Statins, Lipids, and Mutations: Consequences for the Heart and Immune System

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Det kanske är en förenkling som blir värre än Herman Lindqvist,
men det finns ju i alla fall kopplingar.

Edward Blom

Abstract

CAAX proteins are a group of proteins that undergo a three-step protein maturation process that renders the proteins carboxyl-terminus hydrophobic and prone to localize to cellular membranes, where they have their primary function. The first step in this process is called prenylation, which is the covalent attachment of a lipid, either a 15-carbon farnesyl or a 20-carbon geranylgeranyl lipid, to the carboxyl-terminal cysteine residue by the enzymes farnesyltransferase (FTase) and geranylgeranyltransferase type I (GGTase-I), respectively.

Statins are inhibitors of HMG-CoA reductase, the rate-limiting enzyme in the cholesterol biosynthesis pathway, and are widely used in the treatment of hypercholesterolemia. They are thought to improve myocardial function by inhibiting GGTase-I- and FTase-mediated prenylation of the *CAAX* proteins RHOA and RAC1, two known mediators of cardiomyopathy. In the first paper of this thesis, we show that, contrary to popular belief, long-term statin administration causes reduced heart function, hypertrophic cardiomyopathy, and hyperactive RHOA in the hearts of wild-type mice. Similarly, we show that inactivation of the prenylation enzymes GGTase-I, FTase, or both, in heart muscle cells causes severe dilated cardiomyopathy. These findings indicate that statins and prenylation inhibitors might have the capacity to cause heart problems.

In the second paper, we define the mechanism underlying a previous finding that inactivation of GGTase-I in mouse macrophages prevents prenylation of RHO family proteins, paradoxically causes them to become hyperactive, and that this leads to severe rheumatoid arthritis. We find that the RHO-protein RAC1 is responsible for the development of rheumatoid arthiritis. We further show that non-prenylated RAC1 exhibit an increased interaction with the effector proteins TIAM1 and IQGAP1 which trigger GTP loading, activation, excessive inflammatory signaling, and arthritis. Inactivation of RAC1 or IQGAP1 reduces the inflammatory signaling and markedly improves rheumatoid arthritis in GGTase-I deficient mice. We conclude that inhibiting prenylation of RAC1 stimulates effector interactions and cause excessive inflammatory signaling. This finding suggests that prenylation normally restrains innate immunity by limiting RAC1 effector interactions and its activation.

Protein-altering germline mutations are a major cause of dilated cardiomyopathy (DCM). However, recent sequencing studies have shown that rare protein-altering variants are also present in individuals without reported DCM. This complicates the interpretation of genetic testing in the clinic, which is increasingly used for diagnosis. In the third paper, we analyzed genotype-phenotype correlations and variant prevalence in 41 DCM-associated genes in a cohort of 176 Swedish DCM patients, and compared the variants to those of healthy reference individuals. We found 102 rare protein-altering variants, many of which were not previously reported, and further analysis revealed that harboring any variant was correlated with earlier onset of disease and reduced transplant-free survival. Comparing the number of variants found in DCM patients to rare variants in a healthy population showed that, while frameshift and nonsense variant were more common in DCM patients, the prevalence, pathogenicity scores, and location of missense variants were similar in both groups. These findings question the role of many putatively disease-causing variants and suggest that results from genetic testing should be interpreted with caution.

Populärvetenskaplig sammanfattning

CAAX proteiner är en grupp av proteiner som genomgår en tre-stegs mognadsprocess som gör att den c-terminala änden blir hydrofobisk, vilket ökar dess affinitet till cellmembran, där de utövar sin huvudsakliga funktion. Det första steget i mognadsprocessen kallas prenylering, där en kovalent binding bildas mellan en lipid, antingen en 15-kol farnesyl- eller en 20-kol geranylgeranyl-lipid, och det c-terminala cysteinet av enzymen farnesyltransferas (FTas) eller geranylgeranyltransferas typ I (GGTas-I).

Statiner är inhibitorer av HMG-CoA reduktas, vilket är det hastighetsbestämmande enzymet i kolesterolsyntesen, och används framför allt för att behandla högt kolesterolvärde hos människor. Man tror att statinbehandling kan förbättra hjärtfunktion bland annat genom att inhibera prenylering av *CAAX* proteinerna RHOA och RAC1, två proteiner som är associerade till hjärtsjukdom. I den första artikeln i den här avhandlingen visar vi att kronisk statinbehandling istället försämrar hjärtfunktion, orsakar hjärthypertrofi, och överaktiverar RHOA i mushjärtan hos vildtypsmöss. Vidare visar vi att inaktivering av prenyleringsenzym (GGTas-I och FTas, eller båda enskilt) i hjärtmuskelceller orsakar dilaterad kardiomyopati. Fynden visar att statiner och prenyleringsinhibitorer kan orsaka hjärtsjukdom i möss.

I den andra artikeln kartlägger vi mekanismen bakom ett tidigare fynd som visade att inaktivering av GGTas-I i makrofager i möss inhiberar prenylering av RHO-proteiner, hyperaktiverar dem, och orsakar grav reumatism. Här visar vi att RHO-proteinet RAC1 är orsaken till reumatismen. Vidare fann vi att oprenylerat RAC1 har en ökad interaktion med proteinerna TIAM1 och IQGAP1, vilket leder till RAC1-aktivering, ökad inflammatorisk signalering, och reumatism. Om vi inaktiverade TIAM1 och IQGAP1 sänktes den inflammatoriska signaleringen och förbättrade reumatismen i möss som saknar GGTas-I. Vi drar slutsatsen att inhiberad prenylering av RAC1 stimulerar interaktionen med effektor-proteiner och orsakar ökad inflammatorisk signalering. Dessa fynd tyder på att prenylering normalt inhiberar det medfödda immunförsvaret genom att begränsa interaktionen mellan RAC1 och dess effektor-proteiner och därmed hindrar RAC1 aktivering.

