Zelcer et al., 2009; Zhang et al., 2007). These two proteins (in the liver; it is mainly PCSK9) are the main factors that regulate LDLR expression levels post-transcriptionally (Fig. 7). Apart from PCSK9 and Idol, there are some other proteins that can independently regulate LDLR levels such as asialoglycoprotein receptor 1 (ASGR1), cyclase associated protein 1 (CAP1) (that can affect LDLR levels both in a PCSK9-dependent and -independent fashions), and resistin (both in PCSK9-dependent and -independent manners) (Fruchart Gaillard et al., 2023; Melone et al., 2012; Susan-Resiga et al., 2021).

The transcriptional regulation of LDLR is mainly regulated by intracellular levels of cholesterol. When cholesterol is sorted to the late endosomes and lysosomes, it can be transported to the ER, where it interacts with a particular transcription factor protein known as sterol regulatory element-binding protein (SREBP) and regulates the expression levels of LDLR and cholesterol endogenous biosynthesis. While low levels of cholesterol activate the transcription of the SREBP-2 transcription factor and subsequently increase the cholesterol biosynthesis and LDLR expression, high levels of cholesterol in the ER cause the retention of the complex in the ER reducing cholesterol biosynthesis and LDLR expression (Fig. 7) (Ikonen, 2008; Luo et al., 2020).

Expansion of close connections between membranes of endosomes with ER, Golgi, and cell surface, provides proper cholesterol homeostasis, degradation, and localization in various compartments (~80% of early endosomes are in close contact with ER). So far, many proteins proposed to be involved in this contact process, such as aster proteins, annexin A1, and steroidogenic acute regulatory protein (StAR)-related lipid transfer domain-3 (STARD 3), just to name a few (Eden et al., 2016; Friedman et al., 2013; Mesmin et al., 2013; Rowland et al., 2014; Sandhu et al., 2018; Wilhelm et al., 2017).

Exploring the physiological importance of LDLR: extending insights into lipid metabolism and beyond

Since the discovery of LDLR in 1975, many studies have been conducted to better understand the trafficking and function of this interesting protein in the human body. The results of numerous investigations in recent decades unraveled the importance of LDLR not only in cholesterol homeostasis but also in other cellular metabolisms.

LDLR is implicated in various cellular metabolisms due to its major role in cholesterol homeostasis. Interestingly, LDLR can interact with other lipoproteins, such as lipoprotein (a) or Lp(a), in a cholesterol-independent fashion. Lp(a) interacts with cell surface LDLR *via* its apolipoprotein B100 (ApoB100) compound. This interaction shows the regulatory effect of cell surface LDLR in Lp(a) clearance and is protective for atherosclerosis diseases (that are developed by high levels of Lp(a) in plasma) as it reduces Lp (a) levels in circulation. Intriguingly, treatments with monoclonal antibody mAb against PCSK9 (main negative regulator of LDLR) resulted in ~30% reduction in Lp(a) (McKenney et al., 2012; Raal et al., 2014; Romagnuolo et al., 2015; Romagnuolo et al., 2017; Roubtsova et al., 2023). Only recently have novel therapeutic approaches to significantly reduce Lp(a) levels been proposed and validated including the use of ASO or RNAi(Chan & Watts, 2023; Fujino & Nicholls, 2023; Koschinsky et al., 2023)

Furthermore, dysregulation of LDLR pathway can lead to lipid disorder-mediated organ injury. In fact, under specific circumstances (e.g., stimulus inflammation, high glucose, and renin-angiotensin system; RAS stimulation) the mammalian target of rapamycin (mTOR) cascade gets activated and disrupts LDLR pathway. Hence, the interference in cholesterol homeostasis causes lipid accumulation and foam cells. This process can affect various cells, such as hepatocytes, macrophages, VSMCs, and tubular epithelial cells. The downstream results of these modifications are different pathological conditions such as atherosclerosis, non-alcoholic fatty liver disease (NAFLD), and kidney fibrosis. Therefore, it is critical to better understand the trafficking and function of LDLR to prevent/control the adverse effect of many severe pathological conditions (Y. Zhang et al., 2016).

Studying LDLR and its function in cholesterol trafficking, along with investigating its major regulators such as PCSK9, helped scientists to find more therapeutic approaches in various patients with different lipid disorders. In general, current therapeutic approaches for lowering levels of

lipids and risk of cardiovascular diseases (CVDs) (either mono- or combination therapy) are as following (Brandts & Ray, 2023):

1. Treatments that directly lower the levels of cholesterol in the circulation including statins by reducing the biosynthesis of cholesterol *via* inhabitation of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (HMGCR; a rate limiting enzyme in cholesterol biosynthesis), ezetimibe by inhibiting cholesterol absorption in the intestine, and bempedoic acid that is the small molecule inhibitor of ATP citrate synthase (ACLY) in cholesterol biosynthesis pathway.
2. PCSK9 targeting treatments such as antisense oligo nucleotide (ASO; AZD8233), siRNA inclisiran, monoclonal antibodies (mAbs) including alirocumab and evolocumab, synthetic polypeptides like adnectins (BMS-962476 and LIB003), vaccine, and cyclic peptides.
3. Targeting circulating angiopoietin-related protein 3 (ANGPLT3) by evinacumab (a new mAb approach that FDA and EMA approved in 2021) that prevents the inhibitory function of ANPLT3 on lipoprotein lipase (LPL) and endothelial lipase (EDL) activities. This prevention enhances the hydrolyzation of triglycerides and reduces circulating LDLc levels independent of LDLR presence. Other medications that can affect ANPLT3 are siRNA-based (ARO-ANG3) or ASO (vupanorsen).
4. Using omega-3 fatty acids that reduce circulating triglycerides and triglycerides-rich lipoproteins.
5. Inhibiting the cholesteryl ester transfer protein (CETP) and decreasing cholesterol exchange from HDL to LDL and VLDL *via* using small molecules called obicetrapib.
6. Targeting ApoC3 by ASOs such as volanesoren and olezarsen.
7. Targeting Lp(a) either by ASOs (placarsen) or siRNA (olpasiran, LY3819469, and SLN360) approaches.
8. Increasing HDL-mediated cholesterol efflux by increasing ApoA-I levels (using plasma derived ApoA-I; CSL112).
9. Increase the activity of Lecithin–cholesterol acyltransferase (LCAT) enzyme by infusion of recombinant human LCAT (ACP-501).

Although LDLR is known for its broad function in lipid metabolism, its role in the human body is beyond that. An interesting example is LDLR regulatory effect on the immune system.

Before it was known that LDLR is an important player in the regulation of $CD8^+$ T cell antitumor activity *via* its interaction with cholesterol. However, in 2021 researchers found a novel non-canonical function of LDLR on T cell receptors (TCRs). It is known now that $CD8^+$ T cells need to have a proper amount of cholesterol uptake to have efficient priming and clonal expansion in cancerous cells. Indeed, lower cholesterol intracellular levels (due to reduced uptake of cholesterol) will decrease $CD8^+$ T cell proliferation. Interestingly, cholesterol increases the activation and granule production of naive $CD8^+$ T cells and not activated CTLs. These data together show the importance of cholesterol in $CD8^+$ T cell's function (specifically naïve cells) in cancerous cells (Kidani et al., 2013; Yang et al., 2016; Yuan et al., 2021).

After priming and clonal expansion of naive $CD8^+$ T cells and upon stimulation with specific antibodies such as anti-CD3, these cells get activated and transferred into the effector stage, in which they are called activated CTLs. Although the effector function of activated CTLs is neither affected by intracellular nor plasma membrane cholesterol, it is regulated by LDLR expression levels. This non-canonical cholesterol-independent function of LDLR is due to the direct interaction of LDLR with the CD3 subunit of TCR complex. Following this interaction, CD3 is phosphorylated and activates the downstream signaling pathway of TCR. This initiation of TCR signaling is critical for T cell activation and effector function. These surprising data revealed the new direct function of LDLR in immune response activation in cancerous cells. As PCSK9 is the main negative regulator of LDLR, targeting this protein can be a great approach to increase the LDLR levels in immune cells and boost the subsequent immune responses *via* its interaction with TCR complex (Yuan et al., 2021). This study is a great example that shows the importance of studying cellular metabolism pathways more comprehensively and having a broader view of their novel functions in the human body.

I.I. III. Proprotein convertases family (PCs):

The intriguing prediction of the presence of specific secretory mammalian proteases recognizing single or pairs of basic amino acids goes back to the mid-1960s, when scientists were interested in studying hormones such as insulin, melanocyte-stimulating hormones (α-MSH and β-MSH), and β-endorphin. Working on these hormones brought the idea that these hormones are first produced as an inactive precursor protein and then get activated by cleavage (by certain cellular proteases) at the specific paired basic amino acids. Subsequent studies revealed that such processing pathways also implicate in activation of many other secretory proteins. It took more than 15 years for investigators to identify the enzymes responsible for the activation of secretory precursor proteins. This post-translational regulatory mechanism is now called limited proteolysis and is responsible for the processing of various precursors to either activate or inactivate them to have their proper function in the body. In humans, proteases are a group of ~600 enzymes, divided into five distinct categories based on their catalytic mechanism: cysteine-, serine-, threonine-, aspartic-, and metalloproteases. Their function in coordinating numerous physiological processes led to the development of promising therapeutic approaches. Indeed, ~5-10 % of medications target specific proteases (Chretien, 2013; Chretien et al., 1976; Chretien & Li, 1967; Deu et al., 2012; Kappelhoff et al., 2017; Puente et al., 2003; Seidah et al., 1976; Seidah & Prat, 2012; Steiner, 2011; Steiner et al., 1967).

The next major milestone happened in the mid-1980s, when yeast cells lacking Kex2 protein (Kexin; a serine protease of the subtilisin family) in *saccharomyces cerevisiae*, suggested that this is a key protease required for the proteolytic processing of pro-α-mating factor precursor into the bioactive α-mating factor hormone. This breakthrough encouraged researchers to chase Kex2-like homologs in mammals, which led to the discovery of the 9-membered proprotein convertase (PC) family. Identification of all 9 convertases took ~13 years from 1990 to 2003 when the last member of the family (PCSK9) was discovered by Seidah and his group (Julius et al., 1984; Mizuno et al., 1988; Seidah et al., 2003; Seidah & Prat, 2012; Van de Ven et al., 1990).

The mammalian PC family is a group of serine proteases related to bacterial subtilisns and yeast kexin (genes *PCSKs*) that are involved in the irreversible post-translational modification (PTM) of various secretory proteins to regulate their homeostasis. This family has nine members including PC1 (*PCSK1*) and PC2 (*PCSK2*) along with its specific chaperone called 7B2, Furin

(*Fur*), PC4 (*PCSK4*), PC5 (*PCSK5*), PC6/PACE4 (*PCSK6*), PC7 (*PCSK7*), SKI-1/SP1 (*PCSK8*), and PCSK9 (*PCSK9*). The first seven members of this family process their subsequent precursor at basic residues within the motif (K/R)-Xn-(R)↓, where Xn are residues comprising 0, 2, 4, or 6 aa. Conversely, the last two members of PCs cleave their substrates at non-basic residues within the motifs R-X-Aliphatic-Z↓, where X is any residue except Pro and Cys, and Z is any aa except Val, Pro, Cys, or Glu for SKI-1, and (V/I/L) FAQ↓ for PCSK9 (Fig. 9) (Benjannet et al., 2004; Naureckiene et al., 2003; Pasquato et al., 2006; Seidah & Chretien, 1999; Seidah & Guillemot, 2016; Seidah & Prat, 2012).

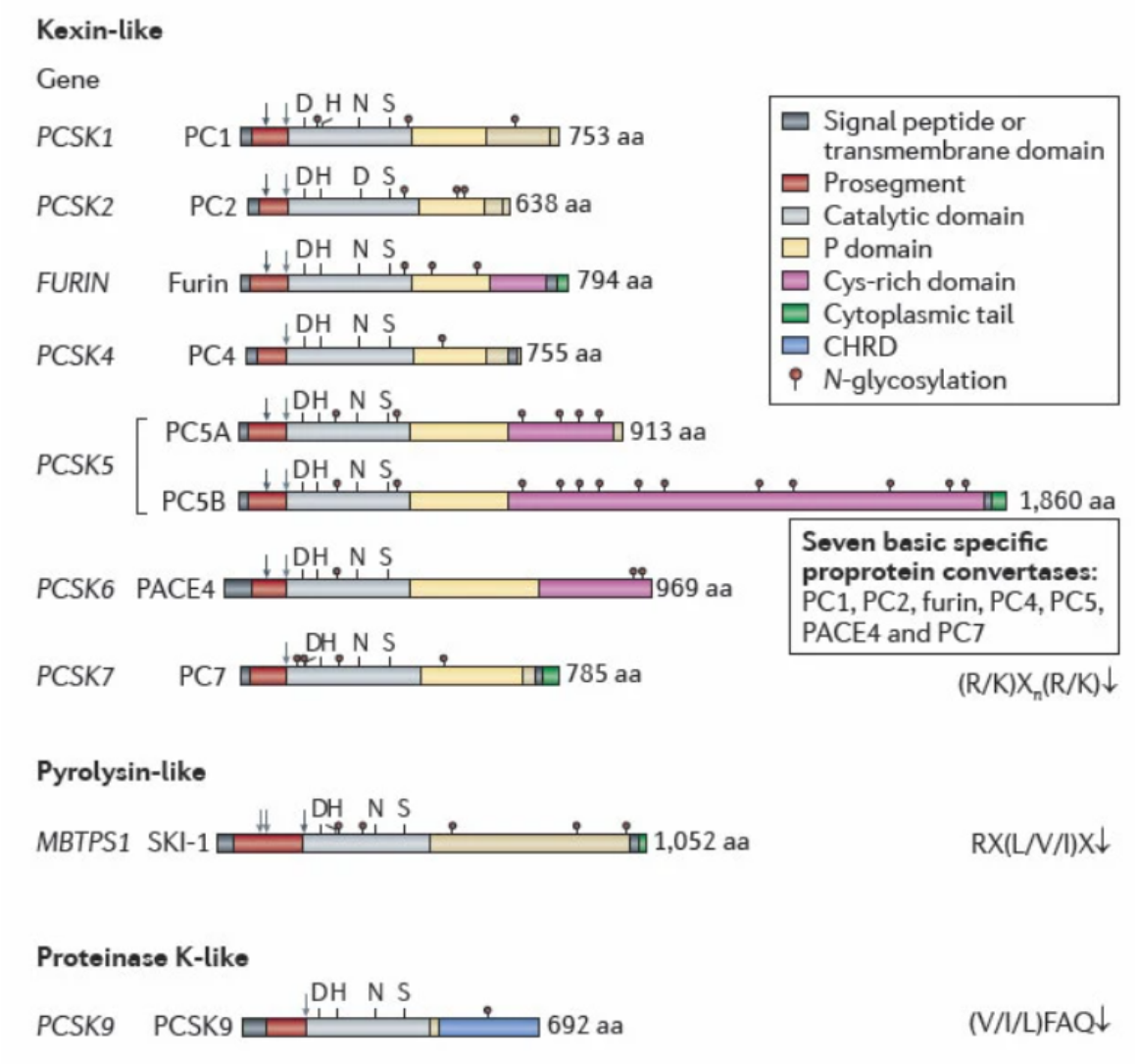


Fig. 9. Proprotein convertases family members (Seidah & Prat, 2012).

Proprotein convertases are sorted from the ER to their destination compartments to interact with their substrates/targets. The tissue expression and subcellular localization of each PC member is distinct as they have their specific group of substrates/targets. Because PCs interact with various types of proteins, dysregulation of their enzymatic activities can lead to a spectrum of varied human pathologies, such as cardiovascular diseases, diabetes, cancer, inflammation, neurodegenerative diseases, and immune diseases. One recent example of PCs' implications in human diseases is the regulatory effect of Furin, SKI-1, and PCSK9 on severe acute respiratory

syndrome coronavirus-type 2 (SARS-CoV-2) discovered during COVID-19 pandemic (Cendron et al., 2023; Essalmani et al., 2023; Essalmani et al., 2022; Seidah & Prat, 2012).

The primary structure of all PCs includes the signal peptide, prosegment, catalytic domain, and C-terminal domain that has a unique sequence in each PC member (Fig. 9). Holistically, the zymogen processing of each PC (except for PC2) starts in the ER, whereby the signal peptide is removed from the nascent protein by signal peptidases followed by N-glycosylation of the zymogen. Although the prosegment acts as an inhibitor, it also functions as a chaperone for the correct folding of the zymogen in the ER. In the next step, the precursor undergoes autocatalytic cleavage of a specific motif within its N-terminal prodomain. This primary auto-processing cleaves prosegment and allows the proprotein to exit ER towards Golgi (TGN; trans-Golgi network) on route to its final destinations. Upon cleavage of prosegment, the latter remains non-covalently associated with the catalytic domain of the protease and prevents substrate binding to this domain in trans. Thereby, the prodomain-protease complex requires a second autocleavage in an acidic environment (Golgi, TGN, endosomes) to remove this inhibitory prosegment chaperone. Then, the mature protease is sorted to its destination compartment(s), and cleavage of specific substrates can then occur to form mature products (Fig. 10). Although the above process is the general maturation mechanism for most PCs, each member undergoes distinct trafficking. For instance, the intact proPC2 forms a complex with its chaperone named 7B2 in the ER and the complex proPC2-7B2 is transported to acidic immature secretory granules to autocatalytically mature into active PC2 that then detaches from 7B2. Apparently, PC4 and PC7 do not need a second step for activation. The subcellular localization of each PC to distinct compartments is summarized in Table 5 (Benjannet et al., 1992; Cendron et al., 2023; Mbikay et al., 2001; Rousselet et al., 2010; Seidah & Prat, 2012).

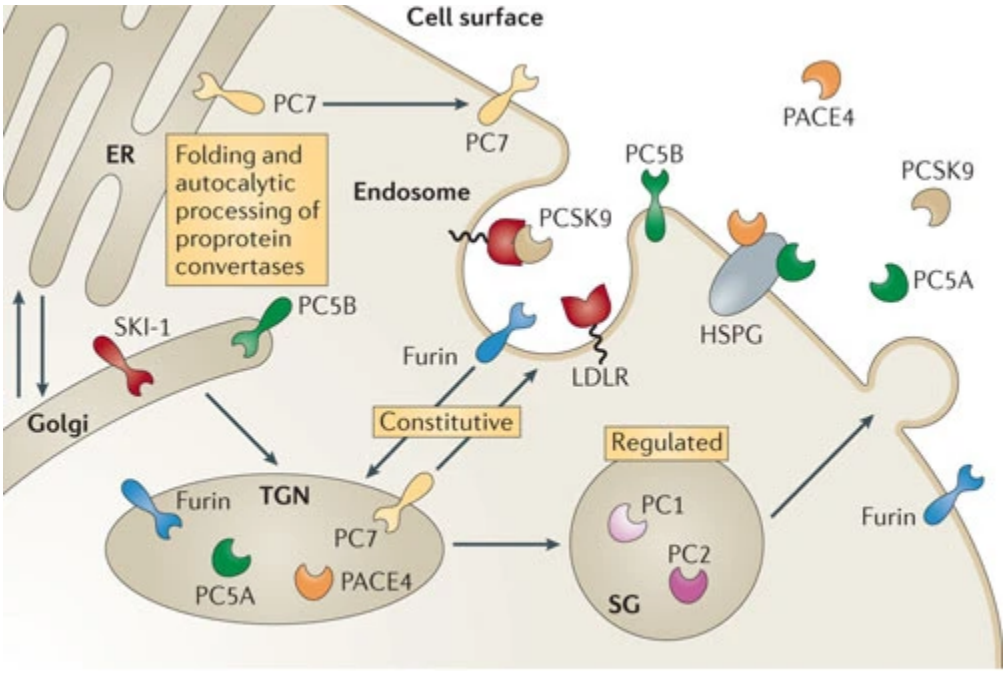


Fig. 10. Subcellular localization of proprotein convertases (Seidah & Prat, 2012).

Since the discovery of PCs, many inhibitors have been developed to target the catalytic activity of these proteins. These efforts led to the development of different types of inhibitors including protein-based (e.g., serpins; natural PI8, engineered forms of α2-macroglobulin, and α1-antitrypsin Portland, α1-PDX), peptide-based (e.g., D-Arg-based peptides, decanoyl-RVKR-chloromethyl ketone; dec-RVKR-cmk, MI-701, MI-052, and MI-1851), and small molecules (e.g., 2,5-Dideoxystreptamine, and bioavailable small-molecule BOS-981; developed by Boston Pharmaceuticals). Although none of these inhibitors have been approved by FDA yet (due to their pharmacokinetic limitations), they provide a great approach to better study the function of PCs and look for their new substrates and activities (Becker et al., 2010; Cendron et al., 2023; Jiao et al., 2006; Paszti-Gere et al., 2022).

Table 5. Expression and subcellular localization of proprotein convertases. adapted from (Seidah & Prat, 2012).

Proprotein convertase	Tissue distribution	Subcellular localization	Secretion
PC1	Neuroendocrine	Acidic regulated secretory granules	Secreted
PC2			
Furin	Ubiquitous	TGN, cell surface, endosomes	Shed
PC4	Germinal	Cell surface?	Shed
PC5	Widespread: adrenal cortex, intestine, kidney, ovary	Cell surface, ECM	Secreted PC5A; shed PC5B
PACE4	Widespread: muscle, heart, pituitary, intestine, cerebellum, kidney	Cell surface, ECM	Secreted
PC7	Ubiquitous	TGN, cell surface, endosomes	Not secreted
SKI-1 (also known as S1P)	Ubiquitous	*cis*- and *medial*-Golgi	Not secreted
PCSK9	Liver, intestine, kidney	TGN, extracellular	Secreted

ECM, extracellular matrix; PACE4, paired basic amino acid cleaving enzyme 4; PC1, proprotein convertase 1; PCSK9, proprotein convertase subtilisin kexin 9; SKI-1, subtilisin kexin isozyme 1; TGN, *trans*-Golgi network.

I.I.III.I Proprotein convertase subtilisin/kexin type 6 (PCSK6):

PCSK6, also known as paired basic amino acid cleaving enzyme 4 (PACE4) or subtilisin-like proprotein convertase 4 (SPC4), is the 6th member of the PC family (here will be called PACE4). PACE4 is less studied compared to other PC members such as Furin and PCSK9. However, recent studies have shown its importance and emerging role in various human diseases such as cancer.

Discovery and tissue expression

PACE4 mRNA sequence was identified in our group in 1991 along with PC4 and PC5/6. The mRNA sequence was isolated from rat testis while hunting for new convertase family members using RT-PCR methods. However, in the same year, Kiefer et al. reported the complete sequence of PACE4 in human hepatoma HepG2 cells (Kiefer et al., 1991; Seidah, 2011). This protein is ubiquitously expressed in all major tissues, namely liver, heart, spleen, kidney, pancreas, and lung (Karlsson et al., 2021).

Structure and trafficking

The *homo sapiens PSCK6/PACE4* gene (gene ID: 5046) is located on chromosome 15 (15q26.3) and includes 23 exons. The full-length human proPACE4 protein (NP_001278238.1) includes 969 amino acids with a molecular mass of ~100kDa. Resembling other PCs, PACE4 also consists of a signal peptide, prodomain, catalytic domain, and C-terminal (Fig. 11). This protein has three distinct cleavage sites at positions A63 (for cleavage of signal peptide), R117 (for second auto cleavage of prodomain), and R149 (for primary auto cleavage of prodomain). The N-terminal of mature PACE4 comprises the active-site residues, including aspartic acid (D205), histidine (H246), and serine (S420). The C-terminal domain of PACE4 is enriched with cysteine residues and is therefore called Cys-rich domain (CRD). The CRD has three N-glycosylation sites (at positions N259, N914, and N932) that are needed for correct folding and subcellular surface localization and interaction of PACE4 with other proteins such as heparan sulfate proteoglycans (HSPGs; specific polysaccharides found at the cell surface or extracellular matrix to mediate several cellular activities by increasing protein-protein interactions) (Seidah, 2011; Seidah & Prat, 2012).

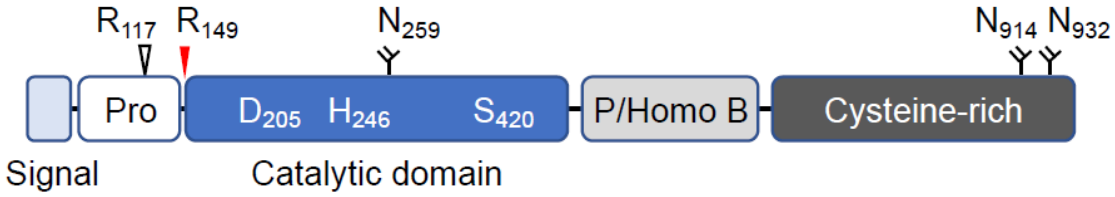


Fig. 11. Schematic presentation of different domains of PACE4. Adapted from (Wu & Chen, 2022)

Like PCs, PACE4 primary autocatalytic cleavage (RVKR↓149) occurs in the ER to form the heterodimer of cleaved prodomain with the rest of PACE4. Thereupon the cleaved prodomain-PACE4 complex is sorted to the TGN on route to the cell surface, where it is retained by either HSPGs or tissue inhibitors of metalloproteinases (TIMPs). At the cell surface, the second cleavage (KR↓117) occurs to release the inhibitory prodomain. Interestingly, the maturation processing of PACE4 is much slower compared to other PCs such as PC1, probably due to its CRD domain as its removal (by generating the engineered PACE4-BCRD construct that lacks the CRD domain) expedites the maturation process (Mayer, Hamelin, et al., 2008; Tsuji et al., 2003). However, the CRD domain of PACE4 is needed for its attachment to HSPGs (Mayer, Hamelin, et al., 2008; Nour et al., 2005).

The direct interaction of PACE4 to HSPGs, enables PACE4 to interact with its subsequent substrates (e.g., endothelial lipases, lipoprotein lipases, and N-cadherin) to either activate or inactivate them (Jin et al., 2005; Maret et al., 2012).

Potential substrates of PACE4

PACE4 and Furin are ubiquitously expressed in many tissues and mostly share common substrates. However, each protein has its specific substrate depending on its cellular localization or tissue expression. So far, PACE4 was shown to have diverse physiological roles in the human body by targeting various types of substrates. Growth factors are one of the best-known substrates of PACE4. For example, platelet-derived growth factor B (PDGF-B) is a critical growth factor in maintaining normal tissue growth and division. Defects in this protein and its unbalanced expression have been correlated with various cancers. In 2005, it was shown that PACE4, along with other PCs (i.e., Furin, PC5, and PC7), is implicated in the processing of proPDGF-B to its

mature PDGF-B form (Siegfried et al., 2005). Insulin-like growth factors (IGFs) are other growth factors that are processed by PACE4 (more specifically, pro-IGF-II) but not Furin. The maturation of IGFs regulates myoblast differentiation *via* phosphatidylinositol 3-kinase (PI3K) pathway (Yuasa et al., 2009). Growth differentiation factor 15 (GDF15) also needs to be proteolytically cleaved by PCs (i.e., Furin, PC5, and PACE4) to get matured and secreted from cardiomyocytes (Li et al., 2018).

PACE4 is implicated in cancer progression and invasion *via* processing certain matrix metalloproteinases (MMPs) such as MMP-9. While PACE4 increases the activity of MMP-9 and reduces the secretion of tissue inhibitor of metalloproteinase 1 (TIMP-1), Furin has the opposite effect and impedes MMP-9 activity (Lapierre et al., 2007). In keratinocytes, PACE4 cleaves and activates membrane type matrix metalloproteinases (MT-MMPs) such as MT1-MMP and MT2-MMP. Upon activation of MT1-MMP and MT2-MMP, collagen type IV gets degraded. This leads to disruption of the membrane architecture resulting in tumour microinvasion (Bassi et al., 2005).

PACE4 can also process and regulate the activity of other serine proteases like Corin, mannose-binding lectin–associated serine protease 3 (MASP-3) and protein C. Corin activity is greatly enhanced upon its cleavage by PACE4. Active Corin processes pro-atrial natriuretic factor into ANF, implicated in the regulation of hypertension and heart disease (Dong et al., 2013; Wang et al., 2018). *In vitro* studies have shown that both PC5 and PACE4 can activate MASP-3, which is necessary to maintain the normal functioning of alternative pathway of the complement system in the innate immunity (Oroszlan et al., 2021). Protein C is another serine protease and a vitamin K-dependent anticoagulant that is cleaved by Furin, PC5/6A, and PACE4 (Essalmani et al., 2017).

Other substrates of PACE4 consist of various cell surface proteins, including insulin receptor isoform B (IRB) and proteinase-activated receptor 1 (PAR1). While Furin cleaved both isoforms of IR (i.e., IRA and IRB), PACE4 only processes IRB when the activity of Furin is impaired (Kara et al., 2015). PAR1 is an inflammation-associated receptor that is cleaved by various PCs (Furin, PC5, PC7, and PACE4) and correlates with HIV-associated neurocognitive disorders (HAND) (Kim et al., 2015). Indeed, it is proposed that PACE4 might be involved in the cleavage and shedding of transferrin receptor 2 (TfR2; either directly or indirectly *via* activating metalloproteinases) and that way involves in iron metabolism (Guillemot & Seidah, 2015; Pagani et al., 2015).

Angiopoietin-related protein 3 (ANGPTL3) and 4 (ANGPTL4) substrates can be cleaved with both Furin and PACE4. The presence of HSPGs increases the protein-protein interaction between PACE4 and ANGPTL3/4 (major inhibitors of lipoprotein lipases; LPLs) and their activation acquired upon protease activity of PACE4. Cleaved ANGPTL3/4 inhibit the activity of LPLs and lead to higher TG levels (Essalmani et al., 2013; Lei et al., 2011).

Apart from the above-mentioned substrates, there are many more proteins that are reported to be processed by PACE4. Some examples of these substrates include lysyl oxidase-like 2 ($LOXL_2$; implicated in several hepatic and vascular fibrotic diseases and cancers) (Okada et al., 2018), group X secretory phospholipase A2 (GX $sPLA_2$; that hydrolyze membrane phospholipids and is cleaved by both Furin and PACE4) (Layne et al., 2015), nodal protein (involves in Left-right asymmetry) (Constam & Robertson, 1999), bone morphogenetic proteins (BMPs; involved in development of human organs) such as BMP-4 (Constam & Robertson, 1999; Cui et al., 1998), bacterial toxins like anthrax toxin protective antigen (PA) and diphtheria toxin (Gordon et al., 1997; Sucic et al., 1999), viral protein Vpr (contribute to HIV diseases) (Xiao et al., 2008), neural peptidase Dynorphin B (Dyn B) (Watanabe et al., 2013), as well as hepatic peptidase hepcidin (master regulator of iron metabolism) (Scamuffa et al., 2008).

Moreover, a naturally identified spliced form of PACE4 (PACE4-alt-CT) results in activation of the pro-growth differentiation factor (pro-GDF) in different cancers, such as prostate cancer. PACE4-alt-CT leads to the expression of a C-terminally modified PACE4 protein that is retained intracellularly (Couture et al., 2017; Seidah & Prat, 2012).

The physiological importance of PACE4

As PACE4 has been implicated in diverse biological processes, it is important to have balanced expression levels of it in various tissues. Indeed, due to the redundancy and secretion of PACE4, it can be considered as an intriguing biomarker for cancer prognosis, either in tissue or circulation. Here, we review the most important findings regarding the involvement of PACE4 in various pathophysiological events.

1. Animal studies

PACE KO mice resulted in deleterious symptoms (e.g., cardiac malformation, bone morphogenesis defect, and severe left/right axis defects) and a 25% death rate. In other words, although the heterozygous PACE4 KO mice are normal and fertile (regardless of the background of the mice),

the homozygous KO (either in C57BI/6J -129/SvEv hybrid or 129/SvEv inbred) results in 25% death rate along with severe phenotypes. These indications are mostly due to the regulatory effect of PACE4 on transforming growth factor beta (TGF-β) superfamily members such as nodal and BMP-4 proteins (Beck et al., 2002; Constam & Robertson, 2000a, 2000b, 2000c). Regardless, its seems that in mice with C57BL/6 background, these deleterious phenotypes are absent or less severe (Malfait et al., 2012a). Homozygous PACE4 KO mice with a C57BL/6 background are fertile and show that the absence of PACE4 alleviates pain in osteoarthritis mice (Malfait et al., 2012a).

In homozygous PACE4 KO mice (with a 25% death rate), the survived three-quarters look normal with no major complications in many critical processes such as immune response, blood coagulation, as well as liver and kidney function both in male and female mice. This suggests that in the absence of PACE4, other PCs, such Furin may compensate its lack for various substrates. Nevertheless, in aged female mice (> nine months), the absence of PACE4 disrupts the function and structure of the ovary (Constam & Robertson, 1999, 2000b, 2000c; Mujoomdar et al., 2011). An interesting observation that shows the sex-depended function of PACE4 in the body. Data from humans with ovarian dysfunction suggested that these phenotypes could be due to the possible proteolytic activity of PACE4 on potential ovarian substrates such as GDF9 and BMP-15 (Dixit et al., 2005; Dixit et al., 2006; Marinakis et al., 2022).

2. Human diseases and mutations

As mentioned in animal studies, it seems that the absence of PACE4 could reduce pain in osteoarthritis models. Interestingly, in humans also, the defect in PACE4 correlates with pain protection in patients with osteoarthritic knees (Malfait et al., 2012b). This phenotype probably is because of the proteolytic activity of PACE4 on major aggrecan-degrading enzymes such as a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS-4) and 5 (ADAMTS-5). Thereby, the defect in PACE4 reduces the activity of ADAMTS-4 and -5 and subsequently decreases the aggrecan degradation that involves in the pathogenesis of the osteoarthritis (Malfait et al., 2008).