Mutationer som orsakar förändringar i proteinuttryck är en vanlig orsak till dilaterad kardiomyopati (DCM) i människor. Nya fynd har däremot visat att ovanliga mutationer också finns i individer utan hjärtsjukdom. Detta gör tolkningen av resultat från diagnostiska genetiska tester i kliniken svåra att tolka. I den tredje artikeln analyserar vi genotyp-fenotyp-korrelationer och mutationsfrekvenser i 41 DCM-associerade gener i en grupp på 176 svenska DCM-patienter, och jämför deras mutationer med mutationer hos friska individer. I gruppen av DCM-patienter fann vi 102 ovanliga proteinförändrande mutationer, varav många sedan tidigare okända hos DCM-patienter. Dessutom fann vi att patienter med proteinförändrande mutationer hade kortare transplantationsfri överlevnad och tidigare sjukdomsdebut. Vidare fann vi att frekvens, beräknad patogenicitet, och position av missense-mutationer var liknande i DCM-patienter och friska individer. Våra fynd ifrågasätter vikten av många förmodade sjukdomsorsakande mutationer i DCM och visar att resultat från genetiska tester borde tolkas med försiktighet.

List of papers

Paper I

Long-term statin administration inhibits protein geranylgeranylation and causes cardiomyopathy in mice

Emil G. Ivarsson, Zhiyuan Zou, Murali K. Akula, Margareta Scharin-Täng, Kristell Le Gal, Christin Karlsson, Johan Sternemalm, Azra Miljanovic, Malin Levin, Jan Borén, Martin G. Dalin, Martin O. Bergo.

Manuscript.

Paper II

Protein prenylation restrains innate immunity by inhibiting RAC1 effector interactions

Murali K. Akula, Mohamed X. Ibrahim*, Emil G. Ivarsson*, Omar M. Khan*, Israiel T. Kumar, Malin Erlandsson, Christin Karlsson, Xiufeng Xu, Mikael Brisslert, Cord Brakebusch, Donghai Wang, Maria Bokarewa, Volkan I. Sayin, and Martin O. Bergo

Nature Communications, In Press.

Paper III

Massive parallel sequencing questions the pathogenic role of missense variants in dilated cardiomyopathy

Martin G. Dalin, Pär G. Engström, Emil G. Ivarsson, Per Unneberg, Sara Light, Maria Schaufelberger, Thomas Gilljam, Bert Andersson, Martin O. Bergo.

International Journal of Cardiology, Volume 228, 1 February 2017, Pages 742-748.

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Abbreviations

α-MHC	Alpha-myosin heavy chain
ASCVD	Atherosclerotic cardiovascular disease
β-MHC	Beta-myosin heavy chain
Bp	Base pair
CoA	Co-enzyme A
CVD	Cardiovascular disease
cTnT	Cardiac troponin T
DCM	Dilated cardiomyopathy
EF	Ejection fraction
ER	Endoplasmic reticulum
FPP	Farnesyl pyrophosphate
FTase	Farnesyltransferase
FTI	Farnesyltransferase inhibitor
GAP	GTPase-activating protein
GEF	Guanine exchange factor
GEMM	Genetically engineered mouse model
GGPP	Geranylgeranyl pyrophosphate
GGTase-I	Geranylgeranyltransferase type I
GGTI	GGTase-II inhibitor
GTPase	Guanosine triphosphatase
HCM	Hypertrophic cardiomyopathy
HDL	High density lipoprotein
ICMT	Isoprenylcystein carboxyl methyltransferase
LDL	Low density lipoprotein
LoxP	Locus of crossing-over
LysM	Lysozyme-M
MAPK	Mitogen-activated protein kinase
MS	Multiple sclerosis
PI3K	Phosphoinositide 3-kinase
PDEδ	Phosphodiesterase-δ
RA	Rheumatoid arthritis
RCE1	RAS-converting enzyme 1
RHOGDI	RHO guanine nucleotide dissociation inhibitor
ROCK	Rho-associated coiled-coil-containing protein kinase
ROS	Reactive oxygen species
TAC	Transverse aortic restriction
TIAM1	T-Cell lymphoma and metastasis 1
ZMPSTE24	Zinc metalloproteinase Ste24 homologue

Introduction

Emil Ivarsson

CAAX Proteins

CAAX proteins are a group of proteins that share a common carboxyl-terminal (C-terminal) amino acid motif, the so-called *CAAX* motif. This sequence of amino acids always starts with a cystein (C), which is followed by two aliphatic amino acids (AA), and ends with a variable amino acid (X). What sets CAAX proteins apart from other proteins is that they all go through a distinct maturation process before being fully functional. At the end of this maturation process, which comprises three steps and starts just after translation, *CAAX* proteins have been imparted with a very lipophilic C-terminus that has high affinity towards membranes, which is where they serve their main function (Figure 1)[1, 2].

Currently, more than 200 proteins are predicted to be *CAAX* proteins and they control or regulate a wide range of functions within the cell: from proliferation and cell division to cytoskeletal organization and migration[1]. This wide-ranging involvement in cellular function also means that some of them are important for the development of diseases such as cancer and cardiomyopathy[3, 4, 5], and therefore, some of the most well studied proteins are indeed *CAAX* proteins. The most prominent examples of such proteins are the RAS and RHO proteins, which are commonly mutated in cancers[6, 7].

The *CAAX* Protein Post-Translational Modification Process

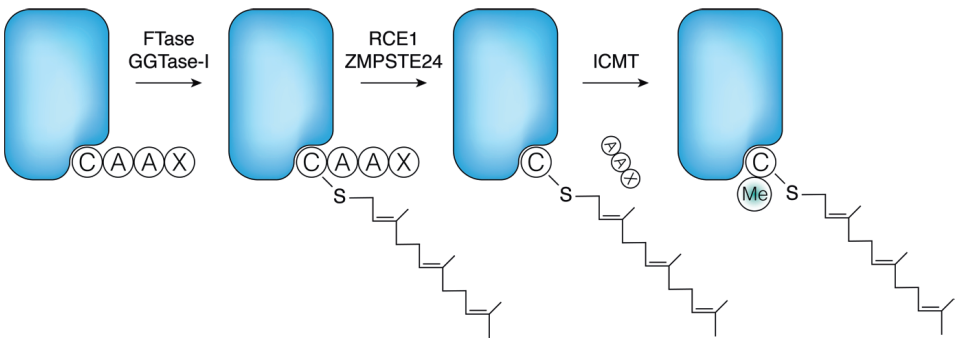

Figure 1. CAAX proteins undergo a three step post-translational modification process.

Step One: Prenylation

The first step in the *CAAX* protein post-translational processing is the covalent attachment of a lipid group to the cysteine residue of the *CAAX* motif, referred to as protein prenylation. In this step, either a 15-carbon farnesyl diphosphate (FPP) or a 20-carbon geranylgeranyl diphosphate (GGPP) is enzymatically attached to the protein by either farnesyltransferase (FTase) or geranylgeranyltransferase type I (GGTase-I), respectively[1]. What decides which one of the two lipids (prenyl groups) is attached to any specific protein is the variable amino acid (X) of the *CAAX* motif. Proteins with CAAX motifs whose X is either an A, M, S, or Q are recognized by FTase, whereas *CAAX* boxes where X is either L or F are recognized by GGTase-I (Figure 2)[8]. While FTase and GGTase-I are highly specific in which proteins they recognize there is a subset of CAAX proteins that can be prenylated by both enzymes. Alternative geranylgeranylation of KRAS and NRAS are two examples of this. Studies have shown that when FTase is inhibited in cells,

and KRAS and NRAS subsequently are not farnesylated, GGTase-I is able to compensate for that loss[8, 9, 10].

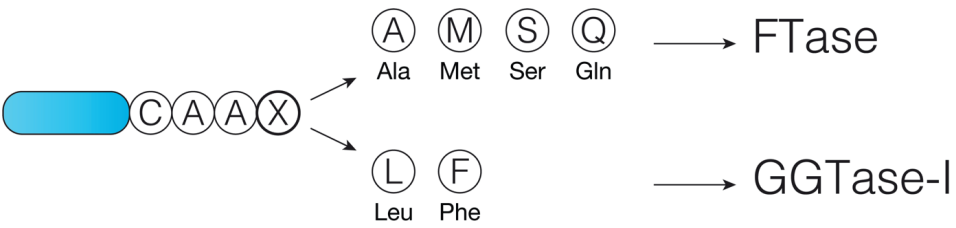

Figure 2. The last amino acid in the CAAX box is the main determinant of which prenyl group will be attached.

There is also a third prenylation enzyme, geranylgeranyltransferase II, also referred to as Rab geranylgeranyltransferase, which does not recognize *CAAX* motifs but geranylgeranylates Rab proteins[11].

FTase and GGTase-I consist of two subunits, of which the alpha-subunit is shared. The beta-subunit is unique for each enzyme and it is this subunit that contains the catalytic sites[12, 13].

Step Two: Endoproteolysis

Once the CAAX protein has been prenylated it is anchored to the endoplasmic reticulum (ER), and the last three amino acids (*AAX*) are cleaved off, leaving the prenylated cysteine exposed. This process is mediated by the ER enzymes RCE1 and ZMPSTE24[14].

Step Three: Methylation

The newly exposed prenylated C-terminal cysteine is subsequently methylated by the ER enzyme isoprenylcysteine carboxyl methyltransferase (ICMT)[15, 16]. The end result of this process is a lipophilic C-terminus with high affinity for membranes, and while it may seem that it does not alter the protein much, methylation has been shown to be crucial for the membrane association of *CAAX* proteins[17, 18].

After this process, the *CAAX* proteins are trafficked to their active intracellular location, a process which is tightly regulated by a set of proteins and chaperones. Examples of these are RHO guanine nucleotide protein dissociation inhibitors (RHOGDIs), which sequester geranylgeranylated RHO proteins[19], and phosphodiesterase-δ (PDEδ), which sequesters farnesylated RAS in the cytoplasm[20, 21]. In which part of the cell the protein ultimately will be transported to is determined by a region upstream of the *CAAX* box. This can either be a polybasic region, as is the case for KRAS, or a set of cysteines that can be palmitoylated[17]. It is thought that this complex process is required for a fully functioning and active *CAAX* protein[1].

Production of Prenyl Groups: The Mevalonate Pathway

The availability of prenyl groups (FPP and GGPP) is crucial for the maturation of *CAAX* proteins. Unprenylated CAAX proteins will not go through the rest of the maturation process and thus will not be fully functional in their normal active site. The prenyl groups are produced within the cell, as a metabolic by-product of the mevalonate pathway, which also produces cholesterol[22].

The mevalonate pathway is an essential metabolic pathway within the cell that provides the building blocks for many biomolecules that are required for proper cell function. It is most commonly known for one of its end products, cholesterol, which is associated with cardiovascular disease at abnormal blood concentrations. Other commonly known products are

vitamin K and coenzyme Q10[22, 23]. Inhibiting the production of cholesterol has been of great interest to the research community because of its association with cardiovascular disease, and several drugs that inhibit enzymes in the pathway have been developed, most notably statins, which have been very successful in reducing lipid concentration in the blood and the risk of cardiovascular disease[24].

The mevalonate pathway starts with the acetyl-CoA molecule and through a series of enzymatic steps produce HMG-CoA. HMG-CoA reductase then reduces the HMG-CoA, yielding mevalonate. This is the rate-limiting step of the mevalonate pathway and it is this enzyme (HMG-CoA reductase) that statins target to decrease cholesterol production in the liver[25]. Next follows a series of enzymatic reactions that ultimately produces FPP. At this point the pathway branches out to produce many different products: FPP can be converted into GGPP, which is then also used in prenylation, or it can be converted to squalene, which is a precursor to cholesterol. Alternatively, it can be converted to a number of other products important for other cellular functions (Figure 3)[22].