So far, various Single-nucleotide polymorphisms (SNPs) on PACE4 were shown to be correlated with penetrance of several complications such as left-handedness (due to PACE4 effect

on nodal and lefty proteins), defects in hand skills, and dyslexia (Arning et al., 2013; Brandler et al., 2013; Constam, 2009).

Additionally, PACE4 can be involved in various cardiovascular events in the human body including lipoprotein metabolism (*via* cleavage of ANGPLT3 and 4) (Essalmani et al., 2013; Lei et al., 2011), hypertension (*via* activating corin) (Wu & Wu, 2003), vascular remodeling in atherosclerosis (*via* activating various MMPs), cardiac repair after myocardial infarction (MI) (*via* TGF-β) (Rykaczewska et al., 2020), and cardiac aging (possibly by affecting DNA-damage inducible transcript 3 (Ddit3) factor) (Zhan et al., 2022). Indeed, PACE4 variants correlate with interatrial septal aneurysms and atrial septal defects, possibly *via* the regulatory role of odd-skipped-related 1 (OSR1) and T-box transcription factor 5 (TBX5) transcription factors (K. K. Zhang et al., 2016).

The upregulated expression of PACE4 has been confirmed in a variety of cancers including skin, prostate, lung, breast, thyroid, and ovarian cancers (Bakrania et al., 2020; Chen et al., 2021; Cheng et al., 1997; Couture et al., 2022; Fradet et al., 2018; Lin et al., 2015; Longuespee et al., 2014; Mbikay et al., 1997; Panet et al., 2017; Patel et al., 2022). Likewise, high expression levels of spliced form PACE4-alt-CT have been observed in cancers such as prostate cancer (Couture et al., 2017; Couture et al., 2022).

Inhibition of PACE4 using Ac-[DLeu]LLLRVK-Amba inhibitor, reduces the tumor proliferation and tumorigenesis both in cells and LNCaP xenograft model of prostate cancer (Couture et al., 2015; Levesque et al., 2015). These findings shed light on the possible new therapeutic merit of targeting PACE4.

I.I.III. II. Proprotein convertase subtilisin/kexin type 9 (PCSK9):

Proprotein convertase subtilisin/kexin type 9 (PCSK9), originally known as neural apoptosis-regulated convertase 1 (NARC1) (Seidah et al., 2003), is one the most interesting and well-studied PC members that is the master regulator of LDLR.

Discovery

Discovery of PCSK9 is owed to the comprehensive investigation of the 8th member of the family (SKI-1/SP1). In fact, the identification and characterization of SKI-1/SP1, as a distinct family member, encouraged scientists to look for novel homologues of this protein. Ultimately, in 2003, Seidah and his group provided the complete cDNA sequences of the last member of the family (they renamed it PCSK9) in human, rat, and mouse using polymerase chain reaction (PCR) methods (Seidah et al., 2003). Fascinatingly, extensive work of Seidah's group on *PCSK9* revealed that its chromosomal location is extremely close to a novel locus (1p34.1p32) identified in large French families with hypercholesterolemia. Previous clinical and co-segregation analyses found the association of this locus with increased levels of hepatic cholesterol in FH patients with no pathogenic defects in either *LDLR* or *ApoB*. Thereby, this specific locus is proposed as a 3rd locus implicated in familial hypercholesterolemia (FH3) (Hunt et al., 2000; Seidah et al., 2003; Varret et al., 1999). These seminal findings culminated in the great collaboration between two teams that were interested in PCs and FH patients. This remarkable collaboration uncovered two specific variants (S127R and F216L) in *PCSK9* in FH families with no mutations in *LDLR* and *ApoB*. This discovery proposed *PCSK9* as a third locus/gene causing FH (FH3) (Abifadel et al., 2003). Soon after, it was demonstrated that PCSK9's effect on cholesterol levels is due to its regulatory function on LDLR (Benjannet et al., 2004; Maxwell & Breslow, 2004; Maxwell et al., 2005; Park et al., 2004). Thereupon, numerous studies have been conducted to better understand the trafficking and regulation of this intriguing protein. However, our understanding of this protein is far from complete, and there are still many gaps that need to be addressed.

Tissue expression and subcellular localization

The pioneering study in 2003 demonstrated that in humans, rats and mice, PCSK9 is mostly expressed in the liver and to a lower extent in the small intestine and kidney, using *in situ*

hybridization (ISH) (of rats at embryonic days; E9, E12, E15, and E17 along with postnatal; P3 and P7) and Northern blot (of human and rat, mouse cell lines) approaches (Seidah et al., 2003).

Structure

A *homo sapiens PCSK9* gene (gene ID: 255738) is located on chromosome 1p32.3 and includes 12 exons encoding a 692 aa PCSK9 protein (NP_777596.2). resembling other PC members, PCSK9 consists of five distinct domains containing signal peptide (aa 1-30), prodomain/prosegment (aa 31-152), catalytic domain (aa 153-422), Hinge domain (aa 423-452), and C-terminal/Cys-His-rich domain (CHRD; aa 453-692). The catalytic domain of PCSK9 exhibits 25% protein sequence identity to SKI-1/SP1 and contains amino acids D_{189}, H_{226}, and S_{386} that are required for the autocatalytic activity of PCSK9. This domain also is the main part of the protein (aa 367-380) that interacts with EGF-A domain of LDLR. Part of PCSK9 prodomain also interacts with LDLR, and mutations in this region (e.g., L108R) decrease PCSK9 binding affinity to LDLR. Although the CHRD does not affect PCSK9 binding to LDLR, it is required for PCSK9's ability to induce LDLR degradation. However, the isolated CHRD alone has no effect on LDLR. The CHRD is composed of three repeat modules/domains termed M1 (aa 453–531), M2 (aa 530–605), and M3 (aa 604–692). The unique function of each module makes this CHRD domain as an interesting topic to study. Interestingly, while the M2 domain is necessary for extracellular function of PCSK9 (as detailed in following sections), it is not required for intracellular activity (Benjannet et al., 2004; Cunningham et al., 2007; Kosenko et al., 2013a; McNutt et al., 2007; Nassoury et al., 2007; Piper et al., 2007; Saavedra et al., 2012; Seidah et al., 2003; Seidah & Prat, 2012; Zhang et al., 2008).

Trafficking and regulation

The processing of PCSK9 is very similar to the other members of the family. Initially, the 72-kDa preproPCSK9 protein (comprising 692 aa) is synthesized in the ER, and signal peptide is cleaved. Upon cleavage of signal peptide, proPCSK9 undergoes autocatalytic cleavage at its N-terminal (at VFAQ152↓ motif) in the ER. Nevertheless, the cleaved prosegment permanently stays non-covalently attached to the rest of the protein, even when it is secreted into the extracellular media. This unique feature of PCSK9 keeps PCSK9 as an inactive protease and prevents any further enzymatic activity on its specific target proteins. Therefore, PCSK9 is the only PC that acts as a protease only once during its autocatalytic processing in the ER, and its regulatory effect on LDLR

and other targets occurs *via* a non-enzymatic mechanism. The heterodimer complex of PCSK9 includes a 14 kDa propeptide (aa 31-152) and a glycosylated 63 kDa (aa 153-692) mature protein (mPCSK9) that is transported to the Golgi and then secreted to the outer media (Benjannet et al., 2004; Luna Saavedra et al., 2013; Naureckiene et al., 2003; Seidah et al., 2003). mPCSK9 in the plasma consists of an active 62 kDa form (towards LDLR degradation) along with an inactive 55 kDa protein produced *via* proteolytic processing of Furin and/or PC5 (at R218↓ residue) (Mousavi et al., 2011; Mousavi et al., 2009).

Function and expression of PCSK9 can be regulated by several factors, such as various transcriptional and translation alternations, different physiological and pathophysiological conditions and many more incentives that can have either direct or indirect effects.

SREBP-1c, SREBP-2, and hepatocyte nuclear factor-1 alpha (HNF1-α) are the main proteins that transcriptionally regulate PCSK9 levels (Jeong et al., 2008; Li et al., 2009). SREBP-2 is the primary transcriptional factor for *de novo* synthesis of PCSK9. When cholesterol levels are low in cells, induction of a series of events activates and sorts SREBP-2 to the nucleus, where it induces the expression of various genes such as *PCSK9*, *LDLR*, and HMG-CoA reductase (*HMGCR*) (Goldstein et al., 2006). Many physiological factors, such as diet, can affect the activity of SREBP-2 and, subsequently PCSK9 expression. For example, it is suggested that ER stress can play an important role in SREBP-2 activation in this process. In contrast, increased ER Ca^{2+} levels (because of factors such as exposure to caffeine) seem to inhibit this process *via* glucose-regulated protein 78 (GRP78) chaperones and block the *de novo* synthesis of PCSK9 (Colgan et al., 2007; Lebeau et al., 2022). Indeed, the expression of certain proteins, such as resistin, can enhance the activity of SREBP-2 and PCSK9 expression (Melone et al., 2012).

Further transcriptional regulation of PCSK9 can be mediated by other proteins such as nuclear receptors such as farnesoid X receptor (FXR), liver X receptor (LXR), peroxisome proliferator-activated receptors (PPARs) α and γ, and the histone nuclear factor P (HINFP). Additionally, physiological, and pathological events, along with pharmacological interventions, can regulate PCSK9 expression levels. Physiological factors include diet (e.g., Mediterranean diet correlates with lower PCSK9 levels), hormones (e.g., growth hormone (GH)), age, gender (higher plasma levels of PCSK9 in women due to the presence of estrogens), diurnal rhythm, and aerobic exercise. pathological conditions comprise inflammation, viral infection, insulin resistance,

diabetes, obesity, atherosclerosis liver, and kidney diseases. Pharmacological interventions including ezetimibe, statins, and fibrates (Baass et al., 2009; Cui et al., 2015; Jeong et al., 2008; Lambert et al., 2008; Langhi et al., 2008; Li et al., 2009; Persson et al., 2010; Peticca et al., 2013; Richard et al., 2012; Saely & Drexel, 2016; Zelcer et al., 2009).

Post-translational modifications also can affect PCSK9 secretion and function. For example, it was shown that PCSK9 can be phosphorylated either at S47 and S688 residues by a Golgi casein kinase-like, or at S47, S666, S668, and S688 residues by a family with sequence similarity 20 member C (Fam20C). Interestingly, although these GOF modifications induce PCSK9 secretion and enhance its function towards LDLR degradation, they are not necessary for PCSK9 activity on LDLR (Ben Djoudi Ouadda et al., 2019; Dewpura et al., 2008). PCSK9 can be also sulfated (at Y38 an Y142) and N-glycosylated (at N533) (Benjannet et al., 2004; Benjannet et al., 2006).

PCSK9 and mutations

So far, many variations of PCSK9 have been identified and functionally characterized in relation to altering circulating levels of cholesterol. Today, it is well known that loss of function (LOF) and gain of function (GOF) variations in PCSK9 correlate with lower (hypocholesterolemia) and higher (hypercholesterolemia) levels of circulating cholesterol, respectively. PCSK9 (S127R & F216L; GOF) and (Y142X & C679X: LOF) are the very first variants that have been deciphered in association with cholesterol circulating levels (Abifadel et al., 2003; Cohen et al., 2005).

GOF and LOF substitutions/deletions can affect several steps in the PCSK9's life cycle, from translation to its interaction with LDLR (Dron & Hegele, 2017). Interestingly, in 2018 gene duplication of PCSK9 have been found in two unrelated Canadian FH families with severely high levels of circulating LDLc (Iacocca et al., 2018). In contrast, in individuals whose PCSK9 secretion is completely lost (e.g., homozygous C679X or Q152H), no pathogenic phenotype has been revealed (Hooper et al., 2007; Mayne et al., 2011).

Investigation about natural mutants on PCSK9 variants enabled researchers to better understand the structural function of different domains of PCSK9. Additionally, the identification and characterization of LOF variation enabled scientists to develop novel therapeutic approaches to lower LDLc levels by targeting PCSK9 protein (Seidah & Garcon, 2022).

PCSK9 D374Y mutation is an interesting GOF mutation that elevates (~10- to 25-fold) the binding affinity of PCSK9 to the EGF-A domain of LDLR (Cunningham et al., 2007; Kwon et al., 2008). The strong and rapid effect of the PCSK9 D374Y variant on LDLR, helped researchers to use this mutation as an atherosclerosis mouse model to study diseases (Seidah & Garcon, 2022).

PCSK9 Q152H is a LOF variation that is usually found in French Canadian individuals. Substitution of glutamine with histidine at position 152 affects the sequence of the autocatalytic cleavage site ($VFAQ_{152}\downarrow$) and prevents further the autocatalytic processing of proPCSK9, which is retained in the ER. Although proPCSK9 accumulates in the ER, it does not induce ER stress; due to its cochaperone-like function that increases the levels of GRP78 and GRP94, which prevent ER stress (Lebeau et al., 2021; Mayne et al., 2011). Intriguingly, in heterozygous subjects, this variant acts as a dominant negative and blocks the exit of WT PCSK9 from ER (Abifadel et al., 2014; Cameron et al., 2009; Cameron et al., 2008; Cariou et al., 2009; Dubuc et al., 2010; Zhao et al., 2006).

Potential Substrates of PCSK9 other than LDLR

Since the discovery of PCSK9, various studies have been conducted to better understand the function of this unique protein convertase (the functional importance of some of these substrates will be explained in the following sections). Now, it is known that although LDLR is the main substrate of PCSK9, it is not the only one. For instance, PCSK9 can target receptors such as LRP1, LRP8, ApoER2, VLDLR, CD81, CD36, angiotensin-converting enzyme 2 (ACE2; a transmembrane protein that serves as the major entry point for SARS-CoV-2 virus that causes COVID-19), and major histocompatibility complex 1 (MHC-I). Conversely, PCSK9 can act as a chaperone for some proteins (ApoB) or increase their protein levels (Niemann-Pick C1-like protein 1; NPC1L1). Some other interactors can act as an inhibitor for PCSK9 function on LDLR. In humans, approximately 30 to 40% of secreted PCSK9 directly binds LDL particles in the circulation. LDL interacts with the N-terminal intrinsically disordered region (IDR) within the prodomain (aa 31–60), and CM1 (M1 module) in the CHRD of PCSK9 and this way inhibits its function towards degradation. Interestingly, some potent GOF variants in PCSK9 (e.g., S127R) might abolish this interaction. Similarly, the interaction of the N-terminal repeat R1 of annexin A2 inhibits PCSK9 function. In contrast, heparin sulfate proteoglycans (HSPGs) seem to facilitate the PCSK9 and LDLR complex formation and potentiate PCSK9 function (Canuel et al., 2013;

Essalmani et al., 2023; Gustafsen et al., 2017; Kosenko et al., 2013b; Kysenius et al., 2012; Le et al., 2015; Liu et al., 2020; Mayer, Poirier, et al., 2008; Poirier et al., 2008; Sarkar et al., 2020; Sarkar et al., 2022; Seidah et al., 2012; Shen et al., 2013; Sun et al., 2018; Tavori et al., 2013). This increasing number of targets of PCSK9 proves that PCSK9's function is beyond LDLR regulation and even lipid metabolism and needs to be studied more thoroughly.

Intracellular and extracellular functions of PCSK9

PCSK9 enhances the degradation of LDLR *via* two distinct pathways: an intracellular pathway and a major extracellular pathway that are summarized in (Fig. 12).

In the extracellular pathway, secreted PCSK9 interacts with the EGF-A domain of LDLR that reaches the cell surface. As it was mentioned earlier, internalization of LDLR and LDLc complex is followed by LDLc sorting to the lysosomes and LDLR recycling to the cell surface (an efficient process facilitated with the help of proteins such as SNX17, Wiskott-Aldrich syndrome protein, SCAR homolog (WASH), and COMMD/CCDC22/CCDC93 (CCC) complex). However, the presence of extracellular PCSK9 disrupts this process and does not allow the receptor to recycle back to the cell surface. In fact, in the presence of PCSK9, the entire complex of PCSK9, LDLR, and LDLc is endocytosed into clathrin-coated vesicles and targeted to lysosomes for degradation (Fig. 11) (Bartuzi et al., 2016; Seidah & Prat, 2012).

Although the exact mechanism, by which PCSK9 and LDLR complex is sorted to the lysosomal compartments is not clear yet, it seems that CHRD plays an undeniable role in this trafficking. Structural and functional assays propose that CHRD is needed to facilitate the interaction of PCSK9 with another yet undefined partner protein (called protein X, which allegedly sorts the complex to the lysosomal degradations). The addition of this so-called protein X completes the formation of the complex at the cell surface and traffics it to the lysosomal degradation compartment. So far, many proteins have been proposed as protein X candidates, such as the ligand binding domain of LDLR, amyloid precursor protein-like protein-2 (APLP2), sortilin 1 (SORT1), LRP1, CAP1, Surfeit locus protein 4 (SURF4), and progestin adipo Q receptor 3 (PAQR3). However, none of them has been validated as an actual protein X. Indeed, many other proteins have been proposed to interact with specific variants of PCSK9 ($PCSK9_{Q152H}$) and, that way, regulate specific physiological conditions such as unfolded protein response (UPR) (i.e., GRP78 & GRP94) (Butkinaree et al., 2015; Devay et al., 2013; Emmer et al., 2018; Fruchart

Gaillard et al., 2023; Gustafsen et al., 2014; Huang et al., 2019; Jang et al., 2020; Lagace et al., 2006; Lebeau, Platko, Al-Hashimi, Byun, Lhotak, et al., 2018; Xu et al., 2015).

Among all proposed candidates, CAP1 seems to be an interesting protein that can affect PCSK9 interaction with the so-called protein X. In 2020, Jang and colleagues proposed CAP1 as a new partner protein that interacts with the CHRD of PCSK9 and boosts its function. The idea of working on this protein came from the initial works of Hampton's group in 2007, in which they illustrated that the CHRD of PCSK9 has structural homology with the C-terminal domain of resistin protein. Resistin is a pro-inflammatory cytokine that interacts with CAP1 protein *via* its C-terminal domain. These fundamental studies brought the idea that the CHRD of PCSK9 also might interact with CAP1, and this may affect the function of PCSK9. Therefore, Jang's group did extensive work and showed that the CHRD (more specifically, M1 and M3 modules) of PCSK9 binds to Src homology 3 binding domains (SH3BD) of CAP1 either in mouse liver tissue or HepG2 naïve cells. Next, they applied both *in vitro* (siRNA against CAP1) and *in vivo* ($CAP1^{+/-}$ mouse) silencing approaches to investigate the importance of CAP1 in PCSK9 function. Interestingly, they discovered that in the absence of CAP1, PCSK9 is no longer able to efficiently degrade the LDLR. Indeed, they proposed that PCSK9-induced degradation of LDLR is *via* caveolin pits and not clathrin-coated vesicles. A surprising finding that is in contrast with previous publications. Hence, this group proposed CAP1 as a new partner protein of PCSK9 that is needed for the proper functioning of PCSK9 and mediates caveolae-dependent endocytosis. In contrast, our laboratory (Seidah's group) recently analyzed the importance of this protein on PCSK9 and revealed that although CAP1 can improve PCSK9 function towards LDLR degradation, it is not crucial for PCSK9's activity. Additionally, our recent work again demonstrated that the endocytosis of LDLR and PCSK9 complex occurs *via* clathrin-coated vesicles and not caveolin pits (Field et al., 1990; Fruchart Gaillard et al., 2023; Hampton et al., 2007; Holla et al., 2007; Jang et al., 2020; Lee et al., 2014; Nassoury et al., 2007).

In the intracellular pathway, the interaction of PCSK9 and LDLR happens in the Golgi (TGN) and before secretion of PCSK9 and cell surface localization of LDLR. Thereby, the whole complex of PCSK9 and LDLR directly sorted to the lysosomal degradations, and LDLR never gets a chance to reach the cell surface (Fig. 11) (Poirier et al., 2009; Seidah & Prat, 2012). While in the extracellular pathway, clathrin-heavy chains (CHCs) are needed for endocytosis, in the

intracellular pathway, clathrin-light chains (CLCs) are required to traffic the complex from TGN to endosomes (Poirier et al., 2009).

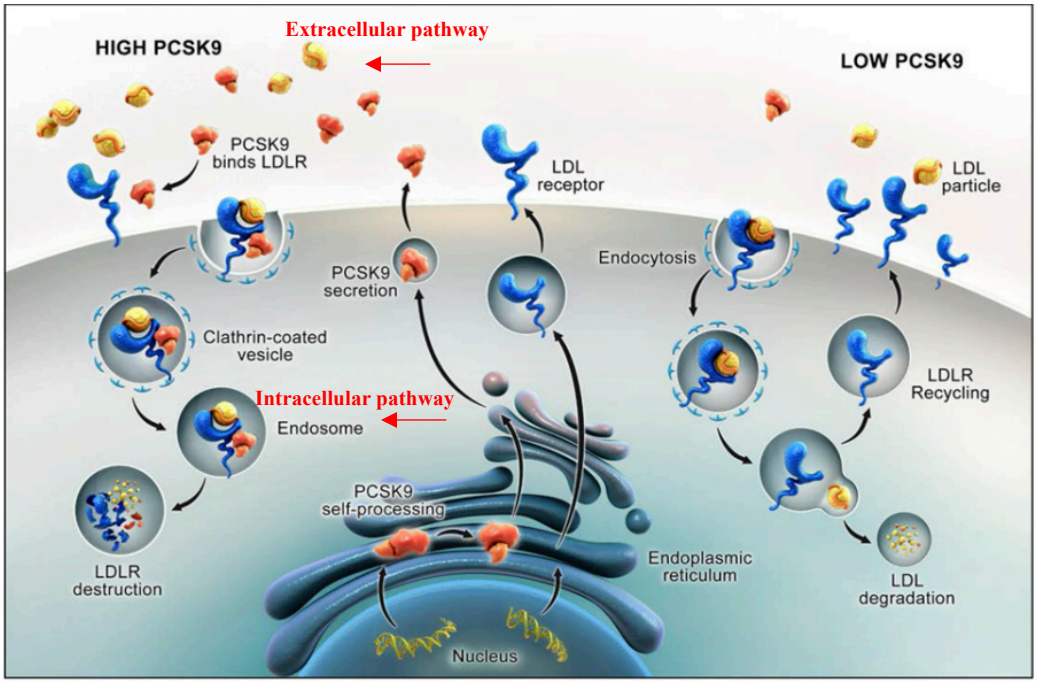


Fig. 12. PCSK9-induced degradation of LDLR. Adapted from (Seidah et al., 2017).

In 2012 structural-functional assays have been done to better distinguish the extracellular and intracellular pathways and look for critical domains involved in each pathway. In one interesting investigation in our laboratory, various structures have been generated to study the importance of CHRD of PCSK9 in either the intracellular pathway or extracellular pathway. To do so, the following constructions have been made: (PCSK9ΔM1, ΔM2, ΔM3, ΔM12, ΔM23, ΔM13). Intriguingly, only PCSK9ΔM2, ΔM12, and ΔM23 were secreted, and the rest are retained unfolded in the ER (Saavedra et al., 2012). These data are consistent with the recent study that shows the importance of CHRD in PCSK9 and secretion. In this study, the authors proposed the CHRD as a critical domain for PSK9 secretion, likely because of its interaction with the SEC24 component of coat protein complex II (COPII) vesicles. They also showed that although the removal of CHRD impairs the PCSK9 secretion, PCSK9 ΔM23 (known as $PCSK9^{1-528}$) has similar maturation and secretion as WT PCSK9 (Deng et al., 2020). Altogether these data suggest the importance of either

M1 or M3 domain for PCSK9 secretion. Interestingly, CAP1 protein also interacts with the same modules of PCSK9 (M1 and M3) (Fruchart Gaillard et al., 2023). To find the critical residues of CHRD and proteins implicated in either PCSK9 secretion or intracellular function (e.g., the possible role of CAP1), further investigations are needed.

Investigation about the intracellular activity of PCSK9 in the presence of CHRD module deletants demonstrates that although PCSK9 ΔM2 is LOF for the extracellular function of PCSK9, it works intracellularly towards LDLR degradation like WT PCSK9. Interestingly, although the M2 domain is not necessary for the intracellular activity of PCSK9, the hinge region seems to be critical for intracellular function as its deletion (PCSK9 ΔHΔM2) abrogates its function. Removal of the hinge region also affects the secretion of PCSK9. Similarly, analyzing the natural mutation R434W showed that this mutation also reduces PCSK9 secretion and extracellular function (~70% compared to WT PCSK9) and completely abrogates the intracellular function of PCSK9. These data show the critical role of the hinge region in the intracellular function (Saavedra et al., 2012). Conversely, other identified mutations on the hinge region (archaic Denisovan PCSK9 H449L) seem to be LOF for the extracellular activity of PCSK9 but have no effect on the intracellular activity of PCSK9. This again shows that these pathways are distinct, and the hinge region might be important for the extracellular pathway, too, as it led to ~2 fold lower binding affinity to the LDLR-EGF-AB domain (Mikaeeli et al., 2020). This data raises the question of whether the charge of the hinge region could favour either pathway (negative for intracellular or positive for extracellular). Consistent with these data, it was shown that the absence of seven histidine residues on CHRD has no effect on the intracellular activity of PCSK9 (Holla, Cameron, et al., 2011; Holla, Laerdahl, et al., 2011).

The dual function (either intracellular or extracellular) is not limited to LDLR and affects other receptors, such as a cluster of differentiation 36 (CD36) that is responsible for the FA uptake of hepatocytes. It has been shown that mAb against PCSK9 compared to siRNA against PCSK9 has fewer adverse consequences as it does not affect the regulatory activity of PCSK9 on CD36 and subsequently prevents lipid accumulation in the liver (that is caused by elevated levels of CD36) (Byun et al., 2022; Demers et al., 2015). These data suggest that the interaction site of PCSK9 with CD36 (probably the CHRD of PCSK9) might be different from LDLR as the presence

of mAb that blocks the catalytic subunit of PCSK9 did not change the ability of PCSK9 to degrade CD36.

Although most of the studies have been focused on the extracellular function of hepatocytes and circulating PCSK9 in CVDs, earlier investigations showed the importance of the intracellular activity of this protein in both hepatic and extrahepatic cells.

The possible intracellular function of PCSK9 is proposed due to the extensive research on specific PCSK9 variants. For instance, it was shown that some variants (PCSK9 S127R and D129G) are GOF (towards LDLR degradation) even though they cause impaired auto cleavage and reduce protein secretion (Benjannet et al., 2004; Homer et al., 2008).

Although PCSK9 mainly has an extracellular function in the liver, it was proposed that in the intestine, it is mostly the intracellular PCSK9 that affects LDLR levels. Investigations on Annexin A2 as a potential inhibitor of extracellular PCSK9 show that this protein mainly expresses in the intestine and not the liver. This suggests that the expression of various regulators of PCSK9 (negative or positive modulators) could determine either the extracellular or intracellular function of PCSK9 in different tissues (Ly et al., 2014; Mayer, Poirier, et al., 2008). Consistent with this information, tissue-specific knockout of PCSK9 in the small intestine showed no significant effect on levels of circulating lipids (Garcon et al., 2020). Thus, although the extracellular function of PCSK9 might be inhibited in extrahepatic tissues such as the intestine, the intracellular PCSK9 may have other function apart from lipid metabolism that is not defined yet.

As was explained earlier, this PCSK9 Q152H variant has no extracellular function as it cannot auto-cleave itself and thus is retained in the ER and is not able to reach the cell surface. While PCSK9 Q152H is retained in the ER, it does not lead to ER storage disease (ERSD). In normal conditions, unfolded protein response (UPR) activity is blocked *via* the interaction of GRP78 with ER transducers activating transcription factor 6 (ATF6), glucose-regulated protein 1 (IRE1α), and protein kinase RNA–like endoplasmic reticulum kinase (PERK). In the presence of misfolded proteins in the ER, GRP78 dissociates from these transducers and interacts with the retained protein. This process leads to the UPR activation and prevents further accumulation of misfolded protein. Contrary to this process, retained PCSK9 Q152H protein interacts with GRP94 instead of GRP78 (GRP78 stays attached to the UPR transducers). This interaction not only inhibits the UPR activation but also acts as a co-chaperone and increases the abundant of GRP94

(Lebeau, Platko, Al-Hashimi, Byun, Lhoták, et al., 2018; Lebeau et al., 2021). Indeed, ER-retained PCSK9 (e.g., L455X) also can act as a chaperone for LDLR in the ER and promote its trafficking to the cell surface. This chaperone-like effect of PCSK9 on LDLR is independent of the C-terminal domain of LDLR (Strom et al., 2014). Apart from LDLR, WT PCSK9 can have a chaperone function on other proteins such as ApoB (Sun et al., 2012).

Many studies have been conducted to understand the intracellular role of PCSK9 in extrahepatic cells. For example, in kidney downregulation of renal PCSK9 seems to associate with renal inflammation and lipid deposition in Adriamycin nephropathy model (Zhang & Li, 2019). In the brain, PCSK9 promotes neuronal apoptosis by reducing apolipoprotein E receptor 2 (ApoER2) expression and reduces cholesterol uptake by degradation of LDLR-related protein 1(LRP1) (Adorni et al., 2019; Wu et al., 2014). In the heart, PCSK9 knockdown in cardiomyocytes (CM-*Pcsk9*$^{-/-}$ mice) correlates with impaired mitochondrial function and subsequently progressive dilated cardiomyopathy-like phenotypes (Laudette et al., 2023).

In a recent study, it has been proposed that PCSK9 targets cell surface receptors such as HLA-ABC and programmed death-ligand 1 (PDL1) in pancreatic beta cells. The authors also confirmed that in beta cells, PCSK9 targets many receptors, such as LDLR and CD36 and has similar trafficking as hepatocytes. However, PCSK9 in beta cells seems to have lower maturation and secretion rate compared to hepatocytes. In beta cells, PCSK9 levels are regulated transcriptionally by SREBP-1 instead of SREBP-2. Indeed, the regulatory effect of PCSK9 on cell surface receptors seems to be a little bit different and while extracellular PCSK9 targets VLDLR and LDLR, intracellular PCSK9 affects CD36, HLA-ABC and PDL1 receptors. These captivating new data proposed the pancreatic beta cells as an interesting model to better understand the extrahepatic role of PCSK9 (Saitoski et al., 2022).

The physiological importance of PCSK9

1. Importance of animal model

Studying PCSK9 knockout (KO) mice (Pcsk9$^{-/-}$) provided us with a great tool to better understand the physiological importance of PCSK9 and its underlying cellular mechanism. Pcsk9$^{-/-}$ results in 42% lower levels of total cholesterol and 80% lower levels of LDLc with a 2.6-fold increased levels of hepatic LDLR. Comparing levels of LDLc in total Pcsk9$^{-/-}$ (42% reduction in LDLc) with liver-specific Pcsk9$^{-/-}$ (27% reduction in LDLc) suggests that hepatic PCSK9 plays a

major role in cholesterol homeostasis. In contrast with the KO mice, in PCSK9 transgenic mice LDLc levels increased dramatically (5-15-fold higher than the normal mice) (Maxwell & Breslow, 2004; Rashid et al., 2005; Roubtsova et al., 2011; Zaid et al., 2008).

2. PCSK9 implication in various human diseases

Cardiovascular diseases and inflammation

Today, we clearly know that one of the major implications of PCSK9 is in FH pathogenesis *via* targeting LDLR receptors (Seidah & Prat, 2012). Since cholesterol is one of the main causative factors in cardiovascular disease development, lowering LDLc levels *via* targeting extracellular PCSK9 either alone or in combination with other treatments, such as statins, could reduce the adverse cardiovascular symptoms (Seidah & Garcon, 2022). In atherosclerosis patients with high levels of circulating LDLc, small dense LDL oxidation forms oxLDL that subsequently leads to lipid accumulation and inflammation in immune cells. Furthermore, it is proved that PCSK9 expression is mediated by oxLDL in immune cells (Tang et al., 2012). In contrast, the extrahepatic expression of intracellular PCSK9 in cardiomyocytes seems to be beneficial (by maintaining the proper function of mitochondria) for cardiac responses in a myocardial infarction (Laudette et al., 2023).

Sepsis is a multiorgan disease that happens when the body's inflammatory responses to infection led to organ/tissue injury and, ultimately, septic shock. Reduced levels of PCSK9 either in humans (LOF variants) or mice ($PCSK9^{-/-}$) is protective against septic shock in an LDLR-dependent mechanism that affects the lipid clearance and its availability for pathogens (Boyd et al., 2016; Grin et al., 2018; Walley, 2016) please add PMID: 27405064).

Steatosis

Although inhibition of PCSK9 is a promising approach to reducing levels of total cholesterol, but its complete KO/removal might lead to some complications. For example, CD36 is one of the major receptors responsible for fatty acid uptake in hepatocytes, and it is important to have balanced levels of this receptor at the cell surface to maintain lipid homeostasis. Complete removal of PCSK9 in hepatocytes increases the CD36 levels at the cell surface and led to the formation of lipid droplets and, ultimately, diet induced NAFLD and liver injury (Lebeau et al., 2019). Furthermore, PCSK9 KO in mice increases the expression of renal CD36 and, consequently, lipid

accumulation and inflammation in the kidney. Increased in CD36 levels in renal epithelia results in steatosis within these cells like hepatocytes (Byun et al., 2022).

Viral infections

The implication of PCSK9 in viral infections, either in LDLR-dependent or independent pathways, is another example of the role of PCSK9 in various pathologies beyond lipid metabolism. Considering the regulatory function of PCSK9 on various cell surface receptors such as VLDLR, CD81 and LDLR that help for the entry of viruses, this protein can have a pivotal role in viral infections. Hepatitis C virus (HCV) is one example that the presence of PCSK9 could block its entry and reduces the risk of infection (Pirro et al., 2017). In addition, PCSK9 levels associate with HCV and in patients with HCV have lower circulating levels (Bridge et al., 2014). Conversely, PCSK9 expression is higher in patients with human immunodeficiency virus (HIV) undergoing antiretroviral therapy (ART) or dengue virus (DENV). HIV infection can cause patients to have various lipid metabolism implications that ultimately lead to the development of cardiovascular diseases. However, therapeutic approaches such as mAb against PCSK9 can reduce the adverse pathologies (Gan et al., 2020; Leucker et al., 2020; Leucker et al., 2018). The recently identified target of PCSK9 is the ACE2 protein which today is an interesting subject to investigate as it's the main entry point for the SARS-CoV-2 virus that has been caused by the recent COVID-19 pandemic. Targeting the ACE2 receptor by extracellular PCSK9 reduces the cell surface levels of this receptor and cell fusion (Essalmani et al., 2023). On the other hand, inhibition of PCSK9 using mAbs (e.g., evolocumab) seems to be a promising approach to reduce the availability of circulating LDLc to reduce viral entry. Recent studies have shown the beneficial role of statins in hospitalized patients with COVID-19 in reducing severity and mortality risks (Seidah & Garcon, 2022). Success in reducing the infection in patients with DENV proposed PCSK9 as an intriguing therapeutic target in viral infections (Gan et al., 2020).

PCSK9 and cancer

So far, PCSK9 expression has been correlated with various cancers such as breast, hepatic and colon cancers either in an LDLR-dependent or independent manner.

Analysis of the implication of PCSK9 on melanoma cancer has been conducted recently using LOF and GOF PCSK9 models both *in vitro* and *in vivo*. It was revealed that the expression levels of PCSK9 positively correlate with cell growth, proliferation, and migration in cancerous

cells, and its high levels reduce the survival rate of melanoma and dysfunction of T cells. In this study, it was shown that, in D374Y tumour models, CD^{8+}T and natural killer NK cells increased dramatically in contrast with Q152H or PCSK9 KO models. The Q152H model showed a greater ability to inhibit melanoma compared to the control, specifically in PCSK9 KO mice. This surprising observation proposes the beneficial role of PCSK9 vaccines to prevent or reduce the occurrence of cancers (Gu et al., 2023). Similarly, PCSK9 inhibition has been shown (both in cell and animal models) to be an effective approach (specifically in combination with statins) to alleviate the adverse symptoms in the colorectal cancer (Wong et al., 2022) and Colorectal cancer liver metastatic (CRCLM) (Miran Rada, 2023).

Very recently, Yang et al. revealed an interesting correlation of PCSK9 with head and neck squamous cell carcinoma (HNSCC). This study showed that PCSK9 expression is higher in patients with HNSCC, and its inhibition represses the stemness-like phenotype of cancerous cells in an LDLR-dependent fashion. Indeed, PCSK9 inhibition improved the positive effect of programmed cell death protein 1 (PD-1) antibody (Yang et al., 2023).

The revolutionary discovery of MHC-I as a new substrate of PCSK9 opened a new therapeutic approach for different types of cancers. In the pioneer study in 2020, researchers applied mAb against PCSK9 (evolocumab) combined with PD-I antibody in breast cancer. The results showed that the addition of inhibition of PCSK9 can dramatically improve the symptoms. In the same study, the authors declared that PCSK9 only affects MHC-I (HLA-C subtype) levels and not MHC-II in B16F10 melanoma cells. However, it was proposed that because of the low expression of MHC-II in these cell lines, the effect of circulating PCSK9 with other cells with higher levels of MHC-II needed to be defined (Liu et al., 2020; Seidah & Garcon, 2022).

Altogether these data proposed PCSK9 as an important protein that could be a great target both for tumour diagnoses and treatments in various cancers.

Potential treatment strategies to inhibit PCSK9

The major implication of PCSK9 in lipid metabolism made it an interesting target for scientists to develop therapeutic approaches to reduce LDLc circulating levels. So far, different mAbs (including the clinically prescribed alirocumab and evolocumab) and analog peptides have been developed to directly impede the interaction of PCSK9 with the EGA-A domain of PCSK9. The

combination of PCSK9 with statin has an additive therapeutic impact in patients with high levels of circulating cholesterol (Khoshnejad et al., 2019; Seidah & Garcon, 2022). Surprisingly, the response efficacy to mAb is higher in men than women, probably due to the presence of 17β-estradiol (E2) in women that leads to the shedding of the ectodomain of LDLR (Roubtsova et al., 2022). Single-domain antibody sdAb against PCSK9 that targets the CHRD domain of PCSK9 inhibits its potential interaction with so-called protein X and reduces LDLR levels (Essalmani et al., 2018). Nucleic acid-based methods, including siRNAs (such as inclisiran to induce mRNA degradation of PCSK9) and antisense oligonucleotides (ASOs, such as AZD8233 and MiR-552-3p binds the mRNA of PCSK9 and blocks it translation) against PCSK9 along with CRISPR/Cas9 gene editing system (that induced the aberrant splicing results in 90% reduction circulating PCSK9) also seem to be interesting approaches that affects both intracellular and extracellular activity of PCSK9 (Musunuru et al., 2021; Ray et al., 2017; Visser et al., 2012). Small molecules, such as Adnectin BMS-962476, also disrupt the LDLR and PCSK9 interaction and result in lower LDLc in the circulation (Mitchell et al., 2014). Vaccines such as the L-IFPTA + vaccine that inhibits the extracellular activity of PCSK9 can be considered as a potential approach to prevent cardiovascular diseases and probably various cancers (Momtazi-Borojeni et al., 2021).

I.I. IV. Major histocompatibility complex (MHC) proteins and their importance in immune response

MHC is a large locus of highly polymorphic genes (located on the short arm of chromosome 6; 6p) that encode cell surface proteins in various tissues. This genetic locus is called histocompatibility system 2 (H-2) in mice and human leukocyte antigen (HLA) in humans. MHC molecules were initially discovered by Gorer et.al. (1936) in mice as essential proteins responsible for the acceptance or rejection of transplanted tissues. Indeed, in adaptive immune response, MHC proteins bind to the peptide fragments that are derived from the pathogens. This interaction initiates a series of events that ultimately lead MHC molecules to present these antigens at the cell surface to be recognized by cytotoxic T cells (CTLs). The antigen presentation of MHC enables T cells to fight pathogenic microorganisms in parallel with B cells. MHC proteins not only help CTLs to identify these pathogenic particles but also provide them with the specificity and memory for each type of pathogen and in future infections with the same pathogen. MHC molecules are classified into three distinct classes called MHC class I, MHC class II and MHC class III. Each encodes specific genes as indicated in (Fig. 13). The focus of this thesis would be on two members of MHC class I molecules, namely HLA-C, and HFE (Blanco & Blanco, 2017; Olson & Raghavan, 2023; Sari & Rock, 2023).

MHC class I usually expresses in most nucleated cells and presents antigens in response to endogenous pathogens (e.g., cancerous cells, and viral components). MHC class II is only expressed in antigen-presenting cells (APCs) (e.g., B cells, dendritic cells, and macrophages) and presents antigens in response to exogenous pathogens. Although both MHC classes are heterodimers, MHC-I consists of two different polypeptides (α and β2M that are coded by two different genes), and MHC-II comprises two homogenous peptides (α and β chain that are encoded with the same gene) (Fig. 14). While MHC-I interacts with the CD8 receptor of cytotoxic T cells (CTLs), MHC-II binds to the CD4 receptor of T helper cells (Blanco & Blanco, 2017; Olson & Raghavan, 2023; Sari & Rock, 2023).

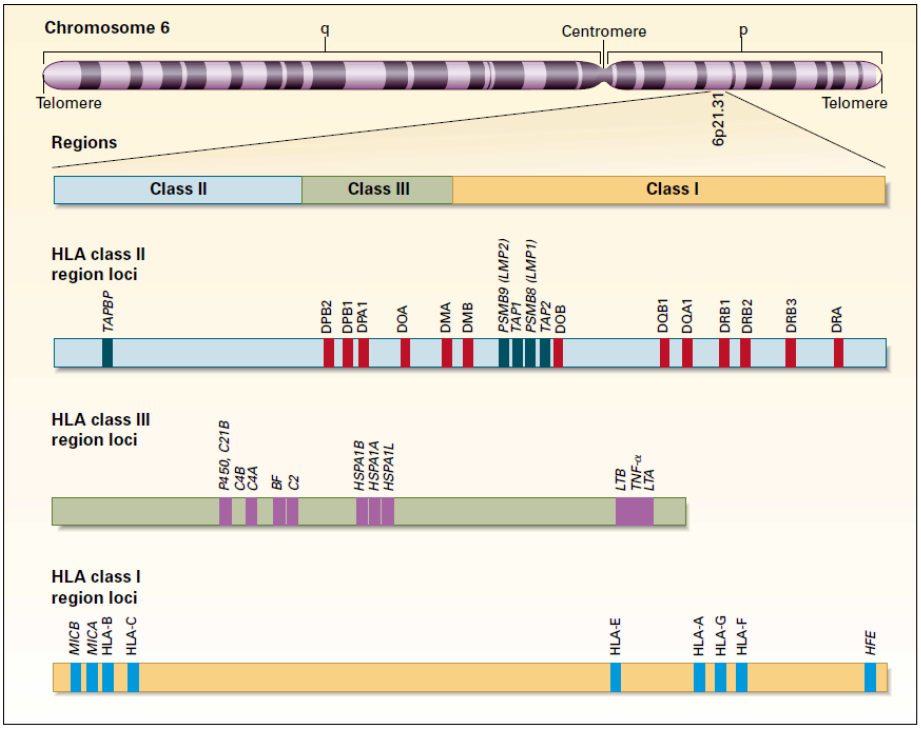


Fig. 13. The locus structure of major histocompatibility complex (MHC) class I, II, and II(Klein & Sato, 2000).

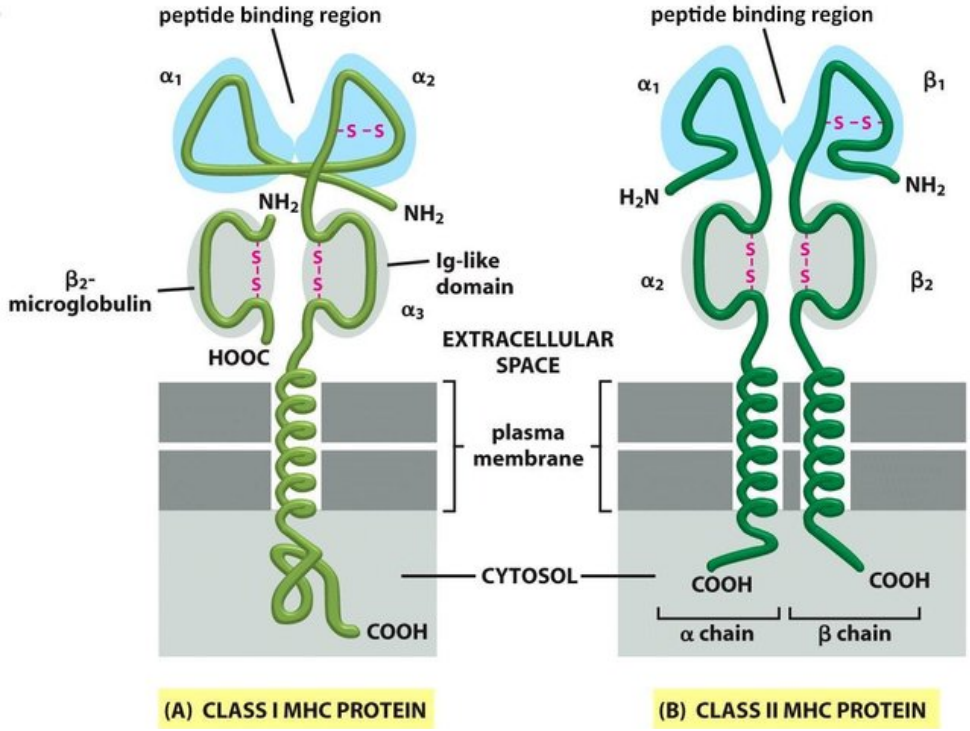


Fig. 14. Structural difference between MHC-I and MHC-II molecules (Manlik, 2016).

MHC class III encodes proteins that involve in the complement system and play an immune role during inflammation. In fact, MHC-III mediates the expression and function of various complement factors (such as C2 and C4) and inflammatory cytokines (such as tumour necrosis factor). MHC-I are heterodimers of two peptide chains, including heavy alpha (α 1,2,3) chain and β2-microglobulin (β2M) light chain that are linked together non-covalently *via* interaction of β2M with α3 domain. The alpha chain is polymorphic that is encoded by the *HLA* gene, whereas β2M has an invariable sequence and is encoded by the *β2m* gene (Blanco & Blanco, 2017; Olson & Raghavan, 2023; Sari & Rock, 2023).

The antigen-presenting pathway of MHC-I starts when cytosolic proteins (derived from endogenous pathogens) are transported to the proteasome complex and degraded to short peptides (8-10 aa in length). These peptides are transported back to the ER by the transporter associated with antigen processing (TAP) transporter. TAP is a heterodimer transmembrane protein consisting of TAP1 and TAP2 polypeptides. At the ER membrane the TAP protein interacts with MHC-I, which is linked to several helper proteins. Apart from the TAP complex, many other proteins, such as calreticulin, Erp57 calnexin, and tapasin, are involved in the peptide-loading process. Calreticulin and ERP 57 bound to the intact MHC molecule (before its interaction with peptide) to provide its stability. Calnexin's function is to provide stability for α chains prior to the attachment of β2m and dissociate from the MHC molecule once the entire molecule is fully assembled, Tapasin connects MHC molecules and TAP protein with each other (Hewitt, 2003; Taylor & Balko, 2022).

When MHC-I permanently binds to one of the peptide fragments, the TAP transporter and other helper proteins dissociate. Subsequently, MHC-I molecules exit the ER through the secretory pathway for transportation to the cell surface. The antigen-loaded MHC-I molecule is pinched off into a vesicle and migrates through the Golgi apparatus to the cell surface. The transportation of MHC molecules *via* the secretory pathway encompasses a series of post-translational modifications, such as some substantial modifications of N-glycan regions on MHC-I, leading to the maturation of the complex. At the cell surface, fusion of the vesicle with the plasma membrane results in the release of the antigen-MHC-I complex. The complex can then be recognized by effector $CD8^+$ T cells. When MHC-I presents the antigen at the cell surface, it binds with CD8 and TCR receptors on cytotoxic T cells. Alpha 3 domain also spans and interacts with co-receptor CD8

at the cell surface. Then, α1 and 2 bind to the TCR, where the TCR assesses the antigenic nature of the peptide. (Fig. 15).(Hewitt, 2003; Koopmann et al., 2000; Olson & Raghavan, 2023; Taylor & Balko, 2022).

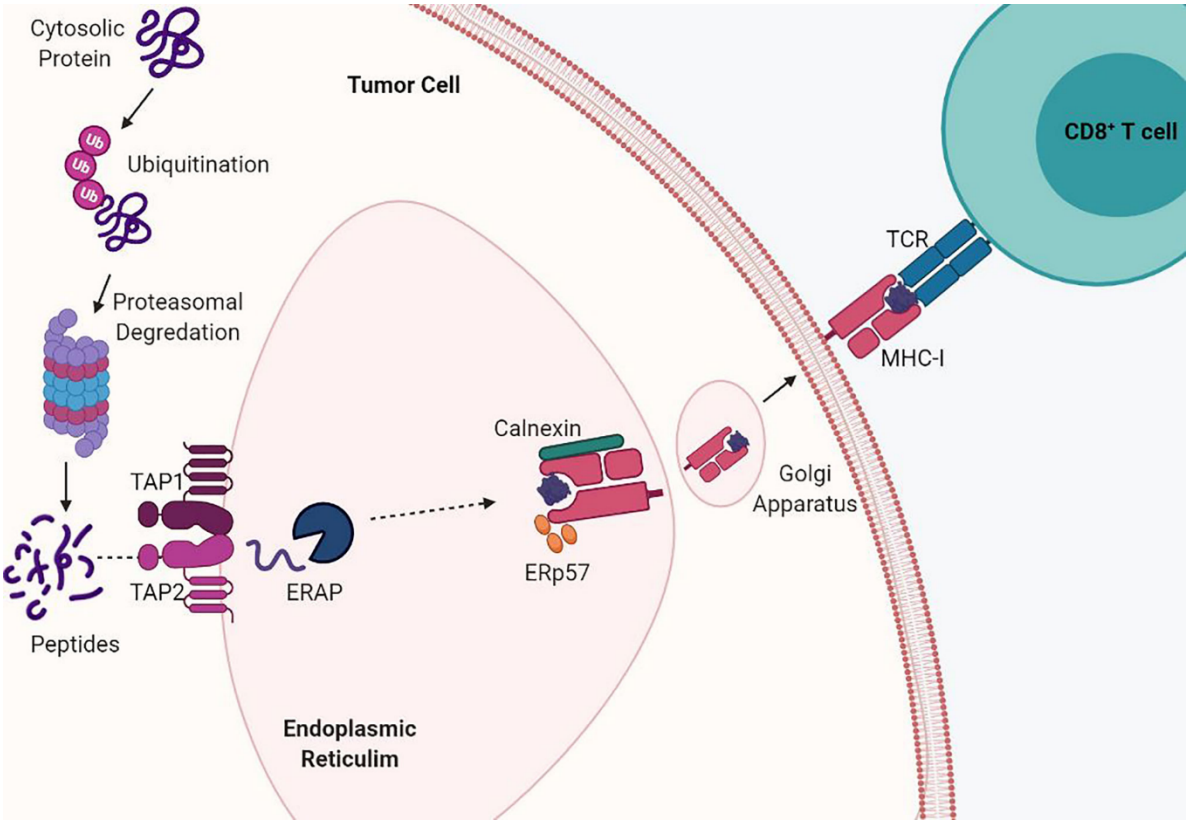


Fig. 15. The antigen presentation pathway of MHC-I molecules. (Taylor & Balko, 2022).

I.I. IV. I. Human leukocyte antigen C (HLA-C) function and regulation

HLA-C emerged about 10 million years ago and probably by duplication of the *HLA-B* gene, as they have a remarkable resemblance in their sequences. In *Homo sapiens*, *HLA-C* is located on the short arm of chromosome 6 (6p), encoding a 45 kDa heavy chain that is non-covalently attached to β2M chaperone. This gene comprises 8 exons, where exons 2-4 code the α1-α3 loops and polymorphisms in exons 2 and 3 provide the peptide binding specificity for this molecule. The heavy chain transcripts comprise 5 domains, including α1, α2 (peptide binding domains), a3 (β2M binding domain), transmembrane domain (exon 5), and cytoplasmic tail (exons 6 and 7). Compared to HLA-A and HLA-B, HLA-C is less expressed in different tissues (about 13-18 times) and at the cell surface, probably due to its lower association with B2M in comparison with the two other paralogues. Indeed, HLA-C exhibits less allelic diversity, notably preserving conservation within the α1 helix (Velastegui et al., 2023). Therefore, it has been proposed that it has a minor function compared to HLA-A and B in the adaptive immune system (Valiante et al., 1997).

HLA-C can interact with both TCR (at CTL cells) and KIR (at natural killer cells) ligands and thus has an essential role in the human immune system. HLA-C plays a central role within the immune system, functioning to protect the host against viral and bacterial pathogens. This protein is also implicated in various other significant processes, including cancer development, the acceptance or rejection of transplanted tissue, preeclampsia during pregnancy, as well as autoimmune conditions like rheumatism, psoriasis, and alopecia (Velastegui et al., 2023).

In 2020, Liu et al discovered PCSK9 as a new regulator of MHC-I both in mice (H2-K) and humans (HLA-A2). This study is a great breakthrough in cancer, that provides a new therapeutic approach in different cancers such as breast cancer. In this study, they have shown that PCSK9 KO in malignant mice dramatically reduces their ability to form tumours. However, this effect was not observed in cells lacking mature T and B cells. In the next step, the researchers asked whether this effect of PCKS9 in cancerous cells is due to its regulatory effect of LDLR, which has great importance in cancer and immune response. The results showed that the effect of the absence of PCSK9 in tumour growth is not dependent on LDLR or cholesterol levels. Interestingly, this group reported that the N-terminal a1-loop of MHC-I H2-K (in mice) and HLA-A2 (in humans) proteins bind to the M2 domain of PCSK9. Indeed, this work showed the importance of the **R**-x-**E** motif on HLA molecules for their interaction with the M2 domain of PCSK9 (Liu et al., 2020). Based

on this investigation, PCSK9 targets HLA molecules for lysosomal degradation in an LDLR-independent fashion (Fig. 16). This interestingly recent information not only unravels the new function of PCSK9 in the immune system but also pronounced the importance of the **R**-x-**E** motif on partner proteins to be able to interact with the M2 domain of PCSK9. However, this study did not provide any information regarding the possible regulatory effect of HLA-C on PCSK9.

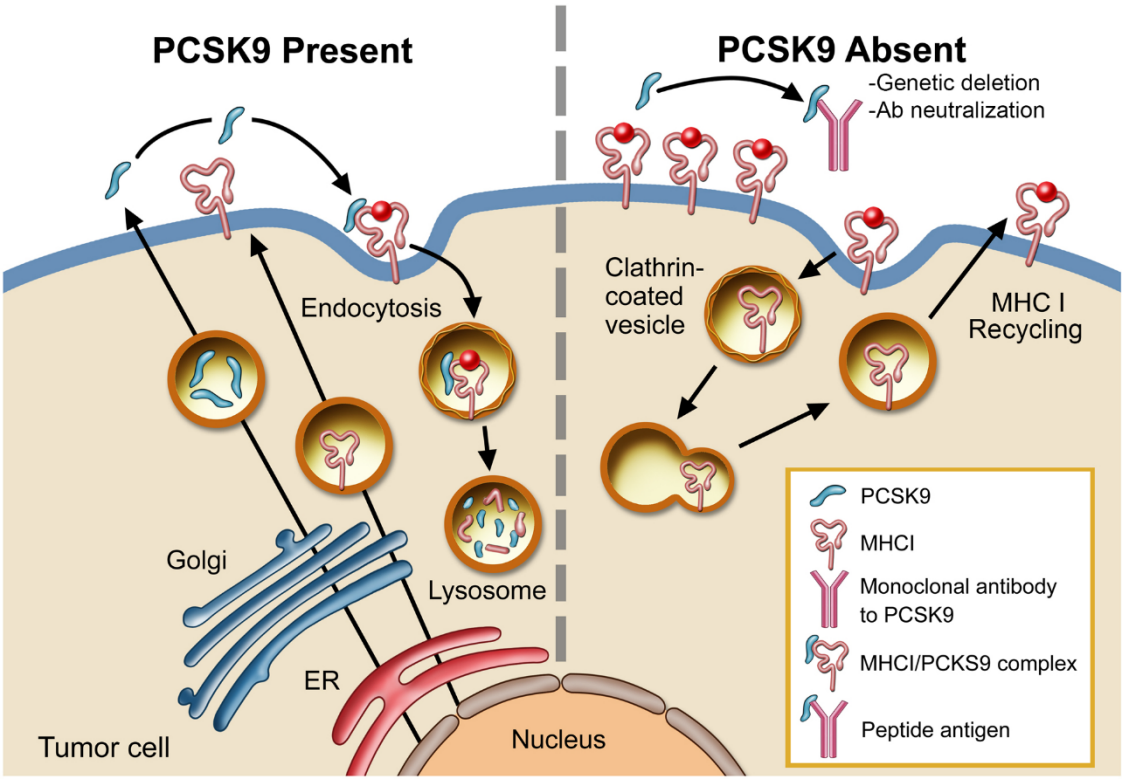


Fig. 16. Schematic diagram showing the interaction of PCSK9 with MHC-I molecule. (Liu et al., 2020).

I.I. IV. II. HFE and its function in iron metabolism

Human hemochromatosis (HFE) is a type 1 transmembrane protein that is coded by the *HFE* gene. *HFE was* initially identified in 1996 through the analysis of linkage disequilibrium within a large group of patients (Feder et al., 1996). Mutations occurring in the *HFE* gene constitute the most prevalent type of hereditary hemochromatosis (HH type 1 with an autosomal recessive transmission), which is very common in Northern European origins (Simon & Bourel, 1978). The HFE C282Y variation is the most prevalent disease-associated mutation, which disrupts the interaction between HFE and β2-microglobulin (Distante et al., 2004). Like other MHC class I molecules, the *HFE* gene is located on the short arm of chromosome 6 (6p21.3) and its protein is composed of three extracellular subunits (α1, α2, and α3), a transmembrane region, and a cytoplasmic tail. Also, HFE interacts with β2-microglobulin, which facilitates its processing within cells and movement to the cell surface. However, it cannot effectively display antigen peptides to immune cells, as it has a smaller groove between its α1 and α2 subunits compared to typical MHC class I proteins (Lebron et al., 1998).

While HFE doesn't directly interact with iron, it can form a complex with TfR1 (Transferrin Receptor 1) at pH 7.5 (West et al., 2001). The interaction of HFE with the homodimer of TfR1 overlaps with the holo-transferrin-binding site. Consequently, HFE competes with holo-transferrin for binding to TfR1, and in the presence of HFE, TfR1 can uptake less iron (Zhang et al., 2003). In circumstances of limited iron saturation of Tf (Transferrin), HFE predominantly remains bound to TfR1 within the plasma membrane, existing in a competitive balance with iron bound Tf. These conditions, cause TfR2 to be directed toward a lysosomal degradation pathway (Fig. 17A). Conversely, in situations of high iron saturation of Tf, HFE dissociates from TfR1 and interacts with the TfR2 complex. These complexes activate the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) signalling cascade, thereby upregulating the transcription and expression of hepcidin (inhibits the iron efflux) (Fig. 17B) (Chloupkova et al., 2010).

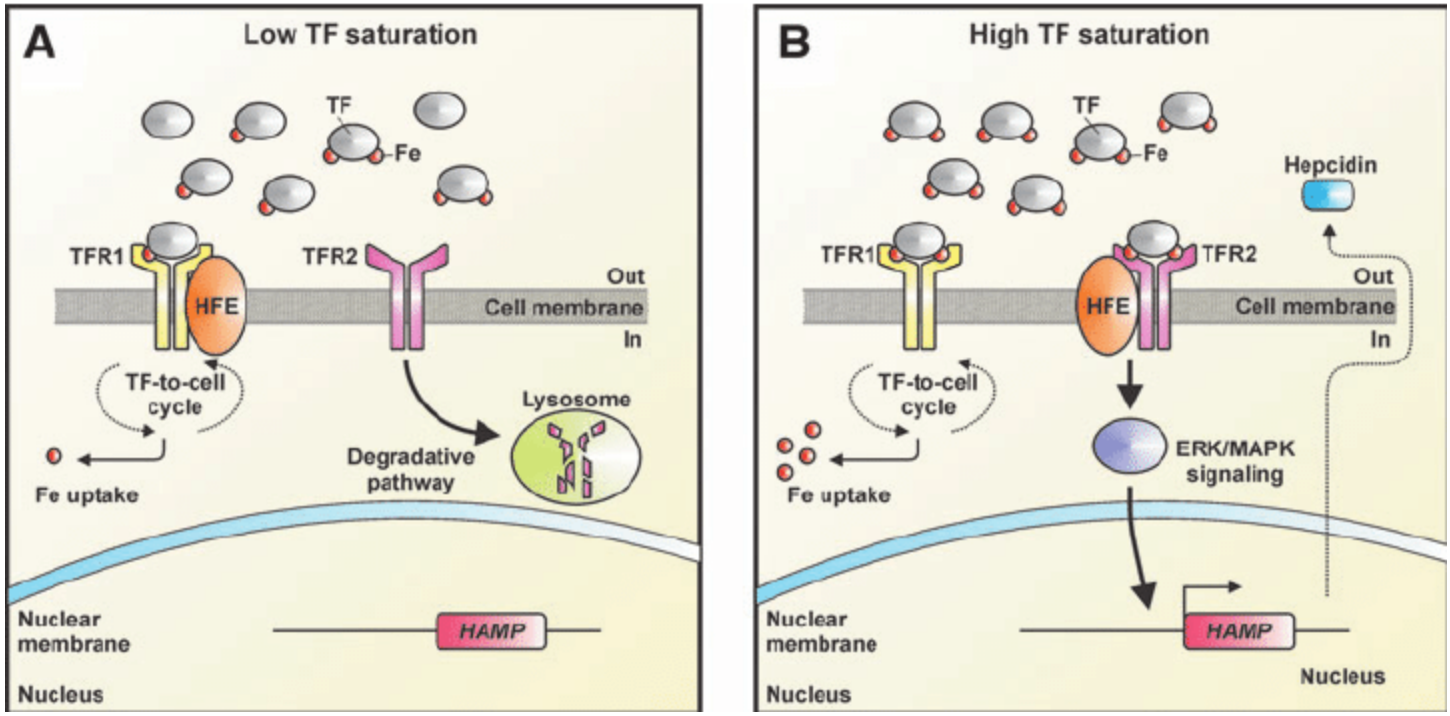


Fig. 17. Iron dependent regulation of Hepcidin by HFE in A) low and B) high circulating iron conditions (Lawen & Lane, 2013).

In 2020, an interesting study conducted by Demetz et al. uncovered a novel role for HFE that extends beyond its established function in iron homeostasis. Within this research, the authors demonstrated that using siRNA against HFE in HepG2 cells resulted in elevated levels of LDLR. Notably, they observed that mice carrying the HFE C282Y mutation, which is akin to knockout (KO) mice due to the absence of β2-microglobulin binding leading to protein retention in the endoplasmic reticulum, displayed higher LDLR levels compared to wild-type mice. Moreover, the authors substantiated that this impact of HFE on LDLR is linked to post-translational modifications, as their qPCR experiments exhibited no effect on the overall mRNA levels of LDLR. The utilization of a Cycloheximide (CHX) assay further confirmed that this effect occurs at the protein level. Essentially, this study introduced a novel regulatory role for HFE in the context of cholesterol homeostasis. Nonetheless, the study did not provide specific data outlining the exact mechanism through which HFE exerts its influence on LDLR (Demetz et al., 2020). Furthermore, a meta-analysis conducted in 2009 revealed a significant association between the HFE C282Y variation and lower levels of cholesterol, specifically LDLc, with a reduction of 15 mg/dl (Pankow

et al., 2008). While these findings have indeed uncovered a new and important role for HFE in lipid metabolism and cholesterol homeostasis, the specific mechanism through which HFE is implicated in cholesterol homeostasis remains an open question.

I.II. Working hypothesis and research objectives:

This research work is motivated by a thorough hypothesis aiming to unravel the complex connections among PCSK9, LDLR, and PCSK9's novel partner proteins in the contexts of cholesterol metabolism and possibly iron homeostasis. The objectives of this Ph.D. project, arise from the complexity of these interactions that goes beyond their usual functions and could provide new insights about lipid and iron metabolism in the liver. Furthermore, this work suggests an unconventional regulation of LDLR in the liver.

The primary research objectives are as follows:

1. Investigation about the importance of the M2 domain of PCSK9 in its function towards LDLR degradation and studying the role of a new M2 interactor of PCSK9, HFE protein: The first research objective is to elucidate the central role of protein X in mediating the internalization trafficking pathways of PCSK9 and LDLR complexes to lysosomal compartments. Furthermore, this study intends to thoroughly explore the potential interaction and role of the HFE molecule in relation to PCSK9. Since HFE has been observed to correlate with LDLR levels through an undisclosed mechanism, and given its structural resemblance to HLA-C, which is known to interact with PCSK9, we hypothesize that HFE might similarly engage with PCSK9. This interaction could potentially influence LDLR levels and subsequently impact cholesterol levels.

2. Deciphering the regulatory role of HLA-C on PCSK9: The second objective of this work is the continuation of our recent publication, which suggests HLA-C as a crucial protein for PCSK9's activity. The primary goal of this study is to validate and solidify the regulatory influence of HLA-C on PCSK9 through appropriate cell-based models. Additionally, within this objective, we aim to investigate the potential competition between HFE and HLA-C in their interactions with PCSK9. Given their analogous structures and likely utilization of the same interaction sites with PCSK9, we seek to compare their binding capabilities.
3. Unravelling PACE4's Impact on PCSK9 and LDLR regulation: The third objective seeks to explore the novel dimension of PACE4's involvement in modulating LDLR levels. Following on earlier mass spectrometry assays that indicate a potential connection between

PACE4 and PCSK9, our goal is to explore how this interaction contributes to lipid metabolism within the liver.

Achieving these objectives has the potential to not only improve our understanding of lipid and iron regulatory networks but also offer innovative perspectives for the development of targeted therapies for a spectrum of lipid-related and iron-related disorders.

Chapter II

II.I. Experimental procedures:

Human plasma samples:

Patients with PCSK9 Q152H mutation as well as normal subjects recruited from the clinical refers at the IRCM (Institut de Recherches Cliniques de Montréal). All the experiments have been done in a blinded way with no access to confounding factors such as age, sex, and BMI. Plasma samples were provided from IRCM clinic (in collaboration with Dr. May Faraj's group) and stored at -80 °C until the analysis. All protocols were approved by IRCM ethical committee.

Mice plasma samples:

The plasma of both Tfr-/- and WT mice were provided from our collaborator Dr. Kostas Pantopoulos. For each genotype both male and female mice were provided. All mice were 8-month-old with C57BL/6J background under normal chow diet. All protocols were approved by IRCM ethical committee.