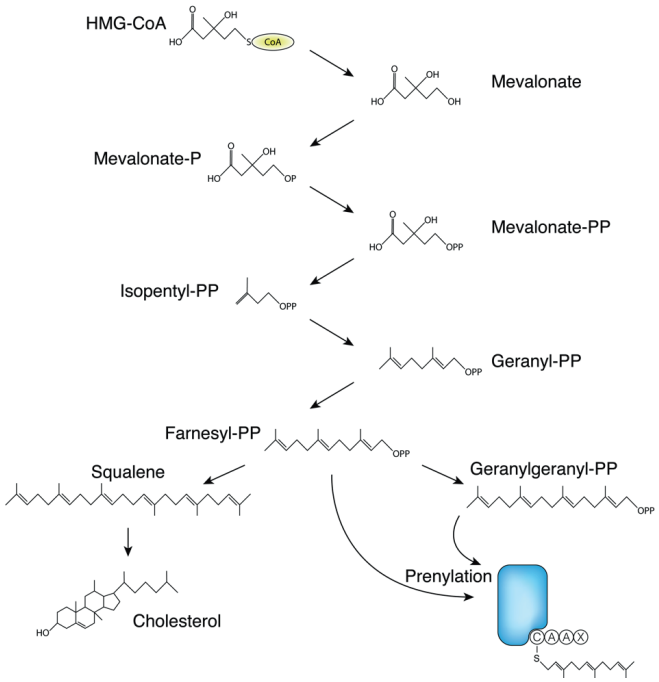

Figure 3. The mevalonate pathway.

RHO Proteins

Many *CAAX* proteins belong to the RHO family of proteins. Prominent examples of these are the geranylgeranylated RHOA, RAC1, and CDC42, which have been implicated in numerous pathologies[4, 7, 26, 27]. RHO proteins belong to the group of small guanosine triphosphatases (GTPases) that transduce intracellular and extracellular signals within the cell, and they are involved in a wide range of cellular functions, primarily through their regulation of the cytoskeleton[28, 29].

These proteins derive their function from their association with phosphorylated guanosine nucleotides. They have two main conformations, an active and an inactive form, which enable them to work as molecular on/off switches. When a RHO protein is bound to a guanosine diphosphate (GDP) it is inactive and signaling downstream is "turned off". In this conformation the RHO proteins can be sequestered in the cytosol by RHO-GDIs. Upon external signaling, e.g. growth factors, RHO-GDIs dissociate from the RHO proteins, exposing them to guanine nucleotide exchange factors (GEFs) for activation. The affinity of GDP to RHO GTPases is high and they dissociate slowly. Therefore, in order to activate RHO proteins, this dissociation is catalyzed by GEFs, allowing RHO proteins to bind cytosolic GTP and enter their active conformation. GTPases have a weak GTP to GDP hydrolytic capacity and they slowly decrease in activity over time, however, if stimulated, GTPase-activating proteins (GAPs) can catalyze this process, directly inactivating them[30, 31, 32, 33, 34].

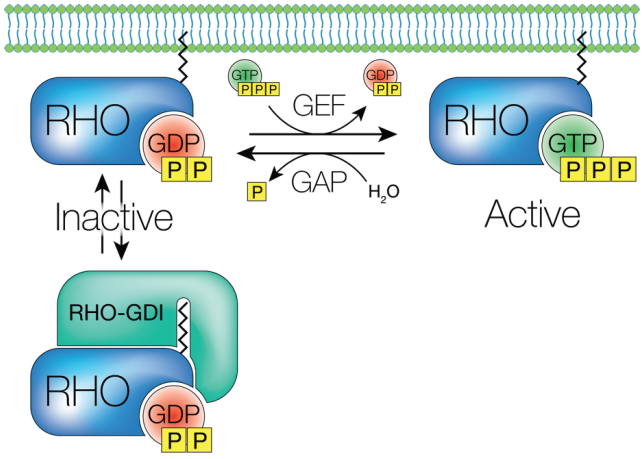

Figure 4. Regulation of RHO GTPases.

The activation and inactivation of RHO proteins is a tightly regulated process, in part through the stimulation of GEFs and GAPs. There are other regulatory proteins, however, such as RAS GTPase-activating like proteins IQGAP1 (IQGAP1) and IQGAP2 (IQGAP2). These proteins control RHO signaling by stabilizing them in their active form[35, 36]. All of these regulatory mechanisms enable the control of a range of cellular functions: from cytoskeletal remodeling to proliferation, and from growth factor production to cytokine excretion[37, 38].

The Role of *CAAX* Proteins in Physiology

The Role of Prenylated Proteins

As outlined earlier, prenylated proteins regulate many different cellular functions, and as a consequence are also involved in several diseases. Most notable is the farnesylated RAS subfamily, which includes three proteins commonly found mutated in cancer: KRAS, NRAS, and HRAS[6, 7]. In response to extracellular stimuli such as growth factors, RAS proteins regulate cell growth, proliferation, and differentiation, mainly through the mitogen-activated protein kinase (MAPK) pathway[39].

Mutations in the genes encoding for ZMPSTE24 (*ZMPSTE24*) or its farnesylated substrate LAMIN A (*LMNA*) can cause a set of diseases commonly referred to as laminopathies[40]. These include various striated muscle diseases, peripheral neuropathy, partial lipodystrophy syndromes, and Hutchinson-Gilford progeria syndrome[41]. LAMIN A is a major component of the intermediate filament network in the cell nucleus, regulating its size, shape, and incorporation of nuclear pores, which are important functions for a fully functioning nucleus[42, 43, 44, 45]. Thus, proper maturation of LAMIN A is crucial for the cell to be able to maintain a healthy nucleus, and by extension, a cell.

The RHO family of GTPases are, as stated previously, a substrate of GGTase-I and they are involved in the regulation of the cytoskeleton[28, 29]. In response to extracellular signals, RHOA, through its effector proteins ROCK and mDia, regulates cytoskeletal remodeling, formation of stress fibers, focal adhesions, cell to cell adhesion, and cell polarity. This makes it essential to cells that need to change their cell shape and/or migrate. In endothelial cells, for example, mechano-sensors reacting to increased shear stress activate RHOA to promote stress fiber formation in order to increase resistance to hemodynamic pressure[46]. Similarly, RHOA relays signals in vascular smooth muscle cells, triggering contraction of blood vessels. Another member of the RHO family, RAC1, is also involved in cytoskeletal remodeling, primarily through lamellipodia formation to promote migration and invasion. The primary signaling pathway for RAC1 is through PAK1, which relays signals to trigger cytoskeletal remodeling, cell adhesions, and gene transcription. It is also involved in the generation of reactive oxygen species (ROS) through activation of the NADPH oxidase complex and regulates phagocytosis, which is essential for the immune response function of macrophages[47, 48].

Interestingly, increased expression of geranylgeranyl diphosphate synthase (GGPPS) has been observed in several animal disease models, further demonstrating the importance of prenylation in normal cell function[49].

The Role of *CAAX* Proteins in Disease

Cancer

As mentioned previously, RAS proteins are common mediators of cancer. Mutations in the pathways that RAS proteins signal through are common, and mutations which render KRAS constitutively active, and constantly signaling downstream, are found in about 30% of all human cancers. This has made RAS proteins in general, and KRAS in particular, important targets in cancer treatment[6, 7].

General consensus states that prenylation is required for the activity of RAS and RHO proteins, which opens up the possibility of indirect inhibition as a strategy in the treatment of disease.

Inhibitors towards FTase and GGTase were first developed as a way of inhibiting RAS activity in cancer in response to the difficulty of direct target inhibition. Since RAS is farnesylated, inhibitors towards FTase (FTI) were first developed and they proved successful in pre-clinical studies.[5] In cells, FTIs showed anti-proliferative and pro-apoptotic effects, and they were able to stop and even regress tumor growth in xenograft experiments. While successful in pre-clinical research, they failed to demonstrate efficacy in clinical trials, and are now mostly used as research tools. The main reason for their inefficacy is thought to be that, while HRAS can only be farnesylated, NRAS and KRAS are able to be geranylgeranylated, which leads to compensatory geranylgeranylation by GGTase-I. Geranylgeranyltransferase inhibitors (GGTIs) in combination with FTIs or dual FTase/GGTase-I inhibitors also work well in cell culture and in some animal models, but exhibited excessive toxicity in human clinical trials. This is likely due to the fact that there are many more substrates for GGTase-I than for FTase, giving GGTIs a greater effect[50].

Heart Disease

RHO GTPases are important both in the physiology and pathology of the heart. Increased activity of some RHO GTPase members is associated with cardiac hypertrophy. This is a physiological state of the heart that is characterized by cardiac myocyte growth, which leads to enlarged muscle fibers and increased muscle mass, which in turn can improve heart function. When the heart experiences pressure or volume overload, hypertrophy is an adaptive compensatory mechanism to be able to handle the new conditions. However, if the stressors are allowed to remain, cardiac hypertrophy can become pathological, leading to decreased contractility and possibly heart failure[51].

RHOA has been implicated in cardiac hypertrophy both in *in vivo* and *in vitro* studies. In animal models of pressure overload there is an increase in the activity of RHOA and its downstream effectors followed by a hypertrophic response. This hypertrophic response is reduced when RHOA signaling is inhibited, suggesting that it is an important mediator of the pathology. Furthermore, RHOA activity is required for the increase in cell size, cytoskeletal reorganization, and gene expression induced by hypertrophic agonists[52]. Mice with cardiomyocyte-specific RHOA overexpression or expression of a constitutively active mutant RHOA develop atrial enlargement and increased heart weight, which ultimately leads to dilated cardiomyopathy (DCM) and heart failure[53], with increased expression of genes associated with cardiac hypertrophy[27]. Interestingly, absence of RHOA in cardiomyocytes in mice does not have pathological effects[54], making it a potential candidate for targeting in drug treatment.

In response to the implication of RHOA signaling in cardiac hypertrophy, inhibitors of two downstream effectors of RHOA, rho-associated coiled-coil-containing protein kinase 1 and 2 (ROCK1/2), have been developed[55]. In animals, these inhibitors have been beneficial in several heart disease models[55, 56]. Clinical trials to evaluate their efficacy in the treatment of human heart disease are currently on-going.

RAC1 contributes to hypertrophic signaling of the myocardium. RAC1 expression is increased in response to hypertrophic agonists. *In vitro* models expressing a constitutively active mutant RAC1 showed hypertrophic cytoskeletal remodeling and gene expression very similar to agonist-induced phenotypes. Further, the phenotypes of agonist-induced hypertrophy were blunted in the absence of RAC1, indicating an important role in hypertrophic pathology. Mice expressing constitutively active mutant RAC1 specifically in cardiomyocytes display one of two different phenotypes: they either develop severe DCM that ultimately leads to heart failure or they develop a slow-onset hypertrophy[27, 57]. Deletion of RAC1 in mice is embryonic lethal[27], however, conditional RAC1 deletion in adult mice ameliorated agonist-induced cardiac hypertrophy[58]. This raises the possibility of RAC1 inhibition as a viable strategy in the treatment of hypertrophy.

Although inhibiting prenylation has been attempted primarily in the context of cancer treatment, prenylation inhibitors have been proposed as RHO GTPase inhibitors in cardiomyopathy as well. Inhibition of FPPS in mice with agonist-induced cardiac hypertrophy attenuated the hypertrophic phenotype[59], and improved pathological cardiac remodeling in animal models of pressure overload[60]. Apart from affecting RHO GTPases, inhibiting prenylation would also have the effect of inhibiting RAS, which is also known to mediate heart disease. In addition, cell based studies have shown that FTIs are able to attenuate the phenotype of agonist-induced hypertrophy[61]. These findings indicate that inhibiting prenylation might be a viable strategy in the treatment of heart disease; however, the role of the prenylation enzymes in this context has not been studied.

Since statins act on HMG-CoA reductase early in the mevalonate pathway, and inhibit the production of cholesterol, it has been suggested that they are able to inhibit prenylation. This has been proposed as an explanation for some of the beneficial side-effects observed with statin treatment, which includes improved heart function and reduced inflammation[23, 62].