Generation of constructs:

Human complementary DNAs (cDNAs) encoding wild-type and mutant forms of LDLR, PCSK9, HFE, HLA-C and PACE4 were created through site-directed mutagenesis. These cDNAs were incorporated into vectors such as pIRES or PcDNA3.1/zeo+ for expression. Additionally, both negative and positive control constructs were included in the experimental setup. To distinguish and track the expressed proteins, various tags like V5 and FlagM2 were introduced to the constructs. These tags enable the visualization and detection of the proteins in subsequent experiments. Before further analyses, the sequence integrity of each mutant construct was rigorously confirmed using complete DNA sequencing. This verification step ensured that the genetic modifications were accurately introduced and that the constructs maintained their intended sequences.

Cell culture and transfection:

Various cell lines were utilized, including IHH (immortalized human hepatocytes), HEK293 (human embryonic kidney-derived epithelial cells), HuH7 (human hepatocellular carcinoma cells), HepG2 (human hepatocellular carcinoma cells), CRISPR HepG2 HLA-C$^{-/-}$ cells, and CRISPR

HepG2 PCSK9$^{-/-}$ cells. These cells were cultured in specific growth media: Dulbecco's Modified Eagle Medium (DMEM) or Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum (FBS). The cells were maintained at a temperature of 37°C in an environment with 5% CO2 to simulate physiological conditions. Transfection was employed to introduce the desired genetic constructs (PCSK9, LDLR, PACE4, HFE, HLA-C and their variants) into the cells. Depending on the cell line, different transfection reagents were used: GenJetTM in vitro, FuGENE®HD (Promega), or jetPRIME (PolyPlus) transfection reagents for IHH, HuH7/HepG2, and HEK293 cells, respectively. The cells were allowed to express the introduced genes for 48 hours post-transfection. For HEK293 cells, a specialized protocol was followed: the cells were coated with poly-L-lysine, then seeded in large flasks (T175) for producing PCSK9 and/or PACE4 media. jetPRIME transfection reagent was used for this process. After 48 hours, the conditioned media containing the secreted proteins were collected and stored at a temperature of -80°C for subsequent analysis. For the media swap experiment different cells were seeded in 12-well cell culture plates, and after 24 hours, they were incubated with serum-free media overnight. Subsequently, the cells were exposed to conditioned media produced from HEK293 cells overexpressing human PCSK9/PACE4.

In-house ELISA measurement of human PCSK9 levels in media:

The secreted concentrations of PCSK9 in the media/plasma were determined using an in-house luminescence-based human PCSK9 ELISA assay, which was conducted as follows: LumiNunc Maxisorp white assay plates were used and coated with 0.5 µg/well of anti-human PCSK9 antibody (hPCSK9-Ab). The coating was carried out by incubating the plates at 37°C for 3 hours and then at 4°C overnight. After the coating, the plates were subjected to washing steps to remove any unbound components. The plates were then blocked using a blocking buffer composed of PBS (Phosphate-Buffered Saline), casein at 0.1% concentration, and merthiolate at 0.01% concentration. This step helps prevent non-specific binding. Calibrators were prepared by creating serial dilutions of known concentrations of a standard PCSK9 solution. Samples, which contained secreted PCSK9 from the cell culture media, were prepared by diluting them at two different dilution ratios: 1:50 and 1:100, using a dilution buffer with (for cell media) and without (for plasma) BSA (Bovine Serum Albumin). The calibrators and samples were added to the coated and blocked plates and allowed to incubate for 30 minutes at a temperature of 46°C. After the

incubation, the plates were washed again to remove any unbound materials. Subsequently, a secondary antibody known as hPCSK9-Ab-HRP (Horseradish Peroxidase) was added to the plates. This secondary antibody helps in detecting the bound PCSK9. The plates were then incubated for 3 hours at a temperature of 37°C while shaking. After the secondary antibody incubation, the plates were washed once more. A substrate solution, specifically SuperSignal™ ELISA Femto Substrate from Pierce, was added to each well of the plate. The substrate reacts with the HRP enzyme to generate chemiluminescent light. The generated chemiluminescence was quantitated using a Pherastar luminometer from BMG Labtech. The concentrations of the secreted PCSK9 in the samples were calculated and adjusted accordingly for each experimental construct, allowing for a comparative analysis of PCSK9 secretion across different conditions or treatments.

Western blotting:

Cultured cells and tissues underwent the following process for protein extraction and analysis: First the cultured cells and tissues were washed to remove any residual media or contaminants. Then, non-denaturing cell lysis buffer was used for protein extraction. The composition of the lysis buffer was as follows: 20 mM Tris-HCl (pH 8), 137 mM NaCl, 2 mM Na2EDTA, 1% NP-40 (Nonidet P-40), 10% glycerol, and 4% protease inhibitor cocktail without EDTA. This buffer helped break down the cells and tissues to release their protein content. The Lowry assay was employed to determine the protein concentrations in the extracted samples. This assay is a commonly used method for protein quantification based on colorimetric measurements. In the next step, the extracted proteins were separated by size using polyacrylamide gel electrophoresis (SDS-PAGE). Two types of gels were used: 6.5% and 8% tris-glycine gels. SDS-PAGE helps separate proteins based on their molecular weight. The separated proteins were then transferred from the gel onto PVDF (Polyvinylidene Fluoride) membranes. This transfer process is known as Western blotting and allows the proteins to be immobilized on the membrane for subsequent analysis. The PVDF membranes were incubated with specific primary antibodies that bind to the target proteins of interest. After the primary antibody incubation, secondary antibodies were applied. These secondary antibodies are labeled with enzymes or fluorophores and bind to the primary antibodies, allowing for the detection of the target proteins. The membranes were analyzed and quantified using a ChemiDoc imaging system from Biorad.

Dil-LDL uptake:

The process for the Dil-LDL uptake assay involved the following steps: Cells were seeded onto plates coated with Poly-L-lysine at a concentration of 50 µg/ml. After 48 hours of cell growth, the cells were exposed to Dil-LDL (Diluted-Low-Density Lipoprotein) for a specific period. The Dil-LDL used in this assay was at a concentration of 6 µg/ml. Dil-LDL is a fluorescently labeled form of LDL, allowing for easy tracking and visualization of its uptake by cells. The cells were incubated with Dil-LDL for 3 hours at a temperature of 37°C. During this time, the cells had the opportunity to take up the labeled LDL particles. After the 3-hour incubation, the media containing Dil-LDL was removed from the wells and cells were washed with ice-cold D-PBS (Dulbecco's Phosphate-Buffered Saline) without ions to remove any residual Dil-LDL that was not taken up by the cells. A final wash step was performed to ensure complete removal of unbound Dil-LDL. Subsequently, 100 µl of PBS was added to each well. PBS provides a buffer for the subsequent measurements. Cells were fixed to be analyzed by confocal microscopy.

Immunofluorescence assay (IF):

For the IF experiment, CRISPR HepG2 PCSK9 KO cells were cultured, and their media was replaced with a media containing 0.3 ng/ml of human PCSK9 (hPCSK9). After the media swap, the cells were allowed to incubate for 24 hours. During this time, the cells were exposed to the hPCSK9. After the 48-hour incubation, the cells were washed twice with PBS (Phosphate-Buffered Saline) to remove any residual substances. Subsequently, the cells were fixed using 4% paraformaldehyde. Fixation helps preserve the cellular structure for further analysis. To prevent nonspecific binding of antibodies, the fixed cells were blocked with a solution of PBS containing 2% BSA (Bovine Serum Albumin). Blocking reduces background staining during subsequent antibody incubations. Then, cells were incubated with a goat anti-human LDLR polyclonal antibody (at a dilution of 1:200) from R&D Systems at a temperature of 4°C overnight. The next day, plates were washed with PBS to remove unbound primary antibody and were then incubated with an appropriate fluorescent secondary antibody. To visualize cell nuclei, the samples were stained with Hoechst dye at a concentration of 1 µg/ml. Hoechst dye specifically binds to DNA and labels cell nuclei. The coverslips containing the stained cells were mounted onto glass slides using Mowiol, a mounting medium. The prepared samples were then visualized using a confocal laser-scanning microscope with a high-powered objective lens (Plan-Apochromat 63 × 1.4 oil)

from Carl Zeiss. Approximately 10 picture was taken from each repeat and condition with same wavelength settings for all confocal channels.

PCSK9 – LDLR (EGF-AB peptide) binding assay:

The CircuLex human PCSK9 functional assay kit (MBL, Cat # CY8153) was used to measure the binding affinity of various mutations of PCSK9 in comparison to the wild-type (WT) PCSK9. This kit utilizes a technique similar to a quantitative sandwich enzyme immunoassay and involves the recombinant LDLR EGF-AB domain (a specific part of the LDL receptor) for binding studies, as an alternative to using antibodies. Media from HEK293 cells containing WT PCSK9 were incubated with HepG2 PCSK9 KO cells that transfected either with HFE or empty vector. Then, samples were collected and serially diluted. These diluted samples were then used for the binding assay. LumiNunc Maxisorp white assay plates were coated with the recombinant LDLR EGF-AB domain. Serially diluted samples of PCSK9 were added to the coated plates containing the LDLR EGF-AB domain. For each concentration of PCSK9 variant, the absorbance at 450 nm (OD) was measured using a SpectraMax i3 plate reader. The obtained OD values were corrected for nonspecific binding and normalized to the maximum absorbance value (OD/ODmax). A binding curve was generated for each PCSK9 variant using a 4-parameter logistic (4-PL) equation. The EC50 value, which represents the concentration of PCSK9 needed for half-maximal binding to the LDL receptor EGF-AB domain, was extracted from the binding curve.

Co-immunoprecipitation assay

In this experiment, the aim was to investigate the interaction between PACE4/HFE and PCSK9/LDLR (V5 tag for HFE and no tag for PACE4 experiments) proteins. For this experiment HepG2 PCSK9 KO were used. cDNAs encoding PACE4/HFE and PCSK9/LDLR proteins were co-expressed in these HepG2 CRISPR PCSK9 KO cells using a transfection method. After 48 hours of transfection, the media/lysate from the cells was collected. The collected samples were then incubated overnight (O/N) at 4°C on a rotator with V5-Agarose Beads. The V5-Agarose Beads were used to "pull down" the PACE4/PCSK9 proteins that were tagged with the V5 epitope. This allowed for the isolation and enrichment of protein of interest from the media sample. The next day, the beads were washed multiple times to remove any non-specifically bound proteins or contaminants. Centrifugation was used to separate the beads from the wash solution. The supernatant was collected and loaded onto an 8% SDS-PAGE gel. SDS-PAGE. After the proteins

were separated in the SDS-PAGE gel, they were transferred onto a PVDF membrane. The bound antibody was then detected using a chemiluminescence-based method. The ChemiDoc imaging system was used to visualize and quantify the signals generated by the antibody-protein interaction.

Modeling of PCSK9/HFE complex:

Global RAnge Molecular Matching (GRAMM*, available at: https://gramm.compbio.ku.edu/) web server was used for molecular docking between HFE complexed with Beta-2-microglobulin (PDB: 1A6Z; chains: A and B; assumed as receptor) and PCSK9's CHRD (PDB: 2P4E; assumed as ligand). HFE residues R67 and E69 of the RVE motif (UNIPROT: Q30201; residues 45 and 47 in the crystallographic structure) were taken as interface constraints for filtering 10 top models.

The comparison of the structural models of the PCSK9/HLAC and PCSK9/HFE complexes was carried out using the PCSK9/HFE model described in this work and the PCSK9/HLAC model published in the article Carole Fruchart Gaillard et al. 2023**using PyMOL. Of the 10 best models obtained, 5 of them present contacts with the M2 of the CHRD of PCSK9. Of the 5 models retained, 2 of them are clearly in competition with HLAC. Of these 2 models, 1 model has similar contacts to HLAC.

In situ hybridization (ISH):

Sense and antisense cRNA probes coding for PCSK9, LDLR, and PACE4 were labeled with 35S-UTP and 35S-CTP to obtain high specific activity. Cryostat sections of mouse tissues were fixed in 4% formaldehyde and then subjected to ISH. The labeled cRNA probes were hybridized with the tissue sections overnight at 55°C. After hybridization, the sections were exposed to photographic emulsion (NTB-2, Kodak) for 6–12 days, followed by development in D19 solution (Kodak). Hematoxylin staining was performed for visualization.

Chapter III

Human homeostatic iron regulator protein (HFE) as a new target and regulator of PCSK9:
The recent papers on the correlation of HFE with LDLR and cholesterol levels raised the question if HFE can have a role in lipid metabolism. Since PCSK9 is the main regulator of LDLR and can interact with MHC-I-like molecules, we proposed that probably the regulatory effect of HFE in LDLR levels is *via* its interaction with PCSK9.

In search for protein X:

Thanks to the massive amount of structural-functional and mutagenesis assays, we now know that although the M2 domain of PCSK9 does not directly bind to the LDLR, its presence is important for the extracellular function of PCSK9 on LDLR. The extensive research on PCSK9's structure revealed that the M2 domain may interact with another yet unidentified protein (called protein X) that is necessary for the extracellular function of PCSK9. Since purified extracellular PCSK9 (recombinant PCSK9) is still active on LDLR protein X cannot be a secretory protein and probably is a transmembrane protein. The so-called protein X needs to have a proper sequence at the C-terminal to send the complex of PCSK9 and LDLR to the lysosomal degradation. Furthermore, the removal of the C-terminal of LDLR (that includes the NY_XP motif needed for the endocytosis of the complex), does not affect PCSK9's ability to degrade LDLR ΔCT. This suggests that there might be another transmembrane protein within the complex with sufficient cytosolic tail to compensate for the absence of C -terminal LDLR and internalize the complex.

To better understand the exact function of this so-called protein X, we needed to know where exactly this protein is needed during the internalization and degradation pathway of the complex. To answer part of this question, we looked at the LDLR localization in the presence or absence of the M2 domain. Thus, we transiently transfected PCSK9-WT-V5, PCSK9-ΔM2-V5, and empty vector encoding EGFA constructs in HEK293 cells to produce the PCSK9-enriched conditioned media in those cells. Then, HepG2 PCSK9 KO CRISPR cells have been incubated with the equal amounts (~ 300 ng/ml) of each protein isoform and fixed to be analyzed by immunofluorescence microscopy (IF) assay. Immunostaining of LDLR confirmed that the addition of extracellular WT PCSK9 dramatically reduces the levels of LDLR both at the cell surface (non-permeabilized conditions) and cytoplasm (permeabilized condition). Indeed, the presence of PCSK9 ΔM2 had

almost no effect on intracellular LDLR, consistent with previous data suggesting this construct as a LOF. Intriguingly, cell surface LDLR (accounts for ~5% of total LDLR) is reduced in the presence of ΔM2 PCSK9 like WT PCSK9. This data suggests that the importance of the M2 domain lies in its role in the trafficking of the complex from early endosomes to lysosomes and might not have a critical role at the cell surface. Therefore, the interaction of the M2 domain of PCSK9 and protein X likely occurs either at the cell surface or later during the pathway (in endosomes or lysosomes) (Fig. 18 A, B).

Moreover, intracellular markers have been used to localize the LDLR either in early endosomes (EEA1 marker) or lysosomes (Lamp1 marker). Here we assessed the effect of WT PCSK9, PCSK9 ΔM2, PCSK9 R549A-E567A (that has been proposed to lose its interaction with the R-x-E motif of HLA-C), and PCSK9 R549A-Q554E-E567A (that further disrupts the interaction of PSCK9 with HLA-C due to the addition of E residue at position 554 that repulses the neighbouring residues on HLA-C at positions E79, 197, and 201) (Fruchart Gaillard et al., 2023).

As expected, these M2 variants showed the same LOF phenotype as PCSK9 ΔM2, which highlights the importance of the R-x-E motif for PCSK9 interaction with protein X. In the control (no PCSK9) and LOF conditions (PCSK9 ΔM2, PCSK9 R549A-E567A, and PCSK9 R549A-Q554E-E567A), the EEA1 signal colocalized with LDLR, is much stronger compared to the condition where WT/functional PCSK9 is present (Fig. 18 C). Thereby, long-term exposure to concentrated WT PCSK9 rapidly enhances the degradation of the LDLR in lysosomes, whereas blockade of the early endosomes to lysosomes sorting of the PCSK9-LDLR complex (using the LOF mutants) leads to accumulation of the LDLR in EEA1 positive vesicles. This suggests that PCSK9 needs protein X for its trafficking from early endosomes to lysosomes. Surprisingly, the LOF variants seemingly show a stronger signal with the LAMP1 marker, too (Fig. 18 C). However, different studies have shown that LAMP1 is not only present in lysosomes, but also can be localized in different subcellular compartments (depending on the tissue) (Cheng et al., 2018). To fully understand the trafficking of LDLR with or without protein X, further experiments are needed to show the localization of LDLR either with hydrolysis enzymes of lysosomes (e.g., cathepsin D) or intracellular markers that are involved in the recycling pathway of LDLR (e.g., Rab5, Rab7, or Rab11).

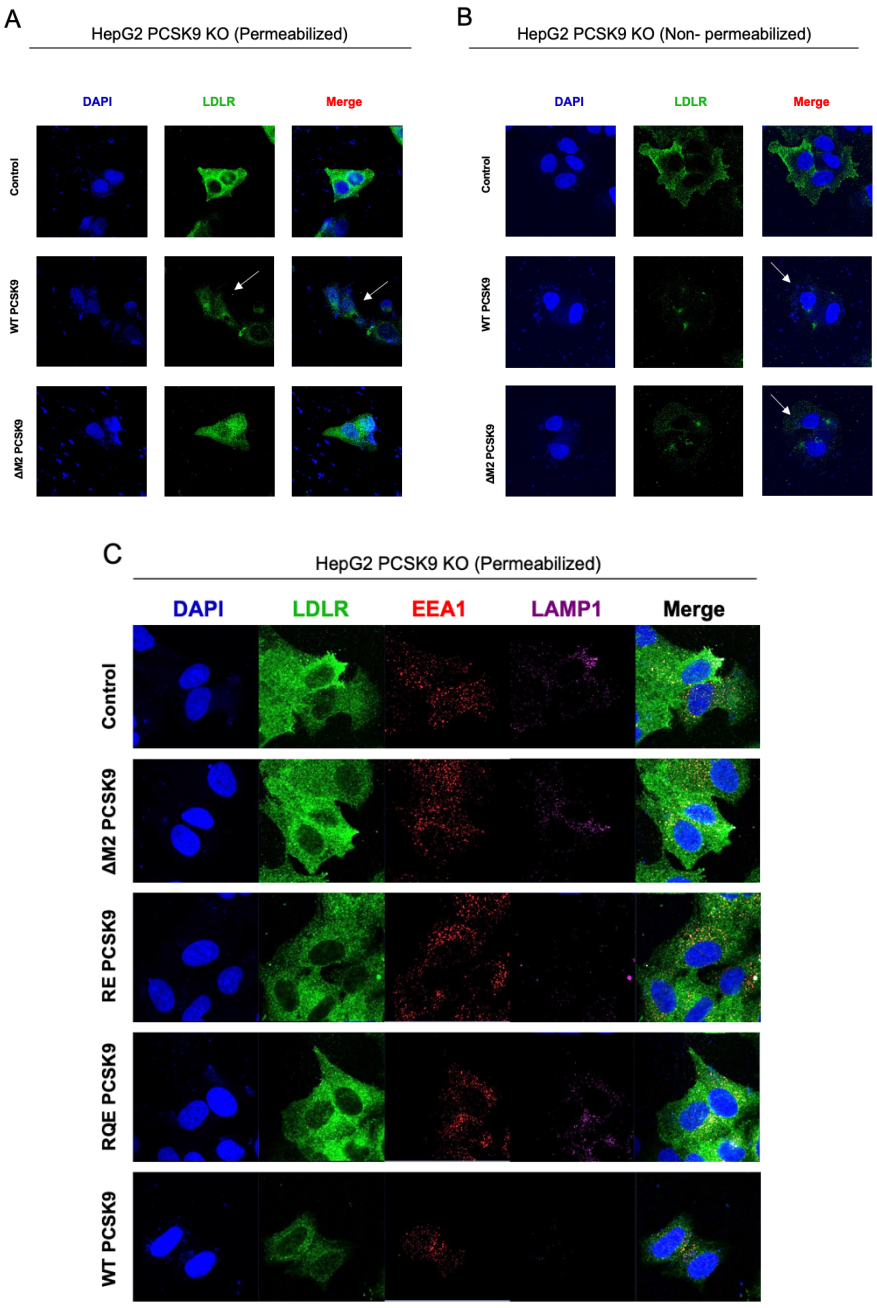


Fig. 18. Extracellular importance of M2 domain of PCSK9 in cellular trafficking of LDLR and PCSK9 complex in HepG2 CRISPR PCSK9 KO cells. Immunofluorescence staining of A) total LDLR (permeabilized conditions) vs B) cell surface LDLR (non-permeabilized conditions) levels in the presence of PCSK9-ΔM2 compared to WT PCSK9. C) Immunostaining of total LDLR along with early endosomal (EEA1) and lysosomal (LAMP1) markers in the presence of PCSK9 ΔM2, PCSK9 R549A-E567A (RE), and PCSK9 R549A-Q554E-E567A (RQE). Blue, Nuclei; green, LDLR; red, EEA1; and purple, lamp1. Imaging has been done using Imaris software. Scale bar, 1μm. Data are representative of three independent experiments.

The next step of our work was to look for the potential candidate proteins that can act as protein X. The recent publications about the interaction and/or implication of MHC-I-like molecules in lipid metabolism grabbed our attention to see if any member of this family can act as a protein X. To do so, we first performed the qPCR experiment to measure the mRNA levels of different members of HLA family, including (HLA-A, HLA-B, HLA-C, HLA-E, HLA-F, HLA-G) along with PCSK9 and β2M in HepG2 naïve, HepG2 CRISPR PCSK9 KO, HepG2 CRISPR HLA-C KO, IHH, and Huh7 cells. The qPCR data showed that HLA-A, B and C are the most frequent members in HepG2 cells. And consistent with previous publications, the mRNA expression of HLA-A and HLA-B is much higher compared to HLA-C, which proposes HLA-A and HLA-B as major MHC-I proteins involved in the adaptive immune system (Fig. 19A-E). Due to the recent evidence on the possible role of HFE and HLA-C on lipid metabolism, we selected these two proteins to investigate. This section will focus on HFE protein, and the next section will look at HLA-C.

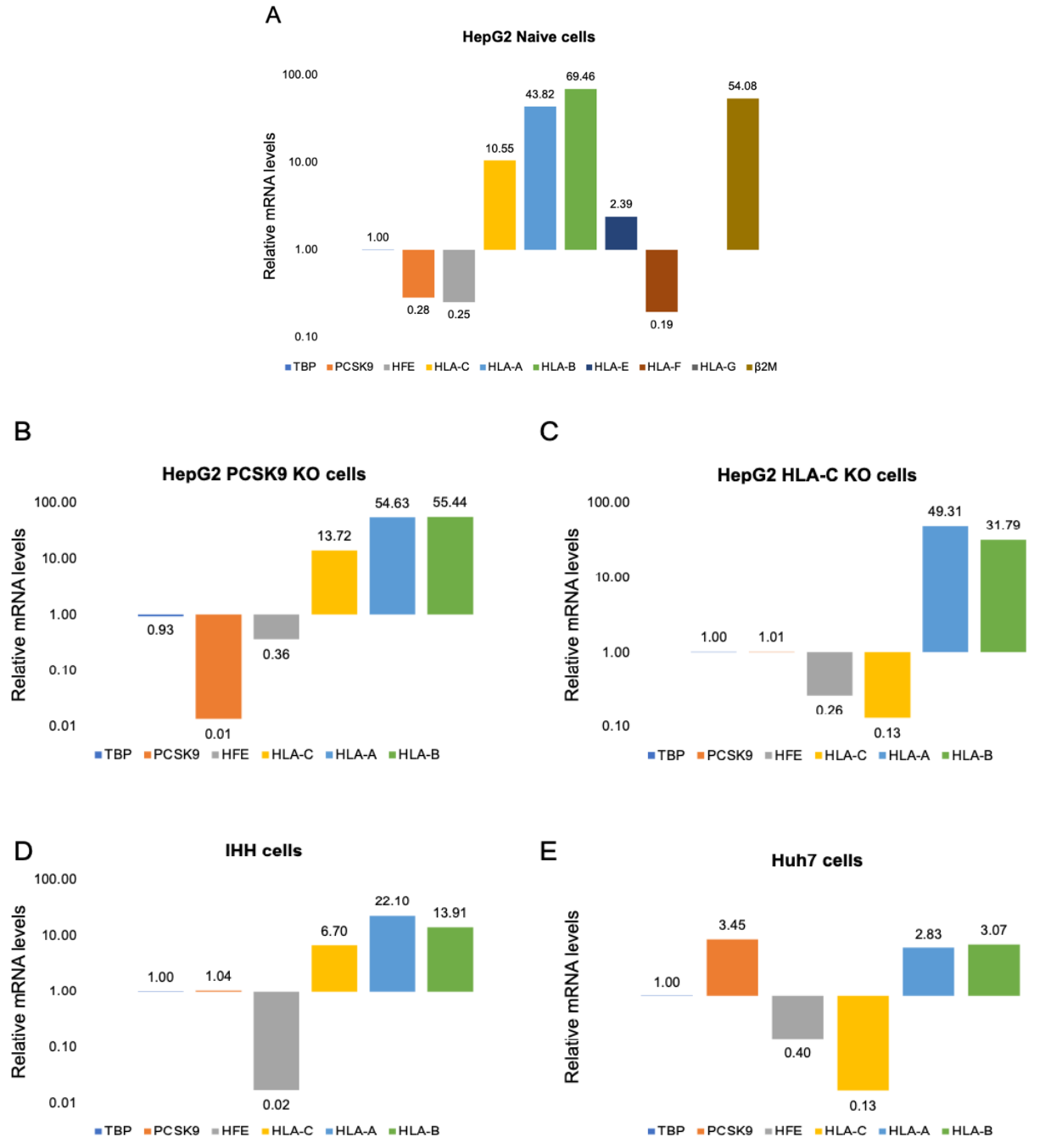


Fig. 19. Comparison of mRNA expression of various HLA family members in different hepatocytes cell lines. RNA was extracted from A) HepG2 naïve, B) HepG2 CRISPR PCSK9 KO, C) HepG2 CRISPR HLA-C KO, D) IHH, and E) Huh7 cells for qPCR analysis. mRNA values were normalized to the mRNA expression of the housekeeping gene (TATA box binding protein: TBP) to calculate the relative mRNA expression of each MHC-I member and compare it to the expression of PCSK9 and β2M.

HFE as a new substrate and regulator of extracellular PCSK9:

In the next attempt, we looked at the possible effect of HFE on PCSK9 and *vice versa*. Therefore, we transiently transfected HepG2 PCSK9 KO CRISPR cells with HFE-flagM2 or its common variant HFE-C282Y-flagM2 with their chaperone β2M-flag-M2. The transfected cells were incubated for 18 hrs with conditioned media enriched with WT PCSK9 or control (empty vector). Then cells were collected and analyzed with WB. Unexpectedly, the data revealed that HFE inhibits the function of PCSK9 on LDLR by ~ 30%. On the other hand, extracellular PCSK9 seems to target WT HFE to the degradation as the presence of WT PCSK9 leads to ~70 % reduction in total levels of over-expressed HFE (Fig. 20A). Nevertheless, PCSK9 is only able to target the WT HFE and not the HFE-C282Y variant. Hence, PCSK9 regulates the function of WT HFE as it reduces the cellular levels of HFE and thus L-ferritin (that is upregulated in HH patients with C282Y variant) (Fig. 20B). Furthermore, the interaction of PCSK9 with HFE requires the PCSK9's M2 domain (like HLA-C), as the PCSK9 ΔM2 domain is not able to degrade HFE. These results were further confirmed by co-immunoprecipitation (Co-IP) assay, where HFE is only pulled down with WT PCSK9 and not PCSK9 ΔM2. Although the HFE-C282Y variant is not degraded by WT PCSK9, it can bind to it (Fig. 20C). Applying different inhibitors of proteasome degradation (MG132), lysosomal degradation (NH_4CL: ammonium chloride), and autophagy (3-MA: 3-methyladenine) suggests that the degradation of HFE occurs in the acidic lysosomes (like LDLR and HLA-C) (Fig. 20D). Altogether, these data, for the first time, suggest HFE as a new negative regulator of LDLR degradation by inhibiting extracellular PCSK9 and as an independent target degraded by PCSK9.

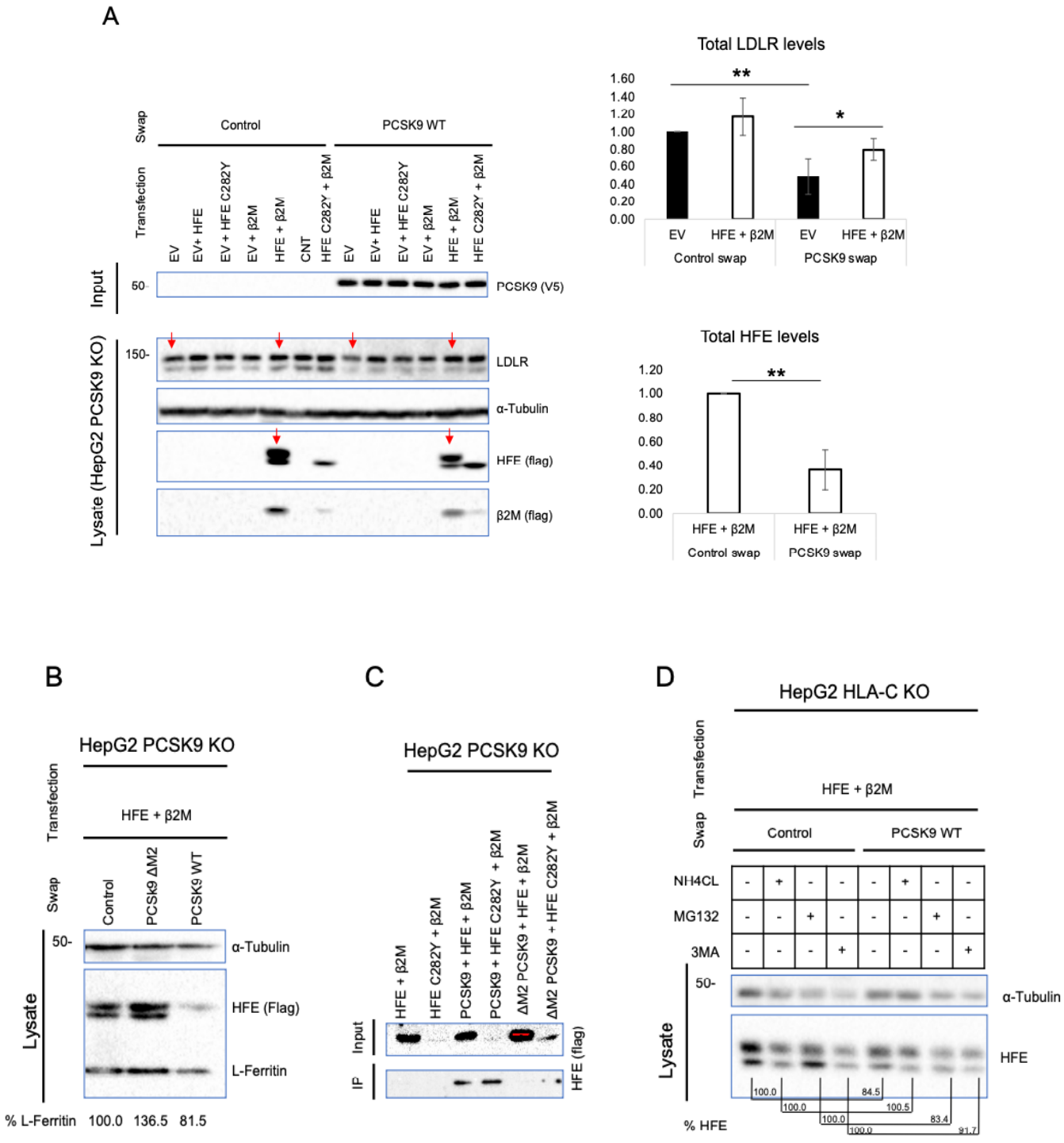

A
Swap
Control
PCSK9 WT
Transfection
EV
EV+ HFE
EV + HFE C282Y
EV + β2M
HFE + β2M
CNT
HFE C282Y + β2M
Input
50-
PCSK9 (V5)
Lysate (HepG2 PCSK9 KO)
150-
LDLR
α-Tubulin
HFE (flag)
β2M (flag)
Total LDLR levels
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
**
*
EV
HFE + β2M
EV
HFE + β2M
Control swap
PCSK9 swap
Total HFE levels
1.20
1.00
0.80
0.60
0.40
0.20
0.00
**
HFE + β2M
HFE + β2M
Control swap
PCSK9 swap
B
HepG2 PCSK9 KO
Transfection
HFE + β2M
Swap
Control
PCSK9 ΔM2
PCSK9 WT
50-
α-Tubulin
Lysate
HFE (Flag)
L-Ferritin
% L-Ferritin 100.0 136.5 81.5
C
HepG2 PCSK9 KO
HFE + β2M
HFE C282Y + β2M
PCSK9 + HFE + β2M
PCSK9 + HFE C282Y + β2M
ΔM2 PCSK9 + HFE + β2M
ΔM2 PCSK9 + HFE C282Y + β2M
Input
IP
HFE (flag)
D
HepG2 HLA-C KO
Transfection
HFE + β2M
Swap
Control
PCSK9 WT
NH4CL
MG132
3MA
50-
α-Tubulin
Lysate
HFE
100.0
84.5
100.0
100.5
100.0
83.4
100.0
91.7
% HFE

Fig. 20. HFE interaction and negative regulatory effect on PCSK9 and vice versa. HepG2 lacking endogenous PCSK9 were transiently transfected with an empty vector (EV), HFE-WT-flagM2, HFE-C282Y-flagM2, β2M-flagM2 and were incubated with conditioned media enriched with extracellular PCSK9 or empty vector (control). A) The effect of HFE on the extracellular activity of PCSK9 has been analyzed by WB (SDS/PAGE on 8% Tris-glycine gel) analysis and looking at total LDLR levels in the Lysates. The extracellular effect of PCSK9 was assessed by media swap experiment. The quantifications reveal that overexpressed HFE significantly inhibits PCSK9's function of LDLR (top panel), and PCSK9 also degrades WT HFE (lower panel). B) L-Ferritin has been measured to see how the presence of extracellular PCSK9 could affect the function of HFE by sending it to degradation. The results show lower levels of L-ferritin in the presence of WT PCSK9 and not ΔM2 PCSK9. C) PCSK9-WT-V5 or PCSK9- ΔM2-V5 were co-expressed with either HFE-WT-flagM2 or HFE-C283Y-flagM2, and cell Lysates were collected for Co-IP assay. For this assay, V5-agarose beads have been used to pull down either WT or ΔM2 PCSK9, and precipitated proteins have been analyzed by WB assay and by using antibodies against flagM2 tag. The Co-In assay shows that only WT PCSK9 interacts with HFE and not ΔM2 PCSK9. D) similar experimental procedure has been done as panel (A), in addition to cellular inhibitors for lysosomal degradation (NH_4CL: ammonium chloride), proteasome degradation (MG132), and autophagy (3-MA: 3-methyladenine). The data suggest the possible lysosomal degradation of HFE. All protein levels were normalized to the control protein, α-tubulin. Data are representative of at least three independent experiments (except for panels C and D). Quantifications are averages ± standard deviation (SD). * $p<0.05$; ** $p<0.01$; ***$p<0.001$ (two-sided t-test). NS: non-significant.