Inflammatory Disorders

Our first line of defense against foreign pathogens, such as bacteria and fungi, is the innate immune system. An immune reaction to a pathogen involves a complex interplay of immune cells, excreted cytokines, and antibodies that all work in concert to overcome an infection. When a pathogen is detected in a tissue, inflammatory cells present there will start to release pro-inflammatory cytokines. These cytokines then recruit more immune cells to the site of infection, which triggers further release of pro-inflammatory cytokines, activation of the complements cascade, and production of antibodies. When the pathogenic threat has been subdued anti-inflammatory cytokines are released by the immune cells, gradually decreasing the inflammatory response[63]. A well-functioning immune system will defend the body against foreign pathogens, but if this process is not properly regulated, and the inflammatory response is too excessive or not triggered by an actual pathogen, it can become pathological. An unregulated immune system is the root of many chronic inflammatory and autoimmune disorders[63, 64].

The essential migratory properties of immune cells, leukocytes, is dependent on proper cytoskeletal rearrangement, which is controlled by the prenylated RHO-GTPases, including RAC1 and RHOA[65].

The RHO-GTPase RAC1 has been implicated in the development arthritis, which is a group of diseases that affects the joints. Arthritis has a number of underlying causes, one of which is aberrant inflammatory signaling, but is most commonly associated with degraded cartilage of the joints due to wear and tear, which is called osteoarthritis. Increased RAC1 activity has been observed in cartilage of osteoarthritic joints, and it is believed that RAC1 mediates the degradation of the cartilage matrix[66].

Rheumatoid arthritis (RA) is an inflammatory type characterized by inflammation in the synovial lining of the joints, gradually destroying the joint cartilage, in which the synovial lining exhibits increased T-cell activity and auto-antibody production. This results in swelling, stiffness, and pain in the joints[67]. Although the exact underlying causes of RA are unclear, RAC1 plays a role in the infiltration of synovial cells into the inflamed tissue, and both RAC1 inhibitors and genetic deletion of RAC1 is able to reduce the invasiveness of synovial cells and reduce swelling in animal RA models[66, 68, 69].

Since RHO proteins are involved in the regulation of migration, phagocytosis, ROS production, and signaling in inflammatory cells[47], targeting GGTase-I has been proposed as a treatment strategy for autoimmune disorders such as RA and multiple sclerosis (MS)[70, 71, 72]. Interestingly, previous findings published by our group have shown that mice with hyperactivation of RAC1 in macrophages develop severe erosive arthritis in joints, by stimulating pro-inflammatory signaling

and increasing pro-inflammatory cytokine production. This was a surprising finding, because the RAC1 hyperactivation was caused by genetic inactivation of the GGTase-I, questioning the role of prenylation in innate immunity. There is a general consensus that inhibiting prenylation of RHO proteins would reduce the inflammatory action of RHO proteins and thereby reduce inflammation, but these findings question that view and suggest that prenylation could be a negative regulator of RHO-GTPase activation[73, 74].

Statins

Statins are a group of drugs that inhibit the rate-limiting enzyme of the mevalonate pathway, HMG-CoA reductase, with the primary intention of limiting the production of cholesterol[75].

Cholesterol is an essential lipid for all human cells, where it serves to build and maintain cellular membranes as well as regulating their permeability and fluidity, and it is estimated to comprise about 30% of all cellular membranes[76]. Due to its hydrophobic nature, the balance of cholesterol supply to all cells in the body is controlled by lipoproteins that transport cholesterol in the blood stream. Broadly, these lipoproteins can be divided into two categories: low density lipoproteins (LDL) that transport cholesterol from the liver to the bloodstream for uptake by other cells, and high density lipoproteins (HDL) that transport cholesterol back to the liver for excretion[77].

When a cell is presented with cholesterol packaged in an LDL-cholesterol particle it is sequestered via LDL-receptors and the cholesterol is distributed within the cell. However, if a cell is already cholesterol saturated the expression of LDL-receptors is downregulated, inhibiting further uptake[78]. If this process is dysregulated, LDL particles will start to accumulate in the bloodstream, and when the concentration of LDL-cholesterol is high enough atherosclerotic plaques, containing LDL-cholesterol, inflammatory cells, and other debris, begin to form in the blood vessel walls[79]. This process greatly increases the risk of cardiovascular disease. The formation of plaques can affect the rigidity of vessel walls, causing high blood pressure, and if an atherosclerotic plaque ruptures and enters the bloodstream it can obstruct arteries, limiting the oxygen supply in that artery, which leads to ischemic injury. If a coronary artery is obstructed, this causes myocardial infarction, which can be lethal[79].

Statins in the Clinic

Cardiovascular disease (CVD) is the leading cause of death worldwide, and the majority of CVD cases can be attributed to atherosclerosis (ASCVD). Although atherosclerosis can be caused by several other factors, high blood LDL-cholesterol concentration is thought to be the main cause[80, 81, 82]. For this reason, inhibitors of HMG-CoA reductase were developed, which have proved to be very effective at lowering blood cholesterol levels, and as such have become the main treatment strategy[83]. Subsequently, they have proved to be effective at lowering the risk of developing CVD and to decrease the progression and mortality rate of ASCVD[82]. It has also become evident that statins are beneficial in primary prevention of CVD, reducing the likelihood of cardiovascular events in patients without diagnosis but who are at risk of developing CVD[84].

Pleiotropic Effects of Statins

Statins have also exhibited beneficial effects on cardiovascular disease, inflammatory disease, and other illnesses, that are independent of the cholesterol lowering effect. Among these are reduced inflammation, improved endothelial function, antioxidant effects, improved plaque-stability, and improved heart function[62, 85, 86, 87, 88, 89]. This has widened the treatment indications for statins, and US guidelines now recommend statins for patients with at least a 5% risk of developing CVD within a 10-year period[90]. Thus, it is currently the most prescribed group of drugs in world, with almost 40 million users 2013 in the USA alone in the year 2013[91, 92].