HFE and PCSK9 interaction:

It was recently reported that PCSK9 interacts with HLA-C molecule *via* its M2 domain with the R-x-E motif of HLA-C. As HFE has a similar structure to HLA-C and includes two R-x-E motifs within its sequence, we hypothesized that HFE could bind to PCSK9 through similar residues. Hence, we modelled the M2 domain interaction with HFE using GRAMM-X web server (http://vakser.compbio.ku.edu/resources/gramm/grammx/). Our 3D structure modelling revealed that PCSK9 uses the exact same residues (R-x-E motif) as HLA-C to interact with HFE. Thus, arginine 67 and glutamic acid 69 on HFE interact with glutamic acid 567 and arginine 549 on PCSK9, respectively. It seems that the other R-x-E motif on HFE is far from the interaction surface and might not be involved in the binding of HFE to PCSK9 (Fig. 21A). Among the contacts highlighted in this model, R549 of the M2 of CHRD is in contact with E69 (8.7 Å) of the α1 domain of HFE. E567 of CHRD M2 is in contact with R67 (8.2 Å) and R78 (5.7 Å) of HFE α1 domain. Moreover, Q554 of the M2 of the CHRD is close to R71 (2.0 Å) of the α1 domain of HFE (Fig. 21A). We further, analyzed the possible interaction of HFE with natural mutations on PCSK9, namely Q554E (LOF for LDLR) and H553R (GOF for LDLR). Our modelling illustrates that due to the positive charge of arginine at position 71 on HFE, the PCSK9 Q554E could act as a GOF for HFE, and negatively charged glutamic acid attracts arginine on HFE. On the other hand, the PCSK9 H553R seems to repulse arginine 71 on HFE (LOF for HFE) (Fig. 21B).

We confirmed these data by mutating WT PCSK9 to introduce/remove the mentioned binding sites. Thus, we alanine mutated the R-x-E motif on PCSK9 along with the production of natural H553R mutation. The produced variants were transfected in HEK293 cells to provide the conditioned media enriched with the mutation of interest. Next, HepG2 CRISPR PCSK9 KO cells were incubated with produced media for 18 hrs, and samples were collected for western blot analysis. This experiment confirmed the 3D modelling as in the absence of the R-x-E motif on PCSK9, HFE is no longer degraded by PCSK9. Predictably, the H553R PCSK9 variant is LOF for HFE (Fig. 21C). However, the effect of H553R PCSK9 on LDLR is not the same as we expected (to be GOF) based on the phenotypes observed in patients with FH. Altogether, these data are consistent with our earlier WB data regarding the interaction of HFE and PCSK9 and indicate a possible competition for PCSK9 between HFE and LDLR.

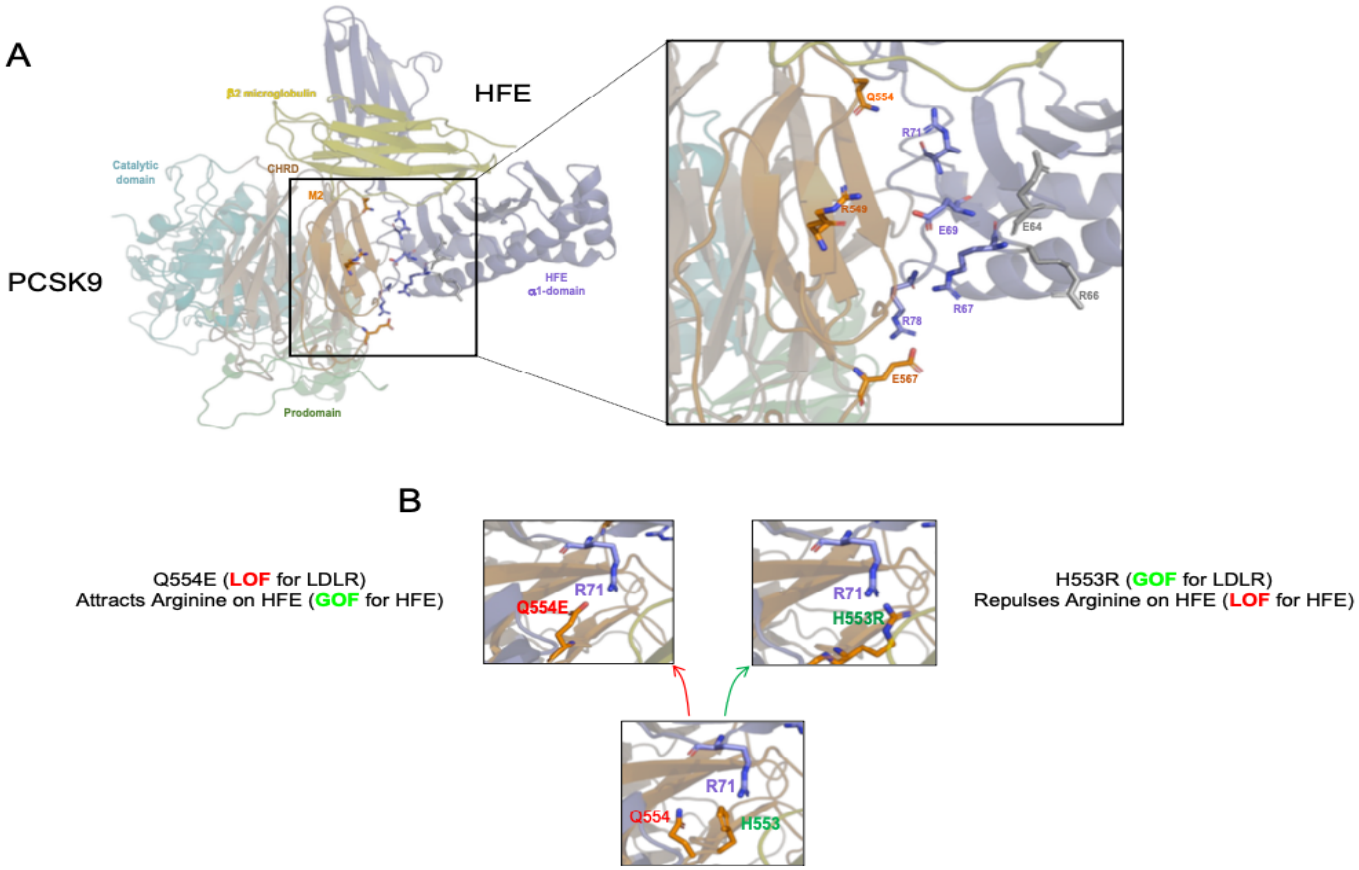

A
HFE
β2 microglobulin
Catalytic domain
CHRD
M2
PCSK9
HFE α1-domain
Prodomain
Q554
R71
R549
E69
E64
R66
R67
R78
E567
B
Q554E (LOF for LDLR)
Attracts Arginine on HFE (GOF for HFE)
R71
Q554E
R71
H553R
H553R (GOF for LDLR)
Repulses Arginine on HFE (LOF for HFE)
R71
Q554
H553
C
HepG2 PCSK9 KO
Swap
Control
PCSK9 WT
PCSK9 ΔM2
PCSK9 R549A-E567A
PCSK9 R549A-Q554E-E567A
PCSK9 H553R
Transfection
EV
HFE + β2M
HFE-R67A-E69A + β2M
Lysate
150-
LDLR
α-Tubulin
HFE(flag)
Relative HFE levels
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
*
**
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Fig. 21. HFE interaction sites with PCSK9. A) Molecular modeling of the interaction of the M2 subdomain of PCSK9 with the α1 domain of HFE that suggests the interaction of the R549-x-E567 motif of PCSK9 with the R67-x-E69 motif of HFE. B) further analysis of the 3D modeling of PCSK9 interaction with HFE, suggests the presence of another interaction site on HFE (R71) with PCSK9 that could be sensitive to PCSK9 natural mutations Q554E (GOF for HFE) and H553R (LOF for HFE). C) HepG2 PCSK9 KO cells were transfected with either, empty vector (EV), WT HFE or HFE R67A-E69A variant and then incubated with conditioned media from HEK293 cells expressing an empty vector (control), WT PCSK9, and proposed LOF variants on PCKS9 (ΔM2, R549A-E567A, R549A-Q554E-E567A, and H553R). Cell Lysates were extracted to be analyzed by WB (SDS/PAGE on 8% Tris-glycine gel). The cell-based data confirmed the predicted interaction sites by 3D structure modeling in panels (A) and (B). All protein levels were normalized to the control protein, α-tubulin. WB Data are representative of two independent experiments. Quantifications are averages ± standard deviation (SD). * $p<0.05$; ** $p<0.01$; ***$p<0.001$ (two-sided t-test). NS: non-significant.

Factors involved in HFE and PCSK9 interaction:

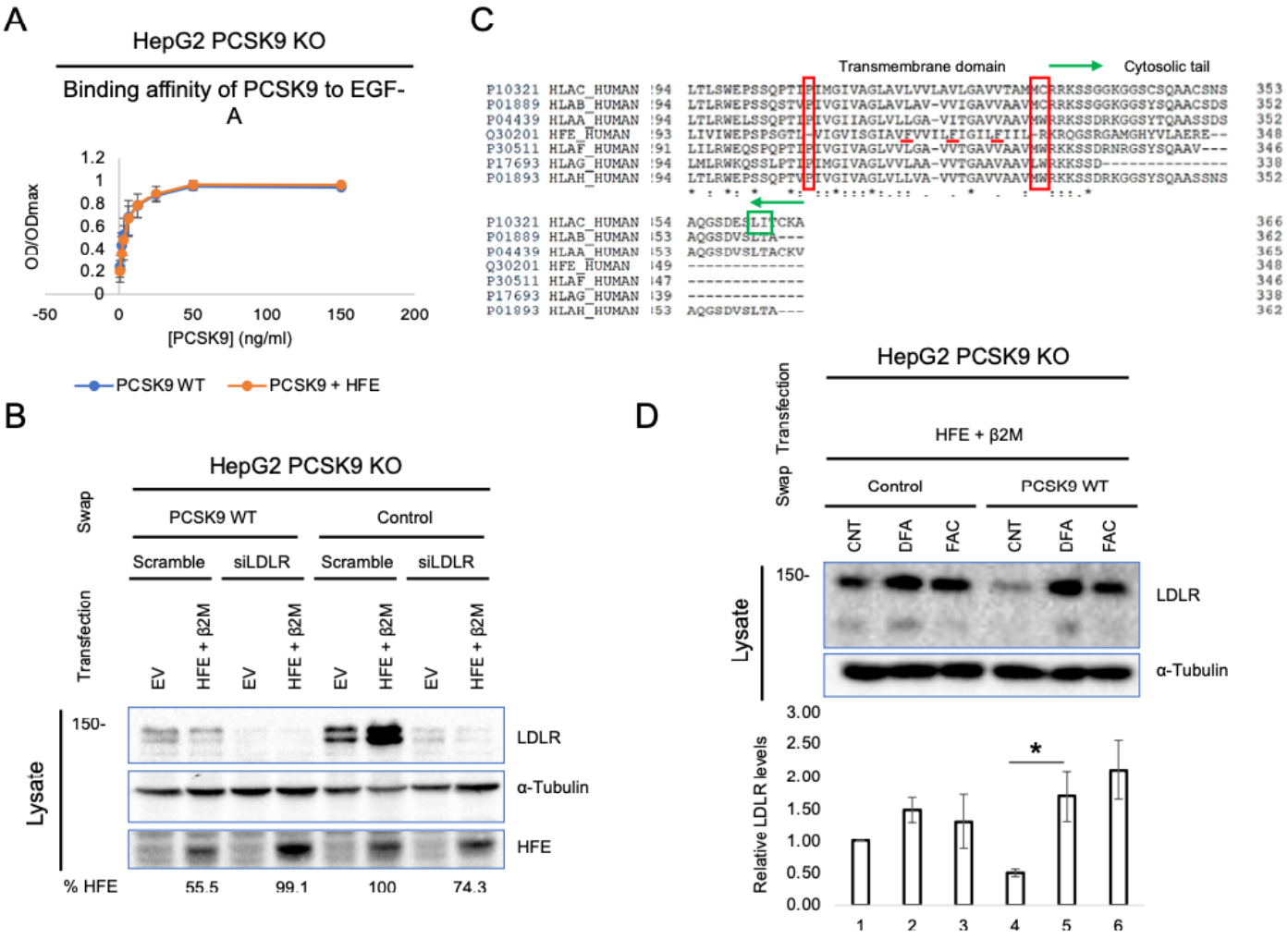
Since the presence of HFE seems to inhibit PCSK9's function on LDLR, we postulated that HFE might compete with LDLR to interact with PCSK9. To test this hypothesis, we first estimated the binding affinity of LDLR to PCSK9 in the presence or absence of HFE using CircuLex human PCSK9 functional assay kit (MBL, #CY8153). The data showed that PCSK9 has the same affinity with the EGF-A domain of LDLR either in the presence or absence of HFE/soluble HFE (sHFE)/chaperone β2M (Fig. 22A). Furthermore, the absence of LDLR (using siRNA approach) inhibits PCSK9 function on HFE (Fig. 22B). Thus, LDLR and HFE do not compete for binding to PCSK9, and HFE degradation requires the presence of LDLR. This is probably because of the short cytosolic tail of HFE in comparison with other HLA subtypes. This is consistent with earlier data that showed that HFE alone cannot internalize into cells and needs to be coupled with another transmembrane protein such as TfR1 to be able to enter the cells. Indeed, the addition of the cytosolic tail of LDLR to HFE construct helps with the endocytosis of HFE in the absence of TfR1 (Ramalingam et al., 2000). Therefore, PCSK9-HFE complex requires LDLR for cellular internalization (Fig. 22C).

At the cell surface, HFE usually is attached to TfR1, and, during iron uptake, it is internalized with TfR1 and then recycles back to the cell surface. However, in conditions such as high levels of iron in circulation, HFE dissociates from TfR1 to activate the ERK/MAP signalling pathway leading to higher expression of hepcidin. Hence, higher levels of iron or the absence of TfR1 could increase the availability of HFE at the cell surface for interaction with extracellular PCSK9 and subsequently may result in a higher inhibitory effect on PCSK9's function on the LDLR.

To test this possibility, HepG2 CRISPR PCSK9 KO cells were transfected with cDNAs coding for HFE-WT-flagM2 and β2M-flagM2 and then incubated with previously produced condition media enriched with PCSK9 WT. Cells were incubated with either 200 µg/ml Iron (FAC: ferric ammonium citrate) or 200 µM iron chelating factors (DFA: deferoxamine) and collected 18 hrs later. As expected, the addition of DFA dramatically increases the total LDLR levels regardless of the presence of extracellular WT PCSK9. This phenomenon has been explained before that the elevation of LDLR levels is due to the presence of (iron regulatory element) IRE at 3'UTR of LDLR that stabilizes and increase LDLR expression (Guillemot et al., 2016). Intriguingly, in the presence of iron (FAC), the function of PCSK9 is completely blocked by HFE (Fig. 22D).

Therefore, disrupting the interaction of HFE with TfR1 results in higher levels of available HFE at the cell surface and greater inhibition of PCSK9.

However, looking at the plasma lipid profile of TfR1$^{-/-}$ mice (by FPLC assay) shows higher LDL levels both in males and females (Fig. 22E). The FPLC experiment shows that in the LDL fractions 20-30, cholesterol is higher in both male and female KO mice. In males, the sum of LDLc is 106.5 (WT) *vs* 130.9 (KO 23%) and in females, it is 142.4 (WT) *vs* 164.9 (KO 16%). This evokes the possibility that in the absence of TfR1, HFE is less able to reach the cell surface from the ER, and hence PCSK9 would be more active.



E

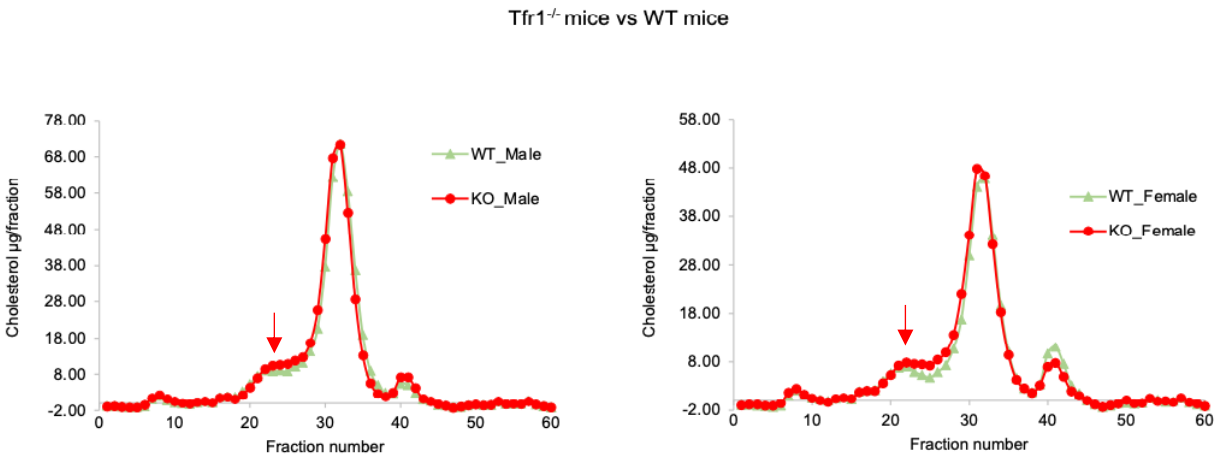


Fig. 22 . HFE trafficking relies on many factors, such as LDLR and circulating iron levels. A) The binding affinity of WT PCSK9 to the LDLR, was measured using the CircuLex human PCSK9 functional assay kit. The results of the affinity curve suggest that the presence of HFE/β2M does not affect the interaction of PCSK9 with the EGF-AB domain of LDLR. B) HepG2 PCSK9 KO cells were transfected with siRNA against LDLR or non-targeting siRNA (Scramble). 24 hrs later, these cells were transfected with either, an empty vector (EV), or WT HFE and then incubated with conditioned media from HEK293 cells expressing an empty vector (control) or WT PCSK9. C) the comparison of cytosolic and transmembrane domains of HFE with other major HLA family members. D) HepG2 PCSK9 KO cells were transfected with either, an empty vector (EV), or WT HFE and then incubated with conditioned media from HEK293 cells expressing an empty vector (control) or WT PCSK9. Following the incubation with conditioned media, cells were treated with either ferric ammonium citrate (FAC) or deferoxamine (DFA) to analyze the function of HFE on PCSK9 in different iron conditions. E) The plasma of female and male mice (WT and $TfR1^{-/-}$), were obtained for FPLC assay. For this experiment, a plasma pool from 6-9 mice (0.3 ml) was analyzed by FPLC (Pharmacia) on a superpose of 6 columns with a flow rate of 0.3 ml/min. All Cell Lysates in panels (B) and (D) were extracted to be analyzed by WB (SDS/PAGE on 8% Tris-glycine gel). Protein levels were normalized to the control protein, α-tubulin. Data are representative of two-three independent experiments. Quantifications are averages ± standard deviation (SD). * $p<0.05$; ** $p<0.01$; ***$p<0.001$ (two-sided t-test). NS: non-significant.

PCSK9 implication in iron homeostasis:

The discovery of HLA-C as a new target of PCSK9 was a breakthrough that demonstrated PCSK9 could be involved in other cellular metabolisms (such as the immune system), and it is not limited to lipid metabolism. Intriguingly, our discovery of another HLA family member (HFE) as a target for PCSK9, proposed a possible implication of PCSK9 in iron metabolism (as HFE is an iron-related protein with no function in immune activity). To test this proposal, plasma samples were provided from patients with the PCSK9 Q152H variant (having very low levels of PCSK9) and control subjects. As PCSK9 Q152H acts as a dominant negative variant, both homozygous and heterozygous forms result in a great reduction in PCSK9 levels that is almost like PCSK9 KO phenotype. In the first step, we randomly selected the plasma of two patients and two subjects and measured their lipid profile by FPLC assay. As predicted, the cholesterol and TG levels of patients were significantly lower than normal subjects, that confirms PCSK9 Q152H is LOF (Fig. 23A). Nevertheless, one patient had significantly higher levels of TG in VLDL fraction 0-10 (Fig. 23A). Whether this phenotype is related to PCSK9 variation or other genetic and environmental factors (such as diet, and mutation in other proteins) needs further investigations. The levels of circulating PCSK9 were measured by in-house ELISA assay and confirmed that the concentration of circulating PCSK9 is much lower compared to controls (Fig. 23B).

To study the iron levels, total iron-binding capacity (TIBC) and serum iron were measured using a Total iron-binding capacity (TIBC) and serum iron assay kit (Abcam, #ab239715). While the TIBC test measures transferrin capacity to bind to iron, serum iron determines total iron levels in the circulation. Although a wide variation was observed in levels of serum iron and TIBC (probably because of different confounding factors such as age, sex, and diet), our data shows a trend toward lower TIBC, and serum iron levels in patients with PCSK9-Q152H variation (Fig. 23C, D). The percentage of transferrin saturation, which is calculated by levels of TIBC, and serum iron also were lower in PCSK9 Q152H subjects (Fig. 23E). These data demonstrate for the first time the possible role of human PCSK9 in iron homeostasis. In agreement, a recent study in 2020, reported the relationship of PCSK9 deficiency with more severe anemia in sickle cell disease (SCD) mice. We could not succeed in looking at soluble HFE (sHFE) in plasma as its concentration is very low in the circulation. As HFE is normally attached to TfR1 at the cell surface and partially inhibits iron uptake by transferrin receptor, we postulate that in the presence of the homozygote

PCSK9 Q152H variant HFE might either interact with PCSK9 in the ER and be retained there (similar phenotype as HFE C282Y variant) or directly goes to the lysosomal degradation by intracellular PCSK9. Either way, if PCSK9 does not allow HFE to reach the plasma membrane, the iron uptake capacity of TfR1 might increases and lead to lower levels of iron in the circulation. That interesting information encouraged us to look at the intracellular interaction of HFE and PCSK9.

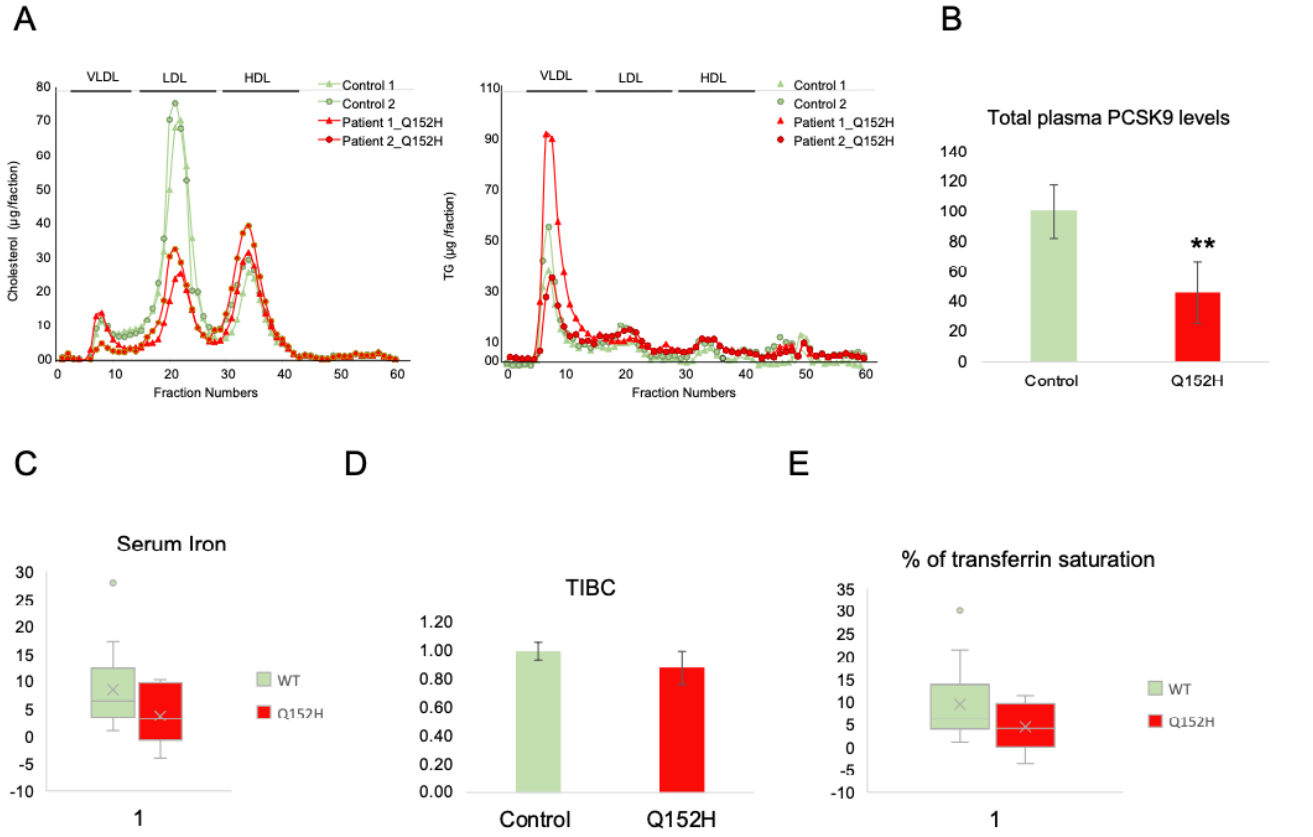


Fig. 23. Possible implication of PCSK9 in iron metabolism. A) The plasma of 7 patients with PCSK9 Q152H and 10 normal subjects, were obtained for FPLC assay. For this experiment, 0.3 ml of each was analyzed by FPLC (Pharmacia) on a superpose of 6 columns with a flow rate of 0.3 ml/min. B) Plasma PCSK9 levels have been measured by in-house Elisa assay to confirm the low levels of PCSK9 in patients with the PCSK9 Q152H variant. Iron indicators were measured using the "Total iron-binding capacity (TIBC) and serum iron assay) kit. The C) serum iron, D) Total iron binding capacity (TIBC), and E) percentage of transferrin saturation have been measured according to the remanufacturer protocol.

Opposing effect of HFE on intracellular PCSK9:

Although the intracellular PCSK9 activity of PCSK9 is not yet understood, many studies suggest that this pathway is distinct from the extracellular function of PCSK9. The presence of the M2 domain of PCSK9 is not critical for its activity inside the cells. Thus, HFE might not have the same inhibitory effect on PCSK9. To better understand this pathway, PCSK9-WT-V5 and PCSK9-ΔM2-V5 were co-expressed with either HFE-WT-V5 or HFE-C282Y-V5 in HepG2 cells lacking endogenous PCSK9. Based on our results, HFE has no significant effect on the function of both WT PCSK9 and ΔM2 PCSK9 (Fig. 24A). However, the presence of HFE dramatically elevated PCSK9 levels both in lysate and media (Fig. 24 B-F). As HFE can change the levels of ΔM2 PCSK9, it could have a different binding domain for intracellular PCSK9. pH of different compartments also could affect the interaction (e.g., PCSK9 Q554 and H553 residue's interaction with R71 residue on HFE). Indeed, in contrast with extracellular PCSK9, overexpressed PCSK9 is not able to degrade HFE (Fig 24A). HFE C282Y variant had the same effect as WT HFE either on LLDR or PCSK9 levels (Fig. 24A: *quantifications are not shown*). These results suggest an opposite function of HFE on PCSK9 activity inside the cells. Therefore, we propose that HFE might have the opposite effects on the function of PCSK9. Thus, while HFE inhibits the activity of extracellular PCSK9, it could act as a chaperone for intracellular PCSK9 and increases the stability/expression/secretion of PCSK9. A similar effect has been reported for PCSK9 itself, where it could degrade LDLR levels, it can also act as a chaperone for cell surface LDLR. Further experiments are needed to unravel the exact mechanism underlying PCSK9 and HFE intracellular interactions.

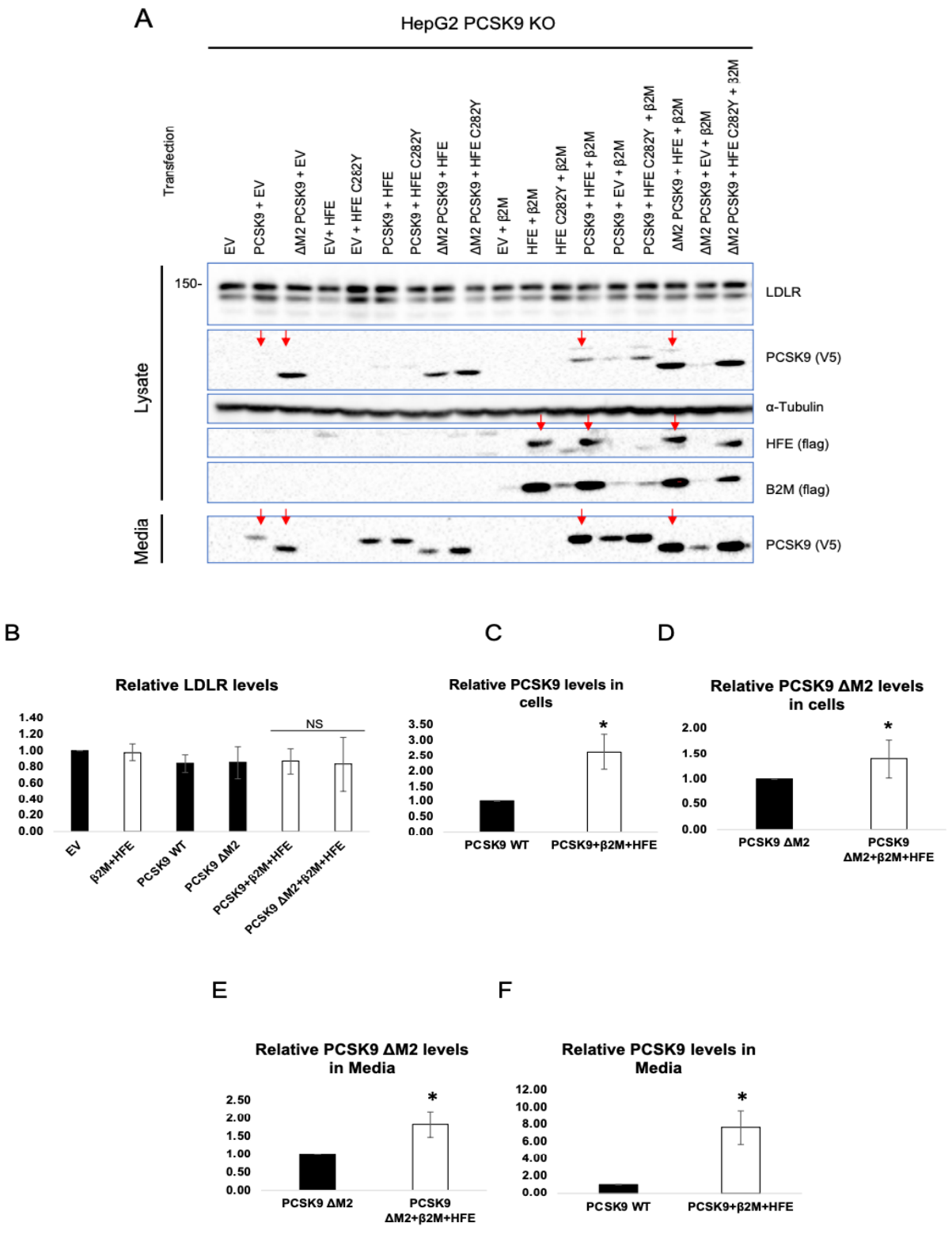

A
HepG2 PCSK9 KO
Transfection
EV
PCSK9 + EV
ΔM2 PCSK9 + EV
EV+ HFE
EV + HFE C282Y
PCSK9 + HFE
PCSK9 + HFE C282Y
ΔM2 PCSK9 + HFE
ΔM2 PCSK9 + HFE C282Y
EV + β2M
HFE + β2M
HFE C282Y + β2M
PCSK9 + HFE + β2M
PCSK9 + EV + β2M
PCSK9 + HFE C282Y + β2M
ΔM2 PCSK9 + HFE + β2M
ΔM2 PCSK9 + EV + β2M
ΔM2 PCSK9 + HFE C282Y + β2M
150-
Lysate
Media
LDLR
PCSK9 (V5)
α-Tubulin
HFE (flag)
B2M (flag)
PCSK9 (V5)
B
Relative LDLR levels
NS
EV
β2M+HFE
PCSK9 WT
PCSK9 ΔM2
PCSK9+β2M+HFE
PCSK9 ΔM2+β2M+HFE
C
Relative PCSK9 levels in cells
PCSK9 WT
PCSK9+β2M+HFE
D
Relative PCSK9 ΔM2 levels in cells
PCSK9 ΔM2
PCSK9 ΔM2+β2M+HFE
E
Relative PCSK9 ΔM2 levels in Media
PCSK9 ΔM2
PCSK9 ΔM2+β2M+HFE
F
Relative PCSK9 levels in Media
PCSK9 WT
PCSK9+β2M+HFE

Fig. 24. HFE and its effect on intracellular PCSK9. A) HepG2 PCSK9 KO cells were transfected with an empty vector (EV), WT HFE, C282Y HFE, WT PCSK9, and ΔM2 PCSK9. 48 hrs post-transfection, lysates, and media were extracted to be analyzed by WB (SDS/PAGE on 8% Tris-glycine gel). Protein levels were normalized to the control protein, α-tubulin. Quantifications showing the B) Total LDLR levels, C) Total WT PCSK9 levels in the lysate, D) Total ΔM2 PCSK9 levels in the lysate, E) Total ΔM2 PCSK9 levels in the media, F) Total WT PCSK9 levels in the media. Data are representative of three independent experiments. Quantifications are averages ± standard deviation (SD). * $p<0.05$; ** $p<0.01$; ***$p<0.001$ (two-sided t-test). NS: non-significant.

In conclusion, our work demonstrates the importance of the M2 domain of PCSK9 and protein X for the intracellular trafficking of PCSK9 and LDLR complex (after internalization) towards lysosomes. Additionally, we found for the first time that extracellular PCSK9 can target cell surface HFE towards lysosomal degradation in an LDLR- dependent fashion. The R-x-E motif of PCSK9 is critical for its association with HFE. Our cell-based assays, as well as human sample analysis, propose the possible implication of PCSK9 in iron metabolism in humans. Indeed, we presented HFE as a novel regulator of both intracellular (positive regulator) and extracellular (negative regulator) PCSK9. Some physiological (e.g., high circulating iron levels) conditions could alter the availability of HFE at the cell surface and increase or decrease its inhibitory effect on PCSK9.

Chapter IV

Deciphering the role of human leukocyte antigen C (HLA-C) on PCSK9 function and its competition with HFE:

The first publication in 2020, identified HLA-C as a new target of PCSK9. In this work, combination therapy of anti-PD1 with evolocumab had significantly higher efficacy in treating tumour growth compared to solo therapy of ant-PDI. This interesting information proposes a new role of PCSK9 in the immune system. However, in this work, there was no information about the possible regulatory effect of HLA-C on PCSK9, and this question remained to be elucidated. Recently in the work of Carole Fruchart and our group, the 3D structure of HLA-C and its interaction with PCSK9 was modelled. Our efforts confirmed the importance of R-x-E motif of HLA-C for interacting with PCSK9 and arginine 68 and glutamic acid 70 on HLA-C bind to glutamic acid 567 and arginine 549 on PCSK9, respectively (Fig. 25A). Removal of R-x-E either on PCSK9 or HLA-C leads to the complete LOF in the activity of PCSK9 (Fig. 25B, D). This compelling observation proposed HLA-C as a potential protein X that is necessary for the function of PCSK9 on LDLR. Furthermore, the natural mutation PCSK9 Q554E (LOF for LDLR) repulses glutamic acid residues 79, 197, and 201 on HLA-C (LOF for HLA-C). In contrast, natural mutation H553R absorbs mentioned glutamic acid residues (E79, E197, E201) on HLA-C. Henceforward, it seems that losing interaction with the potential protein X (HLA-C) leads to the LOF phenotype, as PCSK9 needs protein X for its function. Interestingly, while PCSK9 Q554E loses its interaction with PCSK9's activators (HLA-C), it causes stronger binding with inhibitors of PCSK9 (HFE and Annexin A2). The same relationship also exists for the PCSK9 H553R variant. Additionally, as HFE and HLA-C interact with the exact same residues of PCSK9, and one of them has an inhibitory effect, and the other one increases the activity, we propose the possible competition of HFE and HLA-C in the regulation of PCSK9 activity on the LDLR. Therefore, here we confirmed the critical role of HLA-C as a protein X for the activity of PCSK9 and investigated its potential competition with HFE.

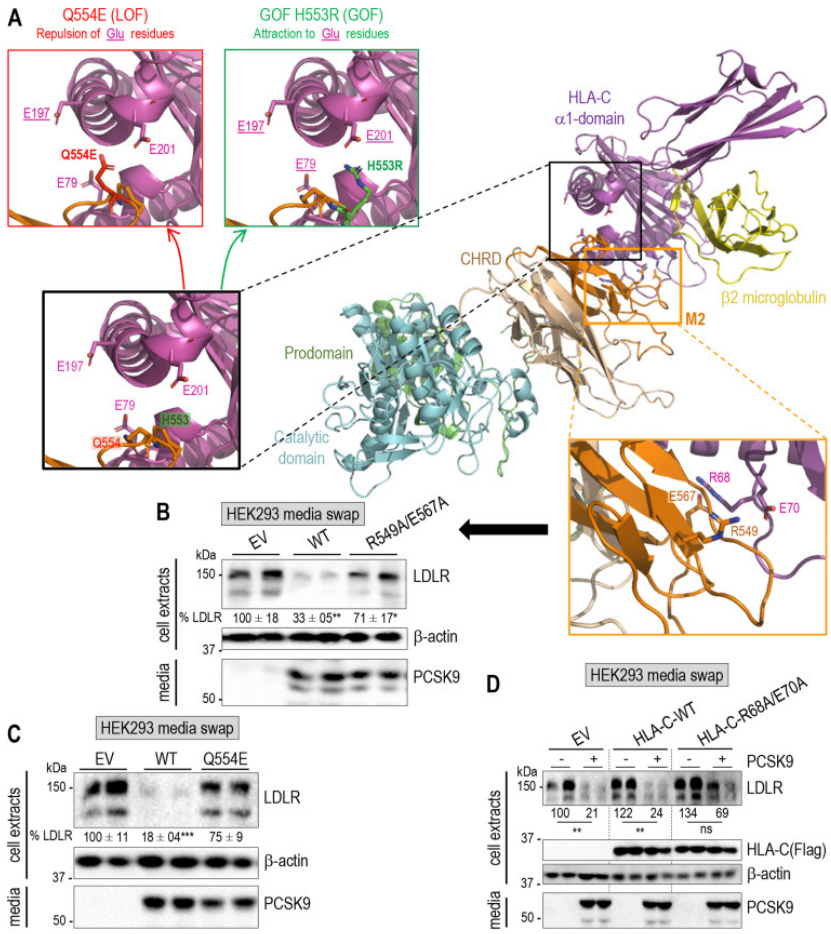


Fig. 25. A-D) The molecular interaction of HLA-C with PCSK9 and confirmation of the interaction sites by cell-based assays. Adapted from (Fruchart Gaillard et al., 2023).

HLA-C interaction sites with PCSK9 and its trafficking:

In the first attempt to further confirm the findings in our previous publication (Fruchart Gaillard et al., 2023), we over-expressed HLA-C-WT-flagM2 or HLA-C-R68A-E70A in HepG2 CRISPR PCSK9 KO cells and looked at the extracellular activity of PCSK9 by producing conditioned media enriched with either PCSK9 cDNA (WT PCSK9, and PCSK9 variants including ΔM2, R549A-E567A, R549A-Q553E-E567A, and H553R) or empty vector (control). The data confirmed the complete LOF activity in all PCSK9 variants, and importance of M2 domain interaction with HLA-C. However, WT HLA-C had no extra effect on WT PCSK9 compared to the control condition (empty vector). We assume the reason for this observation is that the endogenous HLA-C is highly expressed in HepG2 cells (Fig. 19) and already reached its maximum effect on PCSK9. Thus, with low transfection efficiency of HepG2 cells (~20-30%) it would be hard to see the additional effect of over-expressed HLA-C. Interestingly though, HLA-C could dramatically increase the activity of PCSK9 H553R variant on LDLR. This information suggests that this interaction site might be one of the critical sites for HLA-C and PCSK9 relationship. As overexpressed HLA-C might have a different polymorphism versus the endogenous one, it is very possible that this variation of HLA-C (transfected cDNA) had a higher chance to interact with PCSK9 and thus acted as a dominant negative for endogenous HLA-C (Fig. 26).

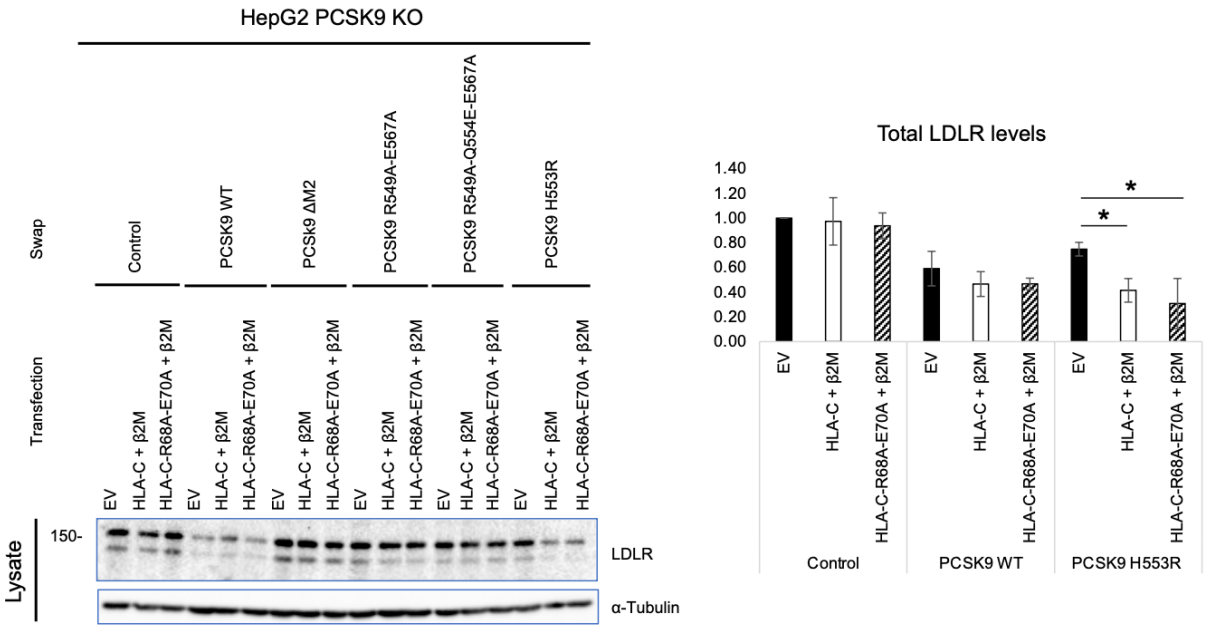


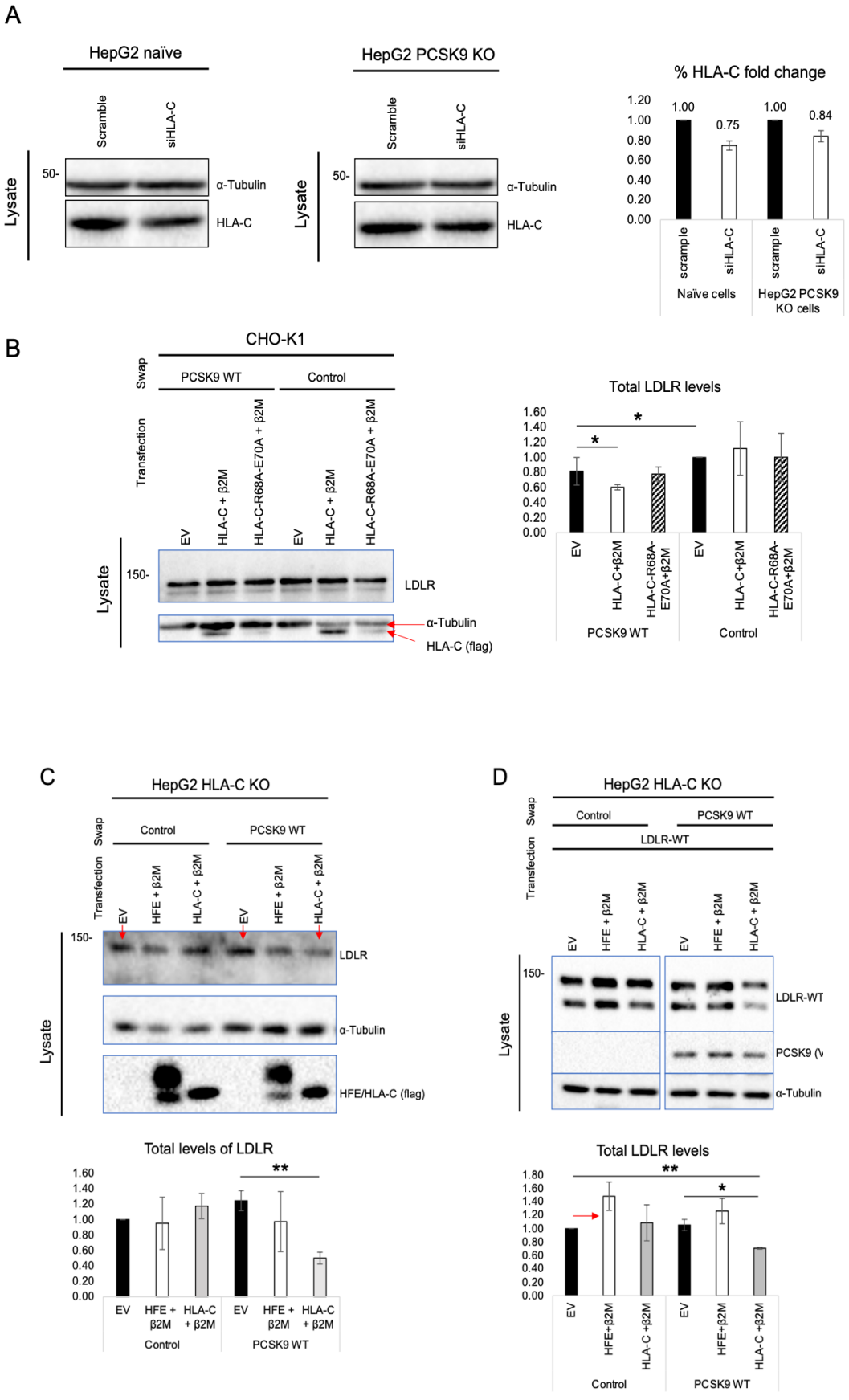
Fig. 26. HepG2 PCSK9 KO cells were transfected with either, empty vector (EV), WT HLA-C or HLA-C R68A-E70A variant and then incubated with conditioned media from HEK293 cells expressing an empty vector (control), WT PCSK9, and proposed LOF variants on PCKS9 (ΔM2, R549A-E567A, R549A-Q554E-E567A, and H553R). Cell Lysates were extracted to be analyzed by WB (SDS/PAGE on 8% Tris-glycine gel). The cell-based data confirmed the predicted interaction sites by 3D structure modeling in panel (A). All protein levels were normalized to the control protein, α-tubulin. WB Data are representative of two independent experiments. Quantifications are averages ± standard deviation (SD). * $p<0.05$; ** $p<0.01$; ***$p<0.001$ (two-sided t-test). NS: non-significant.

HLA-C and its regulatory role on PCSK9:

Our limitation regarding high expression levels of HLA-C, encouraged us to perform the silencing approaches to see if the absence of HLA-C could block the activity of PCSK9. To do so, siRNA against HLA-C have been used both in HepG2 naïve and CRISPR PCSK9 KO cells. Despite using various concentrations of siRNA, we were not able to succeed in knocking down *HLA-C* in either cell lines (Fig. 27A). We think the reason might be, again, because of the high variability and expression levels of HLA-C protein.

In the next step, we selected the sub-clone CHO-K1 cell lines, from the original Chinese hamster overran cells as they do not encode any HLA family members. It was shown before in our gourp that extracellular PCSK9 has no function on LDLR in CHO-K1 cells, but the reason was not known. With our finding regarding the pivotal function of HLA-C on PCSK9, we could suggest that dysfunction of PCSK9 in CHO cells could be due to the lack of endogenous HLA-C. To confirm this hypothesis, CHO-K1 cells were transfected with HLA-C-WT-V5 or HLA-C-R68A-E70A-V5 and incubated with media containing over-expressed PCSK9. Intriguingly, PCSK9's function improved in the presence of HLA-C and not the HLA-C RE variant (Fig. 27B). Although, the effect on LDLR is not large, this data suggests again HLA-C acts as a critical protein for the function of PCSK9. Apart from the fact that the transfection efficiency of this cell line is very low, CHO cells also express the PCSK9's inhibitor Annexin A2, that might compete with HLA-C and limits its function. Indeed, as these cell lines originated from other tissue (rather than human liver), they may have different protein content and affect the interpretation of data. Due to the limitations observed in HepG2 naïve and CHO-K1 cells, and our need to find the best model to study HLA-C and PCSK9 interaction, we decided to generate HepG2 CRISPR HLA-C KO cells.

The HepG2 CRISPR HLA-C KO cells were provided from Ubigene company and tested with qPCR experiment to confirm KO of HLA-C. After the validation experiment, we used this cell line to transfect HLA-C-WT or empty vector and perform the media swap experiment with over-expressed PCSK9-WT conditioned media. Looking at endogenous LDLR levels, clearly showed that PCSK9 has no function as addition of extracellular PCSK9 did not change total LDLR levels (Fig. 27C). Intriguingly, the addition of HLA-C to the condition recaptured PCSK9's function towards LDLR degradation and significantly reduces LDLR levels (~ 30%) compared to the control condition (Fig. 27C). Furthermore, to address the limitation problem of low transfection efficiency, we co-expressed LDLR-WT-V5 with HLA-C-WT-V5 and only looked at transfected LDLR using V5 antibody. In this experiment also, PCSK9 alone had no function on transfected LDLR, but addition of HLA-C activated PCSK9 and decreased LDLR levels by ~ 40%. Altogether, these data show the critical role of HLA-C for PCSK9's function towards LDLR degradation.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siHLA-C
Lysate
50-
α-Tubulin
HLA-C
HepG2 PCSK9 KO
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1.00
0.75
1.00
0.84
scramble
siHLA-C
Naïve cells
HepG2 PCSK9 KO cells
B
CHO-K1
Swap
PCSK9 WT
Control
Transfection
EV
HLA-C + β2M
HLA-C-R68A-E70A + β2M
150-
LDLR
α-Tubulin
HLA-C (flag)
Total LDLR levels
HLA-C+β2M
HLA-C-R68A-E70A+β2M
C
HepG2 HLA-C KO
HFE + β2M
HLA-C + β2M
HFE/HLA-C (flag)
Total levels of LDLR
HFE + β2M
HLA-C + β2M
D
LDLR-WT
PCSK9 (V
Total LDLR levels

Fig. 27. Effect of HLA-C on extracellular activity of PCSK9 towards LDLR degradation. A) HepG2 naïve and HepG2 PCSK9 KO cells were transfected with siRNA against HLA-C (siHLA-C) or non-targeting siRNA (scramble) and then incubated with conditioned media from HEK293 cells expressing an empty vector (control) or WT PCSK9. B) CHO-K1 cells were transfected with either, empty vector (EV), WT HLA-C, or HLA-C R68A-E70A variant and then incubated with conditioned media from HEK293 cells expressing an empty vector (control) or WT PCSK9. C) HepG2 HLA-C KO cells were transfected with empty vector (EV), WT HLA-C, or WT HFE and then incubated with conditioned media from HEK293 cells expressing an empty vector (control) or WT PCSK9. D) HepG2 HLA-C KO cells were co-transfected with empty vector (EV)+WT LDLR, WT HLA-C+WT LDLR, or WT HFE+WT LDLR and then incubated with conditioned media from HEK293 cells expressing an empty vector (control) or WT PCSK9. All cell Lysates were extracted to be analyzed by WB (SDS/PAGE on 8% Tris-glycine gel). All protein levels were normalized to the control protein, α-tubulin. WB Data are representative of two-three independent experiments. Quantifications are averages ± standard deviation (SD). * $p<0.05$; ** $p<0.01$; ***$p<0.001$ (two-sided t-test). NS: non-significant.

HLA-C trafficking and its competition with HFE:

As HFE and HLA-C have opposite roles on PCSK9's regulation but share their interaction sites, we asked this question whether there is any competition between HFE and HLA-C. To answer this question, we needed to co-express HFE with HLA-C and see how the presence of each protein might affect the other one. Since our qPCR data in HepG2 cells demonstrated that HLA-C expression levels are much higher in comparison with HFE, we used HepG2 HLA-C KO cells to demolish the possible dominant effect of HLA-C on PCSK9. Thereby we co-expressed HLA-C-WT-V5 and HFE-WT-V5 in HLA-C KO cells and performed the media swap experiment with PCSK9 WT. Although PCSK9's function on LDLR is lost in HLA-C KO cells, our experiment shows that PCSK9 does not need HLA-C to target HFE and send to the lysosomal degradation (Fig. 27A). In fact, the addition of HLA-C had the opposite effect and inhibits PCSK9's function on HFE. Likewise, the presence of HFE also inhibits the function of PCSK9's towards HLA-C degradation (Fig. 28A). Therefore, HFE and HLA-C are each other's potential competitors, and depending on their concentrations, they can either activate or deactivate PCSK9's function. Some physiological conditions might as well affect the selection of each protein to be the dominant one. The superposition of the 2 models of the selected HFE/PCSK9 and HLAC/PCSK9 indicates that

HFE and HLA-C could be competitors *via* their RxE motif (HFE: R67 and E69; HLCA: R68 and E70) with respect to residues R549 and E567 of the M2 domain of the PCSK9 CHRD (Fig. 27B).

Knocking down LDLR levels using the siRNA technique reveals that although HFE's degradation is *via* LDLR-dependent pathway, PCSK9 does not need LDLR to sort HLA-C to the lysosomal degradation (Fig. 27C). Again, this independency can be because of the cytosolic tail of HLA-C that is longer than HFE and it's probably sufficient for its internalization.

Previous studies reported the activity of PCSK9 on LDLR lacking C-terminal domain. Hence, cytosolic tail of HLA-C could compensate for the absence of LDLR C-terminal domain and leads the complex to the lysosomal degradation. To test this hypothesis, LDLR-ΔCT- V5 co-introduced with HLA-C-V5 in HepG2 HLA-C KO cells with the addition of extracellular PCSK9. Our data suggests that PCSK9 in the presence of HLA-C is still able to degrade LDLR even with the removal of cytosolic tail of LDLR (Fig. 28D, E). Another intriguing observation is that, in the presence of HLA-C more PCSK9 seems to be internalized as there is more PCSK9 in the lysate compared to the control condition (Fig. 28E).

Our next question was to see if HFE and HLA-C take separate endocytosis pathways. Previous publications indicates that the internalization of LDLR is *via* clathrin-coated vesicles. As in presence of HLA-C, PCSK9 degrade more LDLR, we propose that the internalization of HLA-C also would be *via* clathrin-coated vesicles. On the other hand, it is known that the internalization of HFE coupled with TfR1 is through clathrin-coated vesicles. However, looking at the cytosolic tail of HFE shown the presence of hydrophobic amino acids that have been associated with caveola interaction and caveolin endocytosis. Thereby, we propose that HFE and HLA-C might favor different endocytosis pathways when they interact with PCSK9. To test this hypothesis, we used the siRNA approach to knock down either caveolin 1 (Cav1) or clathrin heavy chain (CHC) and see how their absence affect the degradation of HLA-C and HFE by PCSK9. The results of this experiment revealed that HLA-C degradation was inhibited by silencing of CHC only and HFE degradation was affected by knocking down of both CHC and Cav1 (Fig. 27F). Previous publications demonstrated that endocytosis of LDLR is *via* clathrin coated vesicles. Here, we confirmed that HLA-C internalization also is through the same pathway. Thus, in the presence of HLA-C, it is attached to LDLR and PCSK9 and the whole complex is internalized *via* clathrin coated pathway that takes LDLR and HLA-C to the lysosomal degradation. Although normal

trafficking of HFE with TfR1 is through clathrin coated vesicles, some physiological conditions could favour the interaction of HFE and PCSK9 and lead to the internalization of the complex, possibly by caveolin pits. The internalization of LDLR with PCSK9/HFE, somehow leads LDLR to scape and recycle back to the cell surface and HFE alone goes to the lysosomal degradation. The exact mechanism underlying the trafficking of LDLR in the presence of HFE needs further investigation.

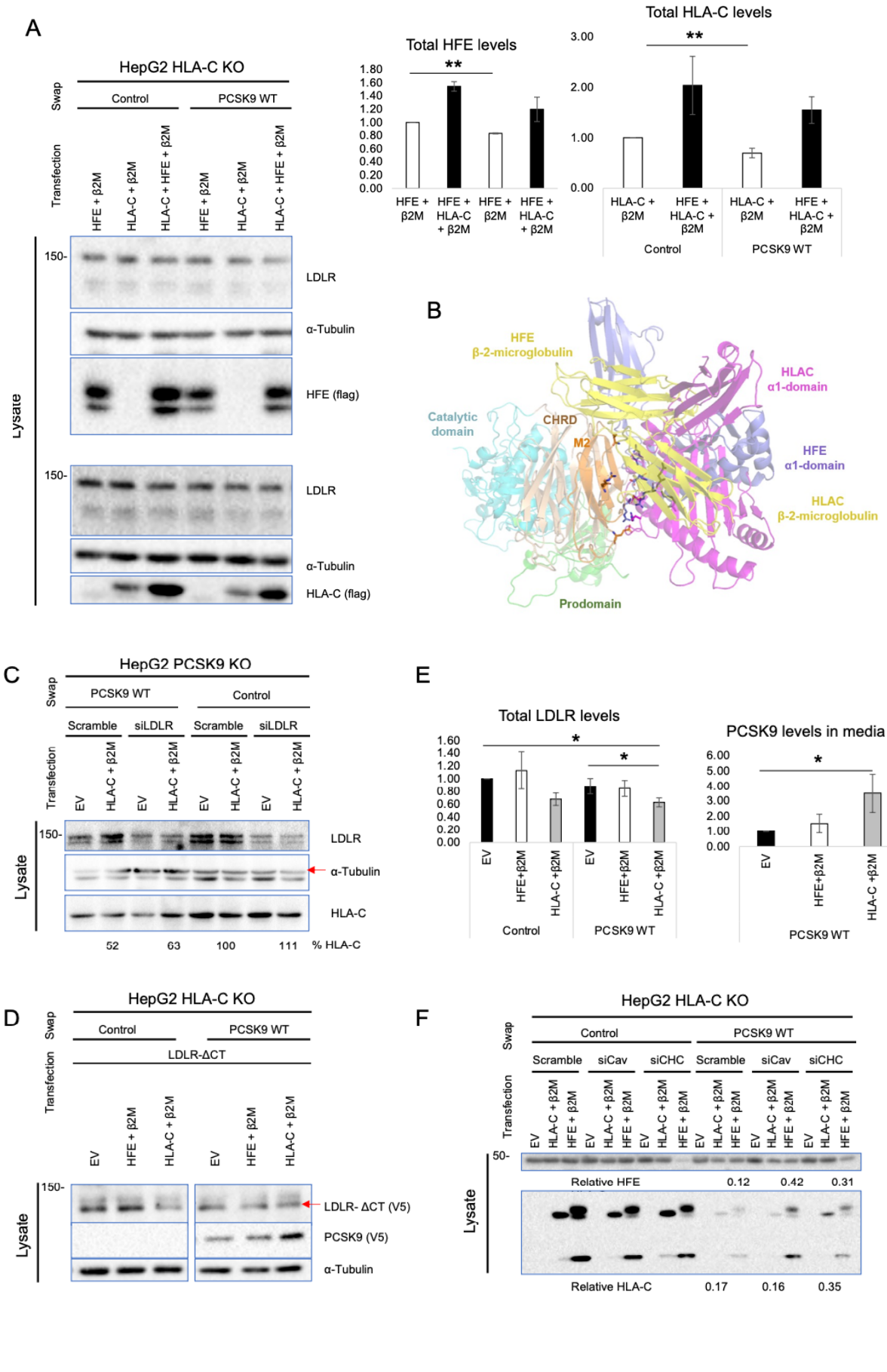
A
HepG2 HLA-C KO
Swap
Control
PCSK9 WT
Transfection
HFE + β2M
HLA-C + β2M
HLA-C + HFE + β2M
Lysate
150-
LDLR
α-Tubulin
HFE (flag)
HLA-C (flag)
Total HFE levels
Total HLA-C levels
**
B
HFE
β-2-microglobulin
HLAC
α1-domain
Catalytic
domain
CHRD
M2
HFE
α1-domain
HLAC
β-2-microglobulin
Prodomain
C
HepG2 PCSK9 KO
PCSK9 WT
Control
Scramble
siLDLR
EV
HLA-C + β2M
HLA-C
52
63
100
111
% HLA-C
D
HepG2 HLA-C KO
LDLR-ΔCT
HFE + β2M
LDLR- ΔCT (V5)
PCSK9 (V5)
E
Total LDLR levels
PCSK9 levels in media
HFE+β2M
HLA-C +β2M
F
HepG2 HLA-C KO
siCav
siCHC
50-
Relative HFE
0.12
0.42
0.31
Relative HLA-C
0.17
0.16
0.35

Fig. 28. HLA-C and HFE distinct trafficking pathways. A) HepG2 HLA-C KO cells were co-transfected with empty vector (EV), WT HLA-C, WT HFE, or WT HLA-C+WT HFE and then incubated with conditioned media from HEK293 cells expressing an empty vector (control) or WT PCSK9. B) 3D modeling of possible competition of HFE with HLA-C. C) HepG2 PCSK9 KO cells were transfected with siRNA against LDLR or non-targeting siRNA (Scramble). 24 hrs later, these cells were transfected with either, an empty vector (EV), or WT HLA-C and then incubated with conditioned media from HEK293 cells expressing an empty vector (control) or WT PCSK9. D) HepG2 HLA-C KO cells were co-transfected with empty vector (EV)+ ΔCT LDLR, WT HLA-C+ ΔCT LDLR, or WT HFE+ΔCT LDLR and then incubated with conditioned media from HEK293 cells expressing an empty vector (control) or WT PCSK9. E) Quantification of the panel (D) that shows total LDLR levels in the lysate, and total PCSK9 levels in the lysates. F) HepG2 HLA-C KO cells were transfected with siRNA against clathrin heavy chain (siCHC), siRNA against caveolin 1 (siCav1), or non-targeting siRNA. 24 hrs later, these cells were transfected with either, an empty vector (EV), WT HFE, or WT HLA-C and then incubated with conditioned media from HEK293 cells expressing an empty vector (control) or WT PCSK9. All cell Lysates were extracted to be analyzed by WB (SDS/PAGE on 8% Tris-glycine gel). All protein levels were normalized to the control protein, α-tubulin. WB Data are representative of two-three independent experiments. Quantifications are averages ± standard deviation (SD). * $p<0.05$; ** $p<0.01$; ***$p<0.001$ (two-sided t-test). NS: non-significant.

Chapter V

Regulation of PCSK9 and LDLR by PACE4:

Expression pattern of PACE4 in different tissues and cells:

Until now, an ample amount of proteomics studies has been conducted to better understand the puzzling trafficking of PCSK9 toward LDLR degradation. The major purpose of these investigations is to search for new interacting/partner proteins for either extracellular or intracellular PCSK9. Regarding the extracellular PCSK9, our previous collaboration with the University of Sherbrook proposed the possible interaction of PCSK9 with one of its family members PACE4 (*data not shown*). This new finding encouraged us to investigate more about this interaction to see whether PACE4 could affect PCSK9 activity on LDLR. Thus, in the first attempt, we performed in situ hybridization assay (ISH) and qPCR experiment to compare the mRNA levels of PCSK9, LDLR, and PACE4 in postnatal mouse and human cell lines, respectively. For the ISH, the fixed mice tissue cryostat sections were provided from postnatal mice at day 10. For the qPCR experiment, RNA of human cell lines, including HEK293, HepG2, Huh7, and IHH cells, were collected. The ISH data shows that PACE4 has a similar expression pattern as LDLR as well as PCSK9 and is highly expressed in the liver and small intestine (Fig. 29A). Like PCSK9 and LDLR, PACE4 expression is higher in HepG2 and Huh7 cells rather than HEK293 and IHH cells. Interestingly, in all cell lines except Huh7 cells, PACE4 expression is higher than PCSK9 (Fig. 29B). This data points out the importance of PACE4 presence in hepatocytes and its possible involvement in major liver metabolisms.