Some of the pleiotropic effects of statins have been attributed to the reduction of FPP and GGPP production, having the effect of dual prenylation inhibitors, and thereby affecting RHO proteins[23, 62]. These findings have mostly been demonstrated in cell-culture experiments, with statin concentrations often significantly higher than those in the blood plasma of patients undergoing treatment[93]. However, non-prenylated proteins have been found in patients that have undergone

high doses of statin treatment for shorter time periods[94]. Non-prenylated proteins have also been detected in mouse experiments where mice were treated with clinically relevant statin doses[95]. It is still unclear, however, exactly at which doses statins are able to inhibit prenylation *in vivo* and if they do so in commonly administered doses.

Effects of Statins on Heart Function

It is well established that statins greatly reduce the risk of cardiac events associated with atherosclerosis[96]. In patients with high cholesterol-levels without heart failure, statins improve myocardial function[97, 98, 99]. Further, they have also been shown to have a more direct positive effect on the myocardium in experimental models, with positive effects on left ventricle remodeling, fibrosis deposition, and hypertrophy[100, 101, 102].

There are cases, however, where statin treatment has shown no benefit, or even reduced myocardial function. Currently, there are no guidelines on the use of statins in patients with chronic heart failure due to contradictory results in clinical trials. Two large clinical trials failed to demonstrate an improved long-term prognosis in this group of patients[103]. Further, a small study looking to evaluate the cholesterol independent effects of statin treatment found reduced myocardial function by evaluating heart function by strain imaging. The study compared the heart function of statin treated patients to cholesterol-level-matched controls who were not under statin treatment[104].

The Effect of Statins on Skeletal Muscles

Statins are generally well tolerated by patients with few side-effects. However, there are some known detrimental side-effects to statin treatment. The most reported complaints are myalgia, cramps, and muscle weakness, which have been estimated to occur in up to 25% of patients[105, 106]. The symptoms range from mild to severe, and one study of patients using high statin doses reported that 4% had symptoms that interfered with their daily activities and 0,4% had symptoms severe enough to be bedridden[107]. The most serious side-effect, however, is rhabdomyolysis, which is characterized by breakdown of skeletal muscle which leads to renal failure, and can be lethal. However, it is a rare occurrence: with only 1,5 fatal incidents reported per 1 million prescriptions[108]. The mechanism behind statin-induced myalgia and rhabdomyolysis still remains unknown.

Cardiomyopathies

Disease of the heart muscle, cardiomyopathy, affects the ability of the heart to properly pump blood around the body. Symptoms generally include shortness of breath, tiredness, and swelling of legs and an increased risk of sudden cardiac death[109, 110].

Cardiomyopathies can be caused by many different factors but is often idiopathic and, although classification is continually changing, they can broadly be divided into primary, where the disease is confined to the heart muscle, and secondary, where it is part of a more systemic disease. The cause of primary cardiomyopathies can be both genetic, acquired, and mixed, which establishes the disease in different ways, although there is an overlap[111].

The most common primary, and genetically caused, cardiomyopathy is hypertrophic cardiomyopathy (HCM), which is characterized by left ventricle hypertrophy without dilation of the chamber. HCM affects approximately 1 in 500 people and they usually experience chest pain and have an increased risk of sudden cardiac death[112, 113]. Arrhythmogenic right ventricular cardiomyopathy is another common inherited heart disease, in which the right ventricular wall starts to thin and balloon due to mutations in desmosomal genes. Less common is restrictive cardiomyopathies, in which the ventricles become too stiff to contract[111].

Dilated cardiomyopathy (DCM), which affects approximately 1 in 2500, is an example of a heart disease with mixed etiology, where around one third of cases are of genetic origin, demonstrating an autosomal dominant inheritance pattern. However, DCM can also be caused by excessive alcohol consumption, various infectious diseases, and nutritional deficiencies. It is characterized by the dilation of ventricles, with normal wall thickness and systolic dysfunction, with reduced contractility and ejection fraction (EF) as a result[114], and it is the leading cause of heart transplantation[115, 116].

Genetic screening in heart disease

A family history of cardiovascular disease increases the risk of disease development. However, it is only with the recent advances in genome sequencing technology that major breakthroughs in our understanding of the underlying genetic causes of cardiovascular disease have been made, which explains this hereditary pattern. Through sequencing technology, single-gene mutations have now been associated with a wide range of cardiovascular disorders, including HCM, DCM, and various arrythmogenic disorders[109, 117].

Genetic screening does not only help us understand risk factors of heart disease but it also helps with identifying the underlying biological mechanisms. Mutations in contractile proteins, such as beta-myosin heavy chain (β-MHC) and cardiac troponin T (cTnT), for example, are very common in patients diagnosed with HCM, which gives insight into the disease development[109, 117]. Mutations associated with DCM have been identified in a wider range of genes, cytoskeletal, sarcomeric, calcium-handling, and many more, making it a much more heterogeneous disease. As such, it is difficult to attribute the cause of DCM to a single gene mutation[118].

DNA Sequencing in DCM

Unlike cardiomyopathy caused by other factors, genetic DCM is a progressive disease, with the potential of early diagnosis by genetic screening, and improved patient outcomes with earlier treatment start. Screening of family members of individuals with DCM has therefore become more common, and as a result many more are diagnosed, and at lower ages[118].

Potential disease causing allele variants have been reported in a large number of genes, but with varying disease burden and penetrance[119]. Sequencing of large gene panels have therefore been employed to diagnose DCM patients, which has made it possible to explain some aspects of the development of the disease[120, 121]. However, it still remains a challenge to properly interpret the sequencing results[119], mainly because it is difficult to differentiate between benign and disease causing variants; healthy individuals also have many genetic mutations, including putative disease-causing variants[122, 123, 124].

Rationale & Aims

The overall aim of this thesis was to define the effects of inhibited prenylation of *CAAX* proteins in the immune system and heart function, but also to shed light on the genotype-phenotype correlations in dilated cardiomyopathy.