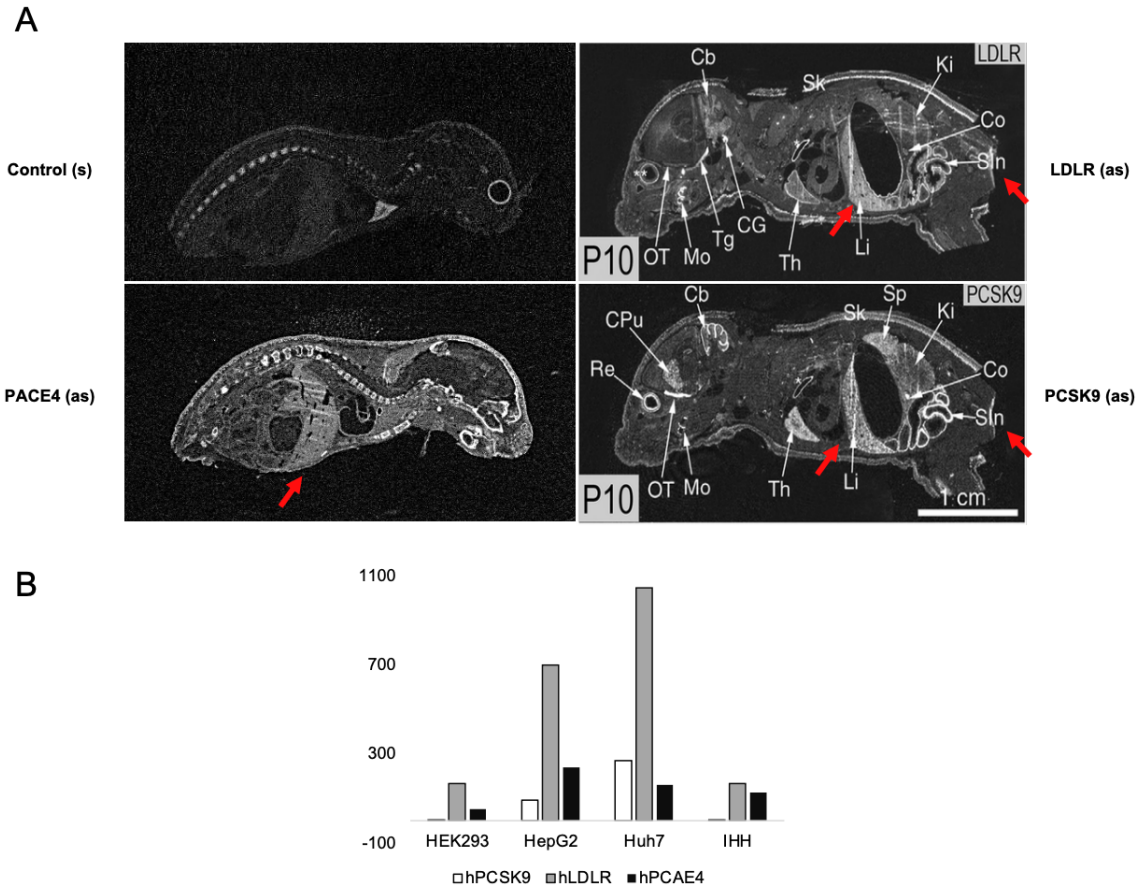

Fig. 29. PACE4 mRNA distribution and its correlation with LDLR and PCSK9. A) Sense and antisense cRNA probes coding for PCSK9, LDLR, and PACE4 were labelled with 35S-UTP and 35S-CTP to obtain high specific activity. Cryostat sections of mouse tissues were fixed in 4% formaldehyde and then subjected to ISH. The labelled cRNA probes were hybridized with the tissue sections overnight at 55°C. After hybridization, the sections were exposed to photographic emulsion (NTB-2, Kodak) for 6–12 days, followed by development in D19 solution (Kodak). Hematoxylin staining was performed for visualization. B) RNA was extracted from HepG2 naïve, HEK293, Huh7, and IHH cells for qPCR analysis. mRNA values were normalized to the mRNA expression of the housekeeping gene (TATA box binding protein: TBP) to calculate the relative mRNA expression of PACE4 and compared it to the expression of PCSK9 and LDLR.

How intracellular PACE4 could affect PCSK9's function and property:

Like other PCs, PACE4 trafficking, and activation starts in the ER, where the first autocatalytic cleavage occurs and sends the heterodimer to the TGN and then the cell surface. At the plasma membrane, PACE4 gets activated *via* second autocatalytic cleavage that removes the inhibitory prodomain. PACE4 can either be secreted to the outer media or retained at the cell surface through binding to HSPG. Hence, like PCSK9, PACE4 could have either intracellular (from ER to the cell surface) or extracellular function (secreted form). Here, our main objective was to see how intracellular or extracellular PACE4 can affect the intracellular or extracellular activity of PCSK9 towards LDLR degradation.

To assess the interaction of intracellular PACE4 and intracellular PCSK9, HepG2 CRISPR cells lacking endogenous PCSK9 were transiently transfected with PACE4 and PCSK9 and then collected for WB assay. As expected, in the normal condition, the presence of overexpressed PCSK9 dramatically reduces the levels of total LDLR (Fig. 30A). However, the addition of intracellular PACE4 significantly blocks the function of PCSK9 on LDLR. Surprisingly, it seems that PACE4 had a direct effect on LDLR and slightly increases its levels independent of PCSK9. However, the effect of PACE4 is much greater *via* inactivating PCSK9's function rather than its direct interaction (Fig. 30B). Although the presence of PACE4 had no significant effect on total PCSK9 levels (Fig. 30C) or the maturation process (Fig. 30D), it remarkedly increased the Furin-cleaved form of PCSK9 by ~3.6 folds (Fig. 30A, E). As the Furin-cleaved form of PCSK9 is inactive on LDLR, that might explain why PACE4 inhibits the intracellular activity of PCSK9.

The same experiment (overexpressing PACE4 in HepG2 CRISPR PCSK9 KO cells) has been performed to analyze the effect of PACE4 on extracellular PCSK9 by producing conditioned media enriched with PCSK9 from HEK293 cells and incubating it on hepatocytes for 18hrs. Despite the immense inhibitory effect of PACE4 on intracellular PCSK9, it has no significant effect on extracellular PCSK9 (Fig. 30F). However, our Co-IP assay demonstrates that both secreted and intracellular forms of PCSK9 and PACE4 interact with each other (Fig. 30G).

In conclusion, our data suggests that although intracellular PACE4 interacts with intra- and extracellular PCSK9, it only inhibits the intracellular activity of PCSK9, possibly through increasing cleavage of PCSK9 by Furin. Indeed, intracellular PACE4 might have a minor direct effect on LDLR.

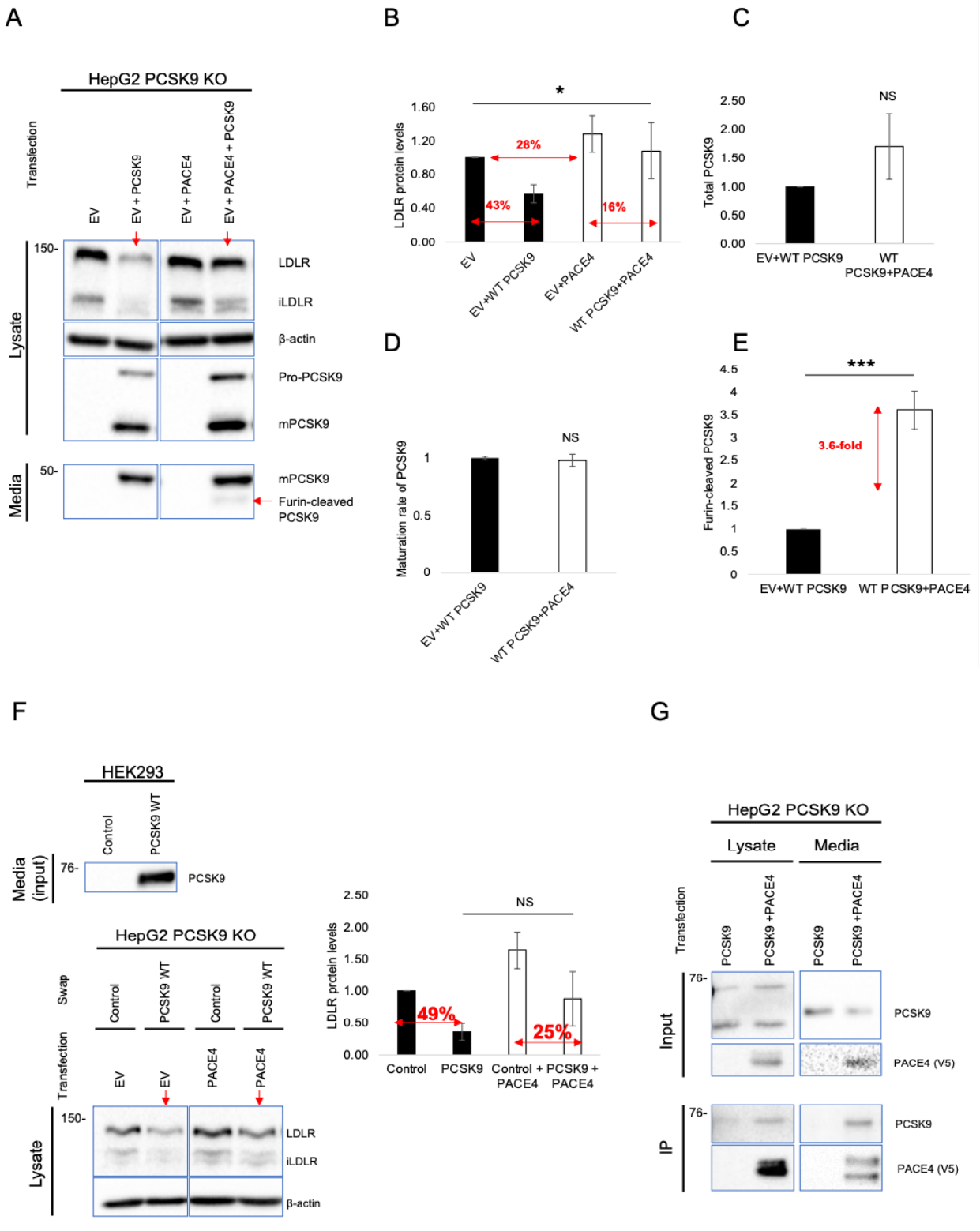
A
HepG2 PCSK9 KO
Transfection
EV
EV + PCSK9
EV + PACE4
EV + PACE4 + PCSK9
150-
Lysate
LDLR
iLDLR
β-actin
Pro-PCSK9
mPCSK9
50-
Media
mPCSK9
Furin-cleaved PCSK9
B
LDLR protein levels
1.60
1.20
0.80
0.40
0.00
*
28%
43%
16%
EV
EV+WT PCSK9
EV+PACE4
WT PCSK9+PACE4
C
Total PCSK9
2.50
2.00
1.50
1.00
0.50
0.00
NS
EV+WT PCSK9
WT PCSK9+PACE4
D
Maturation rate of PCSK9
1
0.5
0
NS
EV+WT PCSK9
WT PCSK9+PACE4
E
Furin-cleaved PCSK9
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
***
3.6-fold
EV+WT PCSK9
WT PCSK9+PACE4
F
HEK293
Media (input)
Control
PCSK9 WT
76-
PCSK9
HepG2 PCSK9 KO
Swap
Control
PCSK9 WT
Control
PCSK9 WT
Transfection
EV
EV
PACE4
PACE4
Lysate
150-
LDLR
iLDLR
β-actin
LDLR protein levels
2.50
2.00
1.50
1.00
0.50
0.00
NS
49%
25%
Control
PCSK9
Control + PACE4
PCSK9 + PACE4
G
HepG2 PCSK9 KO
Lysate
Media
Transfection
PCSK9
PCSK9 +PACE4
PCSK9
PCSK9 +PACE4
Input
76-
PCSK9
PACE4 (V5)
IP
76-
PCSK9
PACE4 (V5)

Fig. 30. Intracellular PACE4 and its interaction with PCSK9. A) HepG2 PCSK9 KO cells were co-transfected with empty vector (EV), WT PACE4, WT PCSK9, or WT PACE4 +WT PCSK9 and then lysate and media were extracted 48 hrs post-transfection for further analysis. Quantifications show the B) Total LDLR levels in the lysate, C) Total PCSK9 levels in the lysate, D) Maturation rate of PCSK9, and E) Levels of Furin-cleaved form of PCSK9. F) HepG2 PCSK9 KO cells were co-transfected with an empty vector (EV) or WT PACE4 and then incubated with conditioned media from HEK293 cells expressing an empty vector (control) or WT PCSK9. G) WT no tag PCSK9 was co-expressed with either WT PACE4 or empty vector (EV), and cell Lysates along with media were collected for Co-IP assay. For this assay, V5-agarose beads have been used to pull down WT PACE4, and precipitated proteins have been analyzed by WB assay and by using antibodies against PCSK9. All cell Lysates were extracted to be analyzed by WB (SDS/PAGE on 8% Tris-glycine gel). All protein levels were normalized to the control protein, α-tubulin. WB Data are representative of two-three independent experiments. Quantifications are averages ± standard deviation (SD). * $p<0.05$; ** $p<0.01$; ***$p<0.001$ (two-sided t-test). NS: non-significant.

Extracellular PACE4 inhibitory effect on PCSK9:

To provide the secreted form of PACE4, HEK293 cells were transiently transfected by PACE4 plasmid and collected 48 hrs post-transfection. The next step was to study the effect of secreted PACE4 either on intra- or extracellular PCSK9. Regarding the intracellular activity of PCSK9, HepG2 CRISPR PCSK9 KO cells were transfected with WT PCSK9 and incubated with PACE4-enriched conditioned media for 18 hrs. As data shows, the addition of PACE4 completely demolishes the activity of PCSK9 on LDLR (Fig. 31A). Conditioned media containing overexpressed PCSK9 and PACE4 were co-incubated for 18hrs to study the extracellular activity of PCSK9 with secreted PACE4. The results suggest that the extracellular activity of PCSK9 was also partially affected by extracellular PACE4 (Fig. 31B). However, the direct effect of extracellular PACE4 was much more evident compared to its PCSK9-dependent effect. This information suggests two distinct pathways for PACE4 activity on LDLR, including PCSK9-dependent and -independent pathways. In the PCSK9-dependent pathway, both extracellular and intracellular PACE4 completely disrupt the intracellular activity of PCSK9 and partially affect extracellular PCSK9. In the PCSK9-independent pathway, PACE4 (mostly extracellular PACE4) increases the total levels of LDLR.

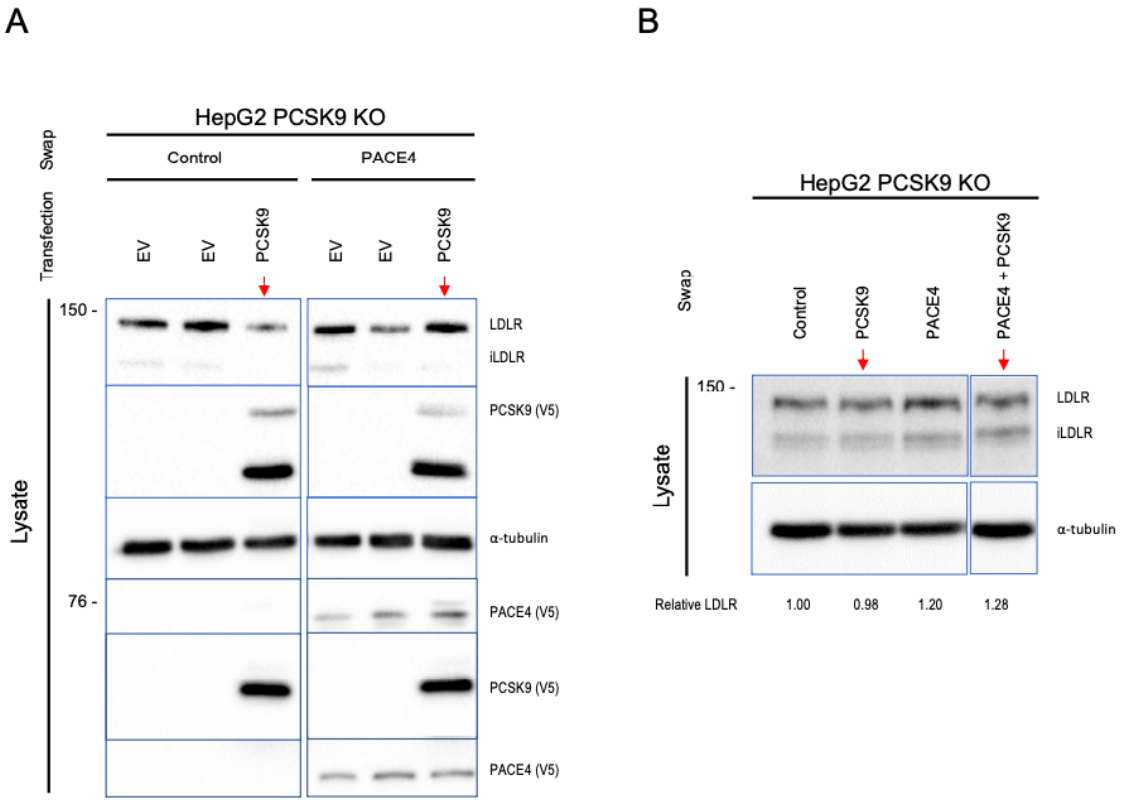

Fig. 31. Extracellular PACE4 activity on PCSK9. A) HepG2 PCSK9 KO cells were transfected with an empty vector (EV) or WT PCSK9 and then incubated with conditioned media from HEK293 cells expressing an empty vector (control) or WT PACE4. The lysate and media were extracted 48 hrs post-transfection for further analysis. B) HepG2 PCSK9 KO cells were co-incubated with WT PCSK9 + control, WT PACE4 + control, WT PCSK9 + WT PACE4. The lysate and media were extracted 24 hrs post-incubation for further analysis. All cell Lysates and media were extracted to be analyzed by WB (SDS/PAGE on 8% Tris-glycine gel). All protein levels were normalized to the control protein, α-tubulin. WB Data are representative of two independent experiments. Quantifications are averages ± standard deviation (SD). * $p<0.05$; ** $p<0.01$; ***$p<0.001$ (two-sided t-test). NS: non-significant.

Direct interaction of PACE4 with LDLR:

To focus on the direct effect of PACE4 on LDLR, we repeated our experiments with a longer exposure time for incubation with secreted PACE4 (72hrs). Interestingly, based on our results, PACE4 could increase the total levels of LDLR up to ~2.7 fold in hepatocytes lacking endogenous PCSK9 (Fig. 32A). Indeed, the immunofluorescent (IF) data illustrates the elevated levels of cell surface LDLR with higher levels of Dil-LDL uptake in presence of PACE4 (Fig. 32B). Since the effect of PACE4 is PCSK9- independent, Co-IP assay needed to be done to find out the possible interaction of PACE4 with LDLR. Then, LDLR-mcherry cDNA was over-expressed in HepG2 CRISPR PCSK9 KO cells and incubated with secreted PACE4 for 72 hrs. Apart from the PACE4 WT construct, we generated two different engineered constructs of PACE4, namely, PACE4-BCRD-V5 (lacking the C-terminal domain of PACE4) and CRD-V5 (C-terminal domain only), to find the domain that is involved in the binding. Removal of the C-terminal domain of PACE4 was shown to lead to the faster trafficking and maturation pathway for PACE4. To see if this removal could also help for PACE4 binding to LDLR, PACE4-BCRD and CRD cDNAs have been used for Co-IP assay. Thereby, LDLR was over-expressed HepG2 PCSK9 KO cells and incubated with either WT PACE4 or engineered PACE4 (PACE4-BCRD or CRD). Lysate was collected, pulled down with V5-agarose beads, and then analyzed with LDLR antibody. Despite the massive effect of PACE4 on LDLR, it does not interact with LDLR. Surprisingly, only the CRD domain of PACE4 pulled down with LDLR. This data suggests that specific conformation of PACE4 protein might affect the interaction of this protein with other molecules (Fig. 32C). Co-incubation of PACE4 with soluble LDLR (sLDLR) also confirmed that there is no direct interaction of PACE4 with LDLR (Fig. 32D). Accordingly, the inhibitory effect of PACE4 seems to be indirect and occurs likely through a third (not identified yet) protein that has a direct interaction with the LDLR.

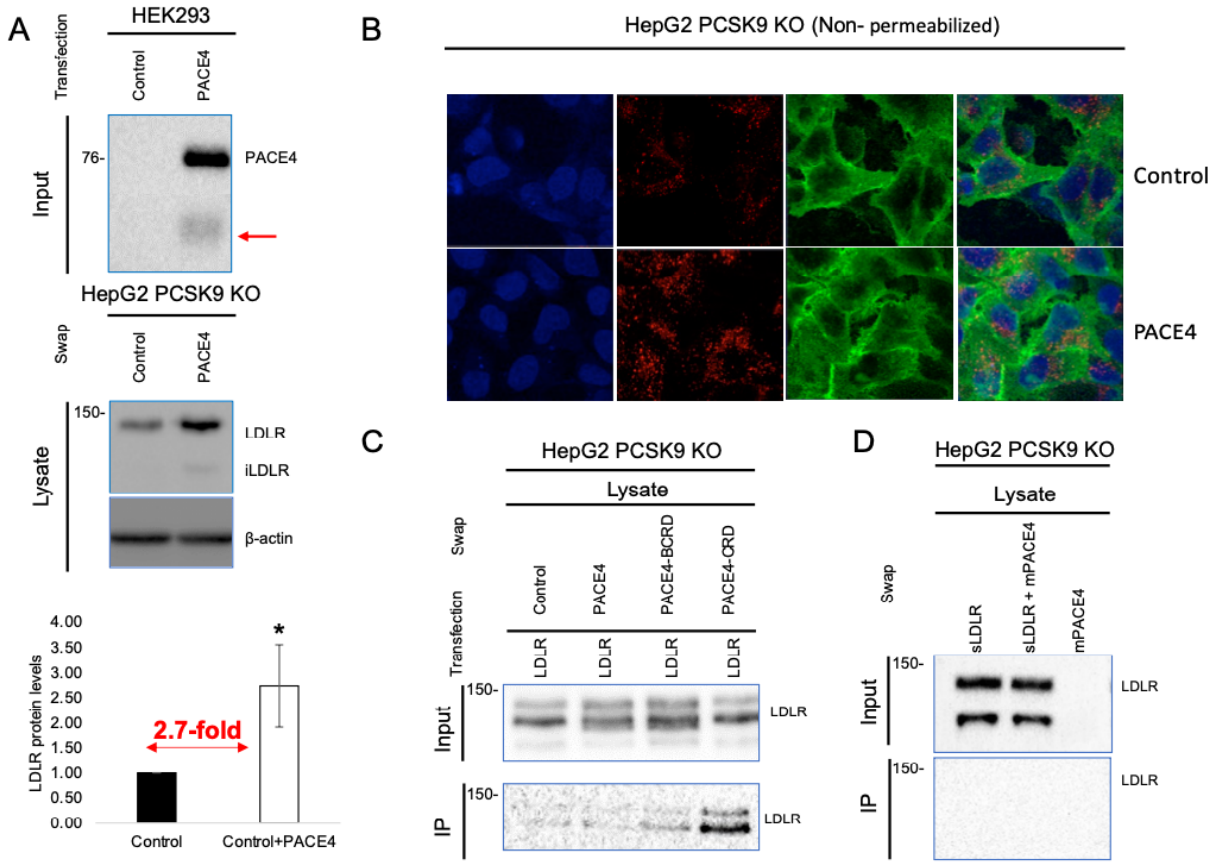

Fig. 32. Direct effect of extracellular PACE4 on LDLR. A) HepG2 PCSK9 KO cells were incubated with conditioned media from HEK293 cells expressing an empty vector (control) or WT PACE4. Then cell Lysates were extracted to be analyzed by WB (SDS/PAGE on 8% Tris-glycine gel). B) Immunofluorescence staining of cell surface LDLR (non-permeabilized conditions) levels in the presence of WT PACE4 compared to control conditions. Immunostaining of total LDLR along Dil-I assay to show the LDL uptake levels. Blue, Nuclei; green, LDLR; red, Dil-I. Imaging has been done using Imaris software. Scale bar, 1µm. C) WT mcherry tag LDLR was expressed and incubated with either WT PACE4 or empty vector (EV), and cell Lysates were collected for Co-IP assay. For this assay, V5-agarose beads have been used to pull down WT PACE4, and precipitated proteins have been analyzed by WB assay and by using antibodies against LDLR. Cell Lysates were extracted to be analyzed by WB (SDS/PAGE on 8% Tris-glycine gel). D) commercially available soluble LDLR co-incubated either with WT PACE4 or control. Then, Lysates were collected for Co-IP assay. For this assay, V5-agarose beads have been used to pull down WT PACE4, and precipitated proteins have been analyzed by WB assay and by using antibodies against LDLR. Cell Lysates were extracted to be analyzed by WB (SDS/PAGE on 8% Tris-glycine gel). All protein levels were normalized to the control protein, α-tubulin. WB Data are representative of three independent experiments. Quantifications are averages ± standard deviation (SD). * $p<0.05$; ** $p<0.01$; ***$p<0.001$ (two-sided t-test). NS: non-significant.

PACE4 mechanism of action:

To investigate more deeply the underneath mechanism of PACE4 on LDLR, we first asked whether this function of PACE4 is enzymatic or non-enzymatic. Thus, we treated our cells with a specific inhibitor of PACE4 (we call it here C23, a.k.a. Ac-[DLeu]LLLRVK-Amba), which has been shown to effectively inhibits the enzymatic activity of PACE4 (Levesque et al., 2015). In the presence of C23, PACE4 no longer increases the LDLR levels (Fig. 33A) and proves that the PCSK9-independent activity of PACE4 on LDLR is *via* its enzymatic activity. Furthermore, knocking down of PACE4 in HepG2 CRISPR PCSK9 KO cells (using siRNA approach), confirmed the PCSK9-independent activity of PACE4 on LDLR (Fig. 33B). Indeed, there was no significant change in mRNA levels of LDLR either in absence (siRNA) or presence (media swap) of PACE4 suggesting the post-translational activity of PACE4 (Fig. 33C, D).

The co-IP data and C23 inhibitor experiment encouraged us to look for the third protein that is implicated in LDLR trafficking and activated by PACE4. Furthermore, we showed iron-related proteins such as HFE could play a role in LDLR trafficking. On the other hand, PACE4 can cleave hepcidin (the critical protein in iron homeostasis that is upregulated in response to high circulating iron levels). Therefore, we were interested to see whether low (using iron chelating factor DFA: deferoxamine) and high concentrations (using FAC ferric ammonium citrate) of iron play any role in PACE4/LDLR relation. Predictably, DFA dramatically increased LDLR levels due to its known effect on 3' UTR mRNA of LDLR (that elevates the stability of expression of LDLR mRNA.) Thus, the high levels of LDLR did not allow us to detect the possible effect of low iron levels on PACE4 enzymatic activity. However, high levels of iron (FAC) also did not seem to have a big impact on PACE4's function (Fig. 34A).

Interestingly, though, it appears that FAC significantly reduces PACE4 protein levels in the media with no effect on mRNA levels of PACE4 (Fig. 34B). This post-translational modification (Fig. 6C) could be a specific response of the body to high levels of iron in circulation to maintain high levels of hepcidin. In other words, as hepcidin is degraded by PACE4 and is critical for iron homeostasis, in some physiological conditions, cells might impede PACE4 to up-regulate hepcidin.

In the next step, we specifically were looking for proteins shown to have a direct effect on LDLR (e.g., CAP1). Although the major function of CAP1 is *via* its regulatory effect on PCSK9, we recently revealed that this protein also has a PCSK9-independent effect on LDLR (Fruchart

Gaillard et al., 2023). Unexpectedly, we observed a similar effect on secreted CAP1 levels in the presence of FAC (Fig. 35A). However, based on our siRNA and media swap approaches, neither secreted CAP1 nor its mRNA were sensitive to PACE4 levels (Fig. 35A, B). Although PACE4 did not affect or cleave CAP1, the Co-IP test unravels the possible interaction of PACE4 with CAP1. With that, we propose two possible regulatory pathways regarding PACE4 enzymatic activity on LDLR. First, PACE4 could directly interact with CAP1 and activates CAP1's direct function on LDLR, and that way stabilizes LDLR levels. Indeed, our previous work clearly showed the importance of CAP1 for the PCSK9/LDLR/protein X complex in a way that the presence of CAP1 exposes the M2 domain of PCSK9 to interact with protein-X (Fruchart Gaillard et al., 2023). Here our second proposal is that the interaction of CAP1 with PACE4 exposes the CRD domain of PACE4 and helps it to interact with LDLR and increases its protein levels. Further functional assay needed to be done to test either pathway.

In conclusion, in this work, we propose a new function of PACE4 in hepatocytes and LDLR trafficking. PACE4 can increase the LDLR protein levels by two distinct pathways: (1) the PCSK9-dependent pathway and by increasing the Furin-cleaved form of PCSK9 and inhibiting its intracellular function, and (2) The PCSK9-independent with the enzymatic activity of PACE4 and its interaction with CAP1.

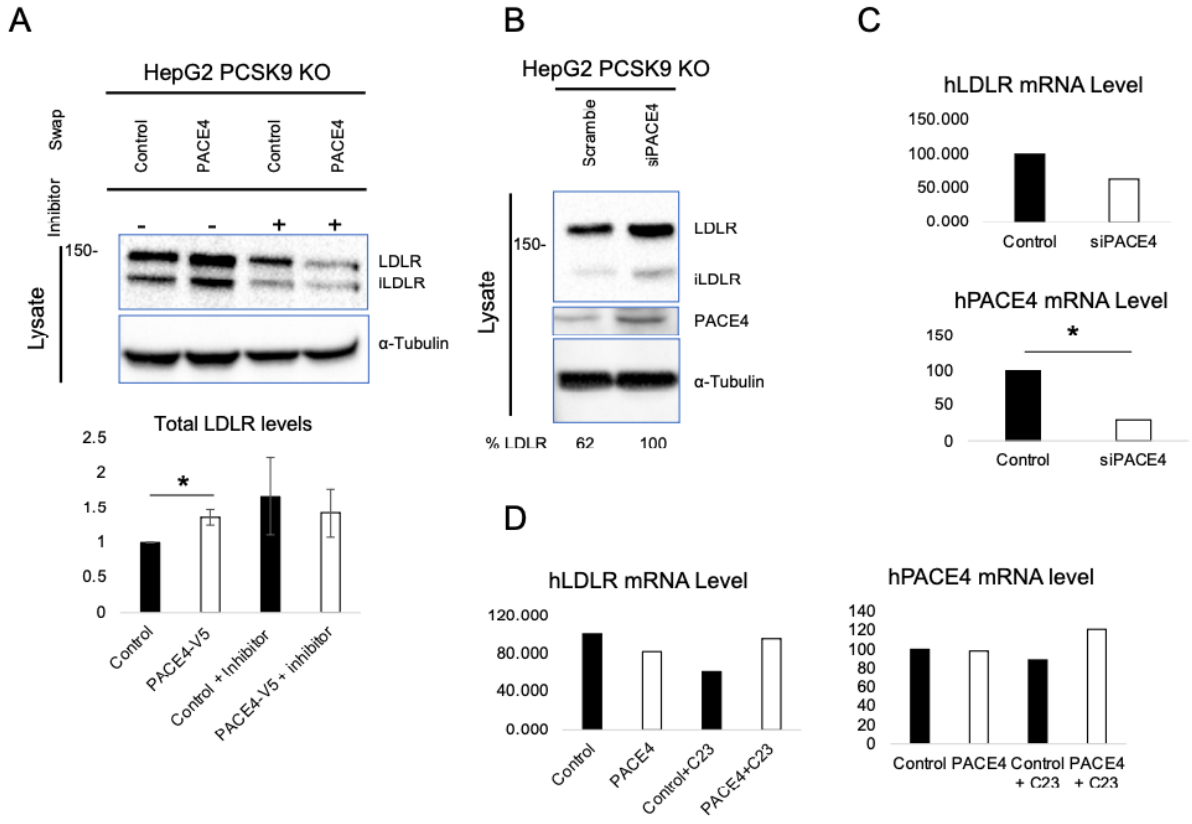

Fig. 33. Mechanism of function of PACE4 on LDLR. A) HepG2 PCSK9 KO cells were incubated with conditioned media from HEK293 cells expressing an empty vector (control) or WT PACE4. After the media swap experiment, cells were treated with PACE4 specific inhibitor C23. 18 hrs after treatment, cell Lysates were extracted to be analyzed by WB (SDS/PAGE on 8% Tris-glycine gel). B) HepG2 PCSK9 KO cells were transfected with siRNA against PACE4 or non-targeting siRNA (Scramble). 24 hrs later, these cells were transfected with either, an empty vector (EV), or WT HLA-C and then incubated with conditioned media from HEK293 cells expressing an empty vector (control) or WT PCSK9. 48 hrs pos-transfection, RNA was extracted from lysate for qPCR analysis. C). Quantifications show mRNA levels of LDLR and PACE4. D) mRNA values of LDLR and PACE4 were also measured in the media swap experiment of PACE4 and in the presence or absence of a C23 inhibitor. Data are representative of three independent experiments. Quantifications are averages ± standard deviation (SD). * $p<0.05$; ** $p<0.01$; ***$p<0.001$ (two-sided t-test). NS: non-significant.

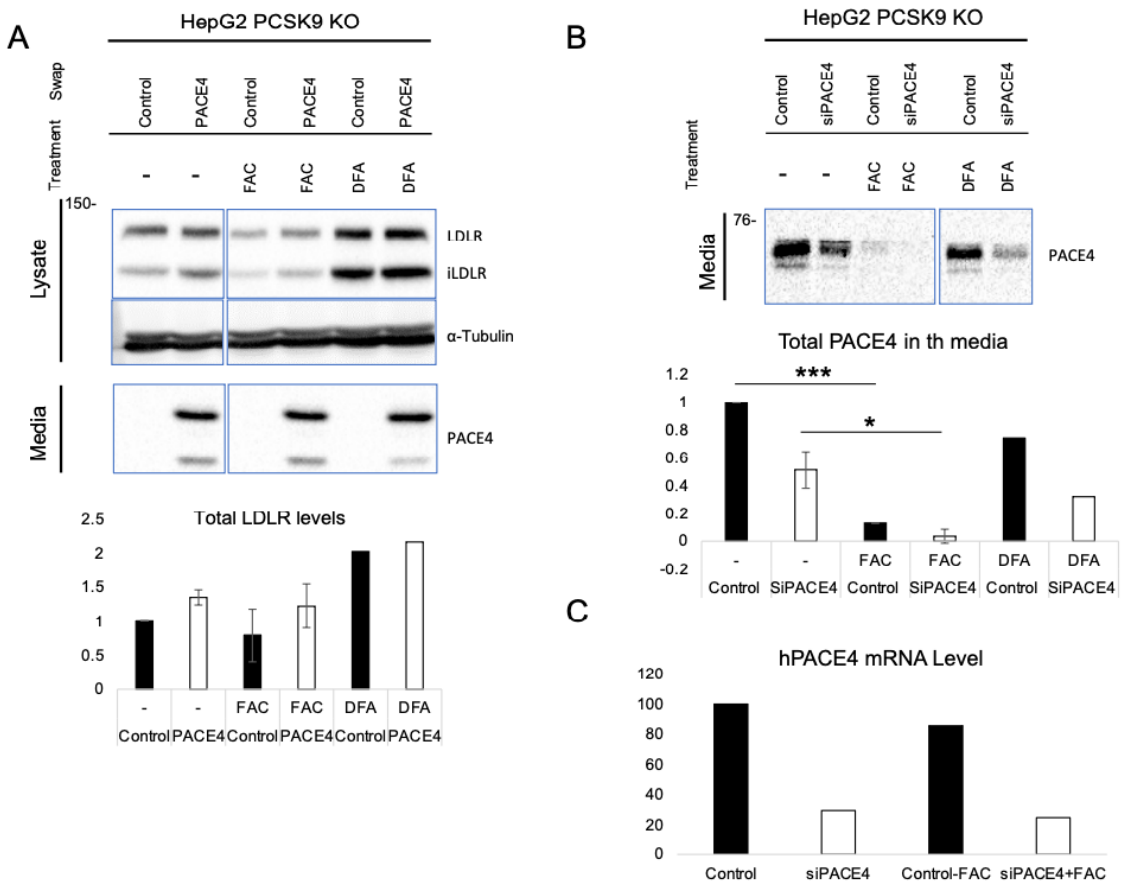

Fig. 34. Role of iron on PACE4 function. A) HepG2 PCSK9 KO cells were incubated with conditioned media from HEK293 cells expressing an empty vector (control) or WT PACE4. Following the incubation with conditioned media, cells were treated with either ferric ammonium citrate (FAC) or deferoxamine (DFA) to analyze the function of PACE4 on LDLR in different iron conditions. B) HepG2 PCSK9 KO cells were transfected with siRNA against PACE4 or non-targeting siRNA (Scramble). Following the siRNA transfection, cells were treated with either ferric ammonium citrate (FAC) or deferoxamine (DFA) and collected for further analysis. C) alternation in mRNA levels of PACE4 in the presence of iron. All Cell Lysates and media were extracted to be analyzed by WB (SDS/PAGE on 8% Tris-glycine gel). Protein levels were normalized to the control protein, α-tubulin. Data are representative of two-three independent experiments. Quantifications are averages ± standard deviation (SD). * $p<0.05$; ** $p<0.01$; ***$p<0.001$ (two-sided t-test). NS: non-significant.

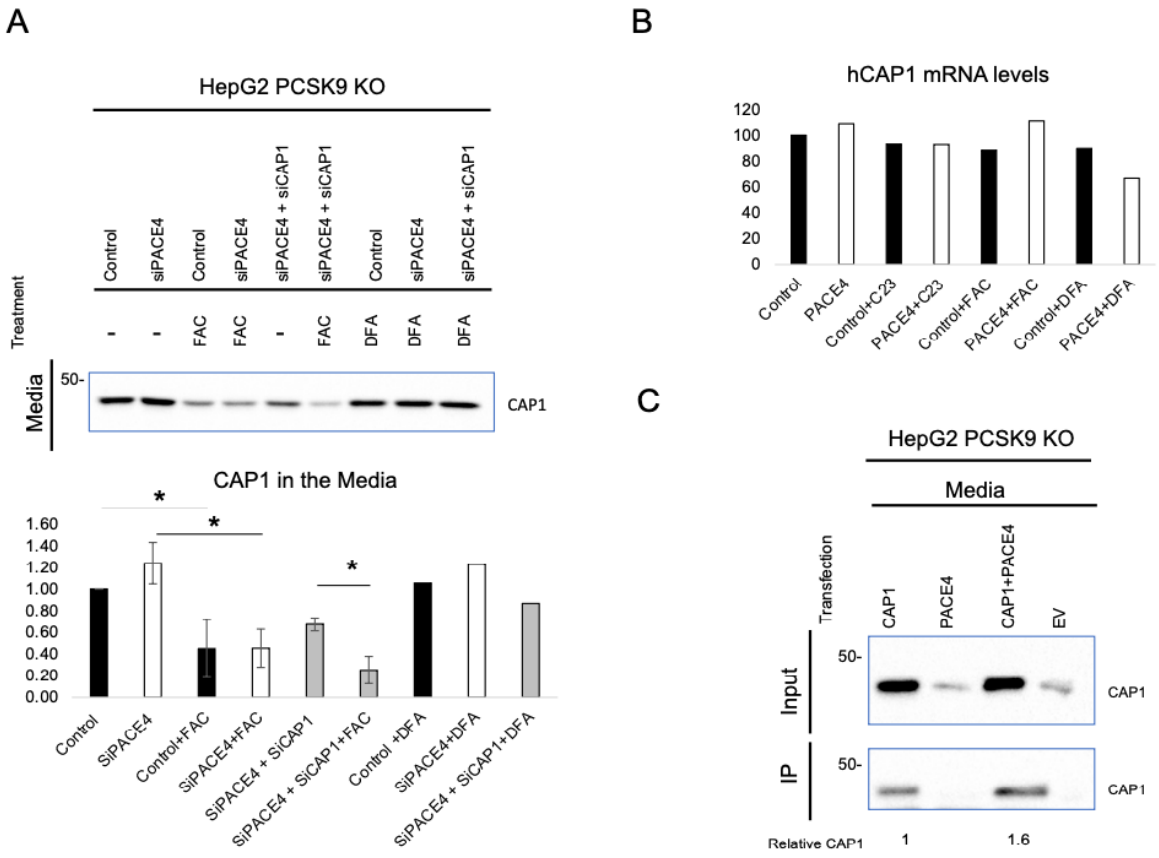

Fig. 35. Interaction of PACE4 with CAP1. A) HepG2 PCSK9 KO cells were transfected with siRNA against PACE4, CAP1, PACE4+CAP1, or non-targeting siRNA (Scramble). Following the siRNA transfection, cells were treated with either ferric ammonium citrate (FAC) or deferoxamine (DFA) and collected for further analysis. B) Alternation in mRNA levels of CAP1 in the presence of iron and PACE4. C) WT CAP1 was co-expressed with either WT PACE4 or empty vector (EV), and cell media were collected for Co-IP assay. For this assay, V5-agarose beads have been used to pull down WT PACE4, and precipitated proteins have been analyzed by WB assay and by using antibodies against CAP1. All Cell Lysates and media were extracted to be analyzed by WB (SDS/PAGE on 8% Tris-glycine gel). Protein levels were normalized to the control protein, α-tubulin. Data are representative of two-three independent experiments. Quantifications are averages ± standard deviation (SD). * $p<0.05$; ** $p<0.01$; ***$p<0.001$ (two-sided t-test). NS: non-significant.

Chapter VI

Discussion:

To grasp the complicated interplay between PCSK9 and LDLR, a crucial complex in the regulation of cholesterol metabolism, researchers have embarked on a challenging journey. While progress has enlightened aspects of this interaction, a complete understanding remains a puzzle yet to be fully solved. Since the inhibition of PCSK9 presents a potent approach for treating cardiovascular disorders (CVDs), understanding the possible implications of this protein on other cellular processes holds the potential to extend the advantages of this established treatment beyond cardiovascular disease. For example, the revolutionary discovery showing that PCSK9 also targets MHC-I has enabled the utilization of mAb against PCSK9 (evolocumab) along with PD-1 antibodies for cancer therapy. This combination therapy has shown the ability to enhance responses in breast cancer cases compared to solo therapy with PD-1 antibody (Liu et al., 2020).

Although proteomics studies identified many proteins that might interact with PCSK9 and LDLR, but it's still tricky to figure out exactly how they all fit together. Given the fact that PCSK9 can connect with different partners, making it challenging to identify the most important interactions that control its effects. Despite these challenges, the recent efforts uncovered important information regarding the trafficking and function of PCSK9 that hold the promise of revealing new insights about cholesterol metabolism and its importance in the liver.

Our recent publication demonstrated the new model of PCSK9 function on LDLR. Accordingly, when the catalytic domain (and part of prodomain) of extracellular PCSK9 binds to the EGF-A domain of LDLR, the complex needs at least two other partner proteins to be internalized *via* clathrin-coated vesicles on route to lysosomal degradation. The suggested proteins include CAP1 and yet-to-be-identified protein X, which respectively associate with the M1/M3 and M2 domains of PCSK9 (Fruchart Gaillard et al., 2023; Jang et al., 2020). CAP1 interaction with the M1 and M3 domain of PCSK9 lead to the exposure of the M2 domain to be available to engage with protein X. Based on previous investigations, protein X was proposed to be a transmembrane protein with a cytosolic tail crucial for internalization and sorting of the complex towards lysosomal degradation. Here, the present work was conducted to enhance our comprehension of protein X and PCSK9. Our objective was not only to define the key characteristics of protein X (including verifying whether the potential candidate protein, HLA-C

is indeed protein X), but also to unveil novel potential regulators or targets of PCSK9, like the HFE protein.

Immunofluorescence microscopy assay revealed that the lack of M2 domain (more specifically residues R549, Q554, E567) and subsequently protein X, results in complete LOF of PCSK9 on LDLR degradation, but has no effect on the endocytosis process of LDLR and PCSK9 complex (Fig. 18). This finding highlights the importance of protein X for directing the complex from early endosomes to lysosomes and not from the plasma membrane to early endosomes. Given that the tested residues (their loss leading to LOF phenotype) play a crucial role in PCSK9's interaction with HLA-C (Fruchart Gaillard et al., 2023), we suspect that this protein might function as protein X. Following performing qPCR experiments to validate the expression of various HLA family proteins (Fig. 19), we focused on studying HFE and HLA-C proteins, due to their established involvement in lipid metabolism (Demetz et al., 2020; Fruchart Gaillard et al., 2023).

VI.I. Human homeostatic iron regulator protein (HFE) as a new target and regulator of PCSK9:

The resemblance of HFE's crystal structure to HLA-C, along with its prior connection to protein LDLR and cholesterol levels, motivated our investigation into whether this protein could engage with PCSK9 like other MHC-I molecules. Additionally, we aimed to determine whether its impact on cholesterol metabolism is mediated through PCSK9 or follows a different pathway.

Interestingly, our cell-based assays, for the first time, showed the interaction of HFE with PCSK9 that negatively regulates PCSK9's function (Fig. 20A, C). Certain physiological conditions, like elevated iron levels, could enhance the inhibitory impact of HFE on PCSK9 (Fig. 22D). This occurs because high iron levels lead to increased availability of HFE, which predominantly associates with TfR1 on the cell surface. Typically, HFE on the cell surface attaches to TfR1, and conditions like elevated iron levels cause HFE to detach from TfR1, consequently triggering hepcidin expression. Moreover, the residues of HFE interacting with TfR1 are located on the α1 and α2 domains of HFE (Bennett et al., 2000). Our 3D modeling also suggests the involvement of the α1 domain (R67, E69, and R71) of HFE to interact with PCSK9 (Chapter 4.1., Fig. 21). These data suggest a potential competition between TfR1 and PCSK9 for interaction with HFE. However, complete knockout of TfR1 in mice did not intensify the inhibitory effect of HFE on PCSK9. Our study of lipid profiles in male and female TfR1 knockout mice indicated higher cholesterol levels compared to normal mice (Fig. 22E). These elevated cholesterol levels could suggest greater PCSK9 activity, resulting from a reduced inhibitory effect of HFE on PCSK9 at the cell surface. Earlier *in vitro* investigations showed that an excess of HFE undergoes degradation without sufficient TfR1, as TfR1 enhances HFE stability (Gross et al., 1998). Therefore, while it seems TfR1 competes with PCSK9, its presence remains crucial for localizing HFE on the cell surface. The key distinction arises when HFE that primarily binds to TfR1, is separated from it under elevated iron levels and may then be free to bind PCSK9, thereby intensifying its inhibitory effect on PCSK9. The absence of TfR1, however, could significantly alter HFE's trafficking to the cell surface. Conversely, previous animal studies on TfR1 knockout mice revealed that the impact of high iron levels on hepcidin expression is the same as in TfR1 KO conditions. Nevertheless, these studies have yet to determine whether HFE's influence on hepcidin arises from the cell surface or intracellular HFE, and how the absence of TfR1 might

influence HFE's stability and cell surface localization. Therefore, further investigations are needed to validate the link between TfR1 and HFE's impact on PCSK9 and to understand the HFE's trafficking and cell surface localization in the presence or absence of TfR1.

Additionally, extracellular PCSK9 led to substantial lysosomal degradation of WT HFE (Fig. 20), suggesting the possible implication of PCSK9 in iron metabolism. Our 3D modeling and cellular analysis revealed that PCSK9's R-x-E motif interacts with HFE's R67 and E69, like HLA-C interactions. However, studying natural mutations (Q554E and H553R) had opposite results on HFE compared to HLA-C, likely implicating R71 of HFE (Fig. 21). With that we are suggesting the potential competition between HFE and HLA-C to interact with the PCSK9's M2 domain. However, no competition was observed between HFE and another PCSK9 target, the LDLR protein. In fact, LDLR's presence appears to be essential for endocytosis of the HFE-PCSK9 complex and degradation of HFE (Fig. 22A-C). This dependence could be because of the short cytosolic tail of HFE that isn't adequate for independent receptor internalization.

While intracellular HFE and PCSK9 seem to not have the same effect on each other as the extracellular forms, HFE increased PCSK9 levels both in lysate and media (Fig. 24) Intriguingly, intracellular HFE's chaperone-like effect on ΔM2 PCSK9 implies distinct binding mechanism, possibly involving acidic pH or other critical residues. This data suggests a dual role for HFE on PCSK9 where intracellularly, HFE could enhance PCSK9 stability, expression, and secretion, acting as an escort/chaperone, whereas extracellular HFE inhibits PCSK9 activity on the LDLR and HLA-C. Future experiments are needed to fully grasp the complications of PCSK9 and HFE intracellular interactions.

Previously the PCSK9's potential impact on iron homeostasis was supported by a study linking PCSK9 deficiency to severe anemia in mice with sickle cell disease (SCD) (Venugopal et al., 2020). Here we explored the possible impact of PCSK9 on iron metabolism under normal conditions by analyzing various iron indicators in individuals with the PCSK9 Q152H variant vs normal subjects. Given that the Q152H variant is retained within the ER and not secreted, we hypothesized that this could lead to reduced degradation of HFE by PCSK9. As HFE is known to reduce the iron uptake activity of TfR1, elevated HFE levels might theoretically diminish iron absorption, resulting in higher circulating iron levels. Surprisingly, our data revealed that the PCSK9 Q152H variant shows a trend toward lower levels of circulating iron compared to WT

PCSK9 (Fig. 23). We postulate that this outcome comes from the Q152H variant being retained in the ER (Lebeau et al., 2021), suggesting an effect distinct from only inhibiting extracellular PCSK9 or knockout conditions. This leads us to propose that PCSK9's presence within the ER and its interaction with HFE serve to retain HFE receptors within cells, subsequently reducing cell surface HFE levels. This, in turn, augments iron uptake and results in lower circulating iron levels. However, due to the variable nature of iron levels influenced by factors like sex, age, diet, and physiological conditions, a comprehensive experiment accounting for these confounding elements and involving a larger sample size is needed. Additionally, a more precise comparison would be comparing plasma iron levels in patients treated with a mAb against PCSK9 (rather than PCSK9 Q152H retained in the ER) with those of normal subjects.

In conclusion, our study highlights the significance of PCSK9's M2 domain and protein X in extracellular PCSK9 and LDLR complex trafficking to lysosomes. Notably, extracellular PCSK9 prompts LDLR-dependent lysosomal degradation of cell surface HFE *via* the R67-x-E69-x-R71 motif. Our findings suggest a potential link between PCSK9 and human iron metabolism. We identify HFE as a dual regulator of intracellular (positive) and extracellular (negative) PCSK9's activity on LDLR. Various physiological conditions, like high iron levels, could influence HFE's impact on PCSK9.

VI.II. Deciphering the role of human leukocyte antigen C (HLA-C) on PCSK9 function and its competition with HFE:

The initial 2020 publication identified MHC-I as a new PCSK9 target, revealing a potential role of PCSK9 in the immune system (Liu et al., 2020), but without details on HLA-C's regulatory impact on PCSK9. In a recent study, both Carole Fruchart and our research team constructed a 3D model of HLA-C and its interaction with PCSK9 (Chapter 4.2., Fig. 1A) (Fruchart Gaillard et al., 2023). Our current research serves as an extension of this recent publication, aiming to validate HLA-C's influence on PCSK9 activity and to identify specific interaction sites mentioned in that study.

Our results reconfirmed the LOF in PCSK9 variants and the importance of the M2 domain's interaction with HLA-C (Fig. 26). Although overexpressed HLA-C didn't affect WT PCSK9, possibly due to high endogenous HLA-C levels in HepG2 cells, it significantly increased the degradation activity of PCSK9 H553R variant on LDLR. This data is consistent with our 3D modeling that suggest the H553R variant as a GOF for PCSK9 and HLA-C interaction.

Facing challenges with HLA-C silencing due to its variable expression (Fig. 25), we turned to CHO-K1 cells lacking HLA family members as well as HepG2 CRISPR HLA-C KO cells. Confirming our hypothesis, PCSK9 function improved in both cell lines upon introducing HLA-C, supporting its critical role (Fig. 27C-D). Our findings indicate that PCSK9 alone, without HLA-C, had no influence on LDLR. However, the presence of HLA-C enhanced its activity. Accordingly, we propose HLA-C as a potential "protein X," pivotal for the extracellular activity of PCSK9.

While HLA-C positively regulates the extracellular activity of PCSK9 on the LDLR, interestingly, PCSK9 doesn't rely on HLA-C for HFE degradation. In fact, the presence of HLA-C or HFE, inhibits the degradation of either protein (Fig. 28A), suggesting a competitive relationship between HFE and HLA-C regarding their interaction with PCSK9 (Fig. 28B).

Conversely, unlike HFE, the reduction of LDLR levels did not impact the degradation of HLA-C by PCSK9 (Figure 28C). Additional data concerning LDLR lacking its C-terminal cytosolic tail indicated that, compared to control conditions, HLA-C internalized extracellular PCSK9 to a greater extent, implicating the cytosolic tail of HLA-C in the internalization process

when the dominant cytosolic tail of LDLR is not present (Fig. 28D). Previous studies have established that PCSK9 and LDLR internalization occurs through clathrin-coated vesicles. Thus, we hypothesized that the presence and internalization of HLA-C with the PCSK9 and LDLR complex might also involve clathrin-coated vesicles. Intriguingly, the presence of aromatic amino acids (such as phenylalanine) in HFE's transmembrane domain hints at a potential interaction between this protein and caveolin vesicles.

Our investigation regarding the endocytosis mechanisms involving HFE and HLA-C revealed distinct patterns. While the degradation of HLA-C was influenced by the removal of clathrin heavy chain (CHC), the degradation of HFE was reduced by the absence of both CHC and Cav1. Consequently, we propose two internalization pathways for HFE: the standard route involving its endocytosis *via* TfR1 through clathrin-coated vesicles, and under specific circumstances, its internalization *via* caveolin-positive vesicle facilitated by PCSK9 and LDLR. Similar pathways have been observed in other receptors, such as the TGF-β receptor. Notably, these authors suggested that receptor internalization for signal transduction occurs through clathrin-coated pits, while its degradation pathway involves caveolin-positive vesicles. They also highlighted those certain physiological conditions, like high potassium levels, that can favor one pathway over the other (Di Guglielmo et al., 2003).

We also propose a similar regulatory pathway for PCSK9, where, under typical conditions, HFE is predominantly internalized through clathrin-coated vesicles with the assistance of TfR1. Additionally, HLA-C binds to the PCSK9 and LDLR complex, prompting their internalization *via* clathrin-coated vesicles, directing LDLR to lysosomal degradation. Under specific circumstances like elevated iron levels, HFE might outcompete HLA-C and undergo internalization *via* the PCSK9 and LDLR complex, potentially in caveolin-positive endosomes. Given that the lack of HLA-C prevents PCSK9's degradation of the LDLR, in absence of HLA-C, only HFE goes to the lysosomal degradation. Under these conditions, the lack of LDLR degradation in absence of HLA-C suggests that the internalized PCSK9-LDLR-HFE complex enters caveolins from where HFE is sent for degradation in lysosomes. However, an unidentified mechanism may also facilitate the recycling of the PCSK9-LDLR complex or LDLR alone back to the cell surface (Fig. 36).

As previous studies also implicated the importance of PCSK9 in iron metabolism under specific iron conditions (SCD disease) we propose that probably the interaction of HFE and

PCSK9 in the liver mostly happens under specific iron situations. However, in cell types or tissues where HFE expression is higher than in the liver, it is possible for this protein to exert a more potent inhibitory effect on PCSK9, regardless of iron levels. A comparable phenomenon was previously demonstrated with Annexin A2, which isn't present in the liver but significantly expressed and inhibits PCSK9 function in the small intestine. Recent findings indicate that intestine specific PCSK9 KO has no discernible effect on overall circulating cholesterol levels. This suggests that PCSK9 might already be sufficiently inhibited in this tissue, implying that its elimination wouldn't yield any additional substantial impact (Venugopal et al., 2020) (Garcon et al., 2020).

Furthermore, when Jang et al. initially reported CAP1's involvement in PCSK9's function it contrasted with earlier research, as they proposed that PCSK9 and LDLR internalization mainly occurs through caveolin-positive vesicles instead of clathrin-coated vesicles (Jang et al., 2020). In their study, they observed LDLR co-localizing with both CHC and Cav1 in the presence of PCSK9. Despite this, they emphasized that the correlation of LDLR co-localized with Caveolin is higher than with clathrin heavy chain. As this proposal contradicts previous findings and considering our suggestion for two distinct pathways for PCSK9 and LDLR complex endocytosis, we speculate that when PCSK9 is incubated with hepatocytes, it might target other molecules like HFE, leading to its internalization potentially via caveolin-positive vesicles, which results in HFE degradation but not LDLR. We propose that over an extended period of PCSK9 exposure, the LDLR that internalizes through HLA-C via CHC vesicles undergoes degradation and becomes of comparatively of lower levels than the LDLR internalized with HFE and Cav1, which avoids LDLR degradation. Consequently, after a certain duration, there might be a higher quantity of LDLR that accumulates in Cav1 vesicles compared to CHC.

In summary, our findings suggest that HFE and HLA-C have a competitive relationship and utilize different endocytosis pathways when interacting with PCSK9, influencing the fate of PCSK9-bound proteins in the cellular trafficking process. These findings also imply the potential utilization of therapeutic approaches targeting PCSK9 in hematochromatosis patients with HFE C82Y variations. Such individuals possess reduced cell surface HFE levels, as most of it is retained in the ER. By removing PCSK9, there is a possibility of enhancing HFE levels at the cell surface, potentially alleviating related symptoms.

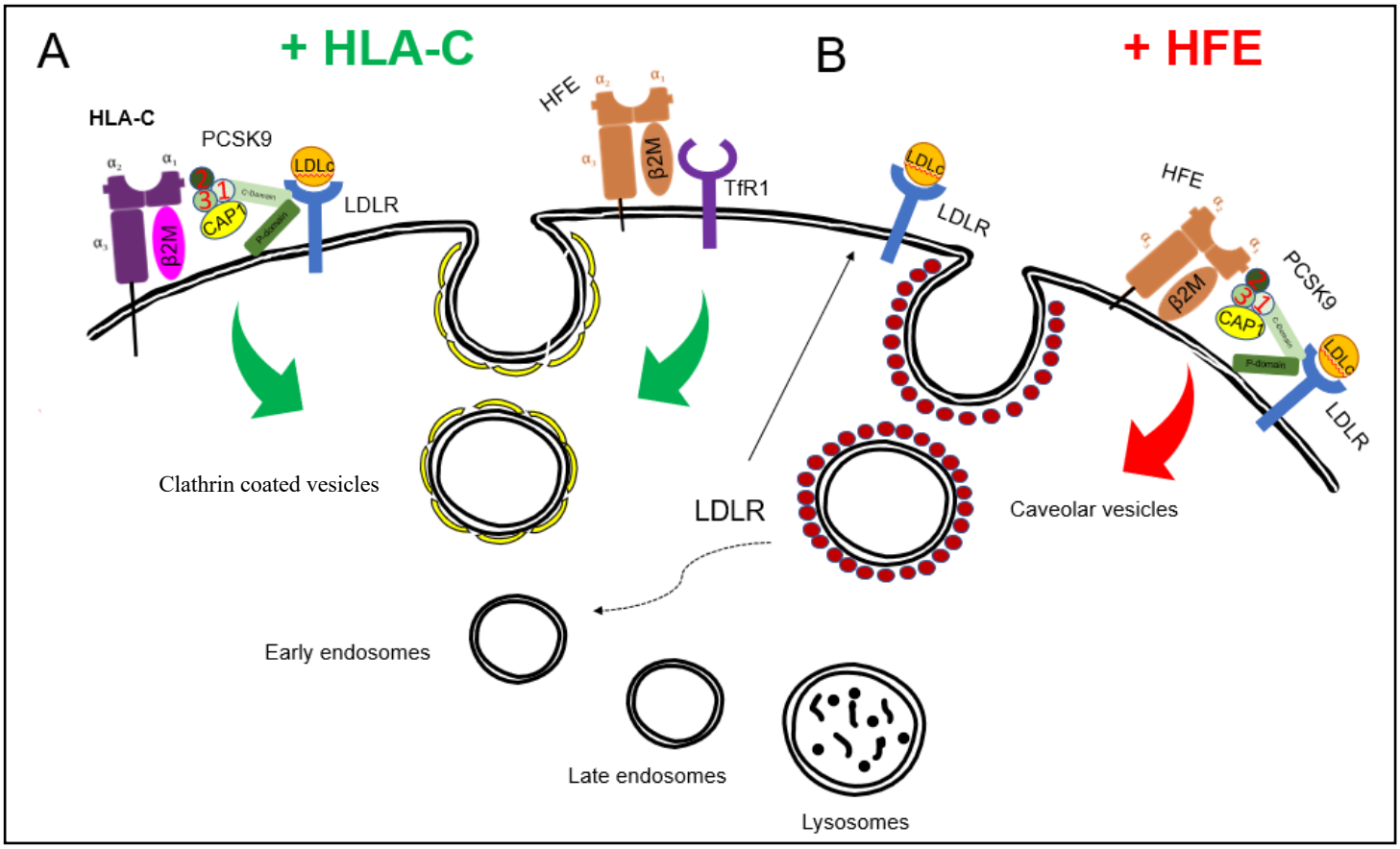

Fig. 36. Schematic of possible internalization pathways of PCSK9 and LDLR complex in presence of A) HLA-C and B) HFE.Fig. 1. Role of iron on PACE4 function. A) HepG2 PCSK9 KO cells were incubated with conditioned media from HEK293 cells expressing an empty vector (control) or WT PACE4. Following the incubation with conditioned media, cells were treated with either ferric ammonium citrate (FAC) or deferoxamine (DFA) to analyze the function of PACE4 on LDLR in different iron conditions. B) HepG2 PCSK9 KO cells were transfected with siRNA against PACE4 or non-targeting siRNA (Scramble). Following the siRNA transfection, cells were treated with either ferric ammonium citrate (FAC) or deferoxamine (DFA) and collected for further analysis. C) alternation in mRNA levels of PACE4 in the presence of iron. All Cell Lysates and media were extracted to be analyzed by WB (SDS/PAGE on 8% Tris-glycine gel). Protein levels were normalized to the control protein, α-tubulin. Data are representative of two-three independent experiments. Quantifications are averages ± standard deviation (SD). * $p<0.05$; ** $p<0.01$; ***$p<0.001$ (two-sided t-test). NS: non-significant.

VI.III. Regulation of PCSK9 and LDLR by PACE4:

Extensive proteomics studies have aimed to decipher PCSK9's complex trafficking mechanisms toward LDLR degradation. Our collaboration with the University of Sherbrooke hinted at a potential interaction between PCSK9 and PACE4, prompting a closer investigation into this relationship's impact on PCSK9's activity. Our experiments in different tissue and cell lines showed a similar mRNA expression pattern of PACE4 with LDLR and PCSK9 (Chapter 4.3., Fig. 1). The high mRNA expression of PACE4 in hepatocytes, indicates the potential involvement of this protein in liver functions. Interestingly, our data for the first time, revealed the negative regulatory effect and interaction of intracellular PACE4 on intracellular PCSK9, possibly *via* increasing the Furin-cleaved form of PCSK9 (Chapter 4.3., Fig. 2).

Surprisingly, although extracellular PACE4 had no substantial effect on PCSK9's function, it significantly increases LDLR protein levels in a PCSK9-independent fashion and *via* its enzymatic activity (Chapter 4.3., Fig. 3,4,5). This suggests two pathways for PACE4's impact on LDLR: a PCSK9-dependent pathway where PACE4 disrupts PCSK9's activity by its cleavage at the Furin-like site at $Arg_{218}\downarrow$, and a PCSK9-independent pathway where PACE4 (mainly extracellular) directly enhances LDLR levels. To explore a direct interaction between PACE4 and LDLR, we performed Co-IP assays. Interestingly, only the CRD domain of PACE4 displayed interaction with LDLR, while WT PACE4 did not directly bind to LDLR (Chapter 4.3., Fig. 4C, D). This suggests that PACE4's specific conformation might affect its interaction with other molecules. Thus, PACE4's inhibitory impact on LDLR likely occurs indirectly, possibly through another yet-to-be-identified protein that directly interacts with LDLR. In search for possible partner molecules for the direct interaction of PACE4 with LDLR (Chapter 4.3., Fig. 6), we discovered the importance of iron levels in the secretion of PACE4 in hepatocytes.

Previous research has demonstrated PACE4's capability to degrade hepcidin (Scamuffa et al., 2008). Consequently, we propose that this response might be a cellular mechanism to counter high iron levels. This scenario involves maximizing hepcidin expression while diminishing PACE4 levels, possibly inhibiting its cleavage impact on hepcidin. However, iron increased the CAP1 levels in the media that suggest the possible global effect of iron in protein secretion. Such a phenomenon was previously suggested for affecting ApoE levels in adipocytes (Britton et al.,

2018). In this study, the authors compared the levels of signal-peptide-containing proteins with exosome-secreted proteins, revealing that iron primarily affects proteins secreted through the classical pathway. Another intriguing discovery in our work was the unexpected interaction between PACE4 and CAP1. As mentioned previously, CAP1 has a direct impact on LDLR and can modify PCSK9's conformation to facilitate its interaction with protein X (Fruchart Gaillard et al., 2023). Consequently, we hypothesize that PACE4's PCSK9-independent regulation of LDLR might occur through two distinct pathways. One possibility is that PACE4 activates CAP1's direct influence on LDLR. Alternatively, PACE4's interaction with CAP1 might expose its CRD domain, linking with LDLR and elevating LDLR levels. Nevertheless, further investigations are required to fully elucidate the precise mechanism by which PACE4 directly regulates LDLR levels.

To conclude, this study introduces PACE4 as a novel regulator of both PCSK9 and LDLR, potentially impacting lipid metabolism. Exploring the potential direct impact of proteins such as PACE4 on LDLR opens doors for innovative therapeutic strategies specifically in patients who exhibit resistance to PCSK9-based therapies.

VI.IV. Conclusion and summary:

In conclusion, our work not only sheds light on key cellular mechanisms linked to lipid metabolism but also offers potential for pioneering therapeutic approaches. This extends to cases of hypercholesterolemia where patients exhibit resistance to PCSK9 inhibition, necessitating the identification of PCSK9-independent regulators of LDLR. Additionally, this holds relevance for conditions like human hemochromatosis, where targeting extracellular PCSK9 could elevate cell surface HFE levels, opening doors for innovative treatments. Furthermore, the generation of soluble forms of MHC-I molecules in CHO cells presents a promising avenue for novel therapeutic targets in conditions like FH and HH. One potential approach could involve producing and administering soluble HFE proteins, offering a potential treatment strategy. Future research endeavors, by both *in vitro* and *in vivo* studies, are needed to comprehensively grasp how lipid and iron processes interact within the liver. This necessitates a detailed exploration of the key proteins driving each of these processes.

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