Specific Aims

Paper I

Statins are widely used in the prevention of cardiovascular disease, yet, their direct effects on myocardial function remain unknown. In this paper, we seek to define the effect on heart function of long-term statin treatment in mice.

Paper II

Genetic inactivation of GGTase-I in macrophages hyperactivates RHO-GTPases and causes severe rheumatoid arthritis in mice. In this paper, we test the hypothesis that RAC1 drives disease development and define the mechanism behind the hyperactivation of non-prenylated RAC1 and other RHO family proteins.

Paper III

Genetic alteration is an important cause in the development of dilated cardiomyopathy. However, recent studies have shown that rare putative disease causing variants are also present in healthy subjects. In paper III, we investigate genotype-phenotype correlations in Swedish DCM patients, and compare detected variants in that group to those in reference cohorts.

Research Strategy

In this section, some of the central research methods used in this thesis are presented. A more detailed description of used methods is included in each paper.

Mice as a Research Tool

The use of animals to better understand the world in which we live has a long history, with the earliest recorded animal experiments conducted by the ancient Greeks. Animals offer us the ability to study biological mechanisms without the need for direct intervention in humans, and they unlock a great number of tools for us to do so. Thus, most major biological breakthroughs have involved animal experiments in one way or another[125].

Important to all experimental research is the control of all influencing factors. Animals give us the ability to control the environment, even to a genetic level, in which an experiment is performed, which makes them incredibly useful in the study of biology and disease.

In this thesis, the common house mouse was used as a research animal.

Studying Disease in Mice

Mice are useful in medical research for several reasons. The physiology and anatomy of a mouse is very similar to that of humans. This is further reflected by the genetic similarities between the species, as 99% of all mouse genes have a human homologue. With the tools for genetic engineering available today this makes the mouse a powerful research asset, providing us with the ability to model most human diseases. Further, their small size makes them very easy to house and they have a short breeding cycle, with a gestation period of only 3 weeks, and reach sexual maturity at about 5 weeks of age. This makes the experimental setup of mouse experiments relatively easy[126, 127].

Genetically Engineered Mouse Models

The ability to alter the genetic information in mice has had a major impact on our understanding of human biology and diseases[128]. With this tool we are able to study the effects of genetic deletions and mutations that underlie our biology and the diseases that they cause, on a whole-organism level.

Genetically engineered mouse models (GEMMs) can be generated in two principal ways: pronuclear injection of DNA into a cell of a mouse embryo or injection of modified embryonic stem cells into mouse blastocysts. When performing pronuclear injection, DNA is integrated randomly into the genome of the cell, creating a transgene. This method is used to add genetic material to the mouse or to over-express endogenous genes[129]. The other method allows for a more precise alteration of the mouse genome. By using methods such as homologous recombination or CRISPR/CAS9 mutagenesis it is possible to change the genome of a cell in more deliberate and predictable ways. When a desired mutation has been achieved in a population of stem cells, they are then re-injected into a blastocyst of another mouse, giving rise to chimeras from which the mutation is further selected for[130].

The CRE/LoxP System for Conditional Gene Alteration

The most common method to alter genes in mice is by using the CRE/LoxP-system, which is a relatively easy and time-efficient method to delete genetic material in organisms.

The system is based on two factors: first, the region of DNA to be excised (e.g. an exon) is flanked by a pair of 34 bp sequences, referred to as locus of X-over-sites (LoxP-sites). Second, the LoxP-sites are recognized by the enzyme CRE-recombinase, which excises the LoxP-flanked region, leaving only one LoxP-site[131, 132]. The combination of these two elements makes this system a powerful research tool, as the expression of CRE-recombinase and presence of LoxP-sites can be controlled separately (Figure 5).

If CRE-recombinase expression is controlled by a tissue-specific promoter, the LoxP-flanked regions will only be excised in that specific tissue, enabling the study of genes only in the tissue of interest. CRE-recombinase expression can also be controlled temporally, for example by induction with tamoxifen injections. This is useful because it can be used to avoid unwanted effects at certain developmental stages of mice, such as embryonic lethality.

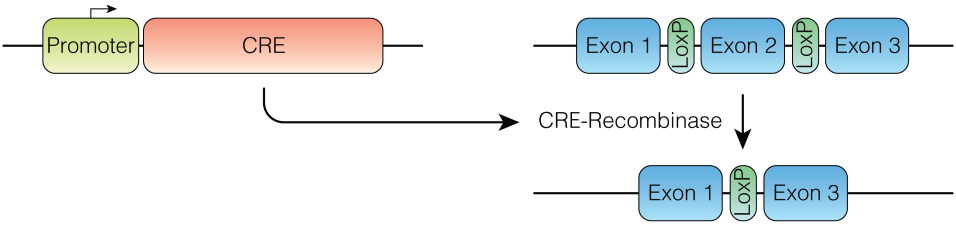

Figure 5. The CRE/LoxP system.

Two tissue specific CRE-expression models were used in this thesis, one with CRE-recombinase expressed in cardiomyocytes and the other in macrophages.

Knockout of the Prenylation Enzymes and RHO GTPases

In Paper I, we used deletion of *Fntb* and *Pggt1b* to knockout the beta subunits of FTase and GGTase-I to study the effects of prenylation in the heart. In paper II, the same GGTase-I knockout was used to study macrophages.

In paper II, mouse strains with knockout alleles of the RHO GTPases RAC1[133], RHOA[134], and CDC42[135] were used with or without the knockout of the prenylation enzymes, whereas in paper I, only RAC1 and RHOA knockouts were used.

Mouse strains harboring these knockout alleles were mated with mice harboring tissue-specific promoter-driven CRE-recombinase expression.

Cardiomyocyte specific CRE-expression

To achieve allele knockouts in cardiomyocytes, *Cre* expression is controlled by the promoter of alpha-myosin heavy chain (α-MHC-Cre)[136]. α-MHC is a contractile protein that is only expressed in cardiomyocytes, allowing for cardiomyocyte-specific expression of CRE-recombinase.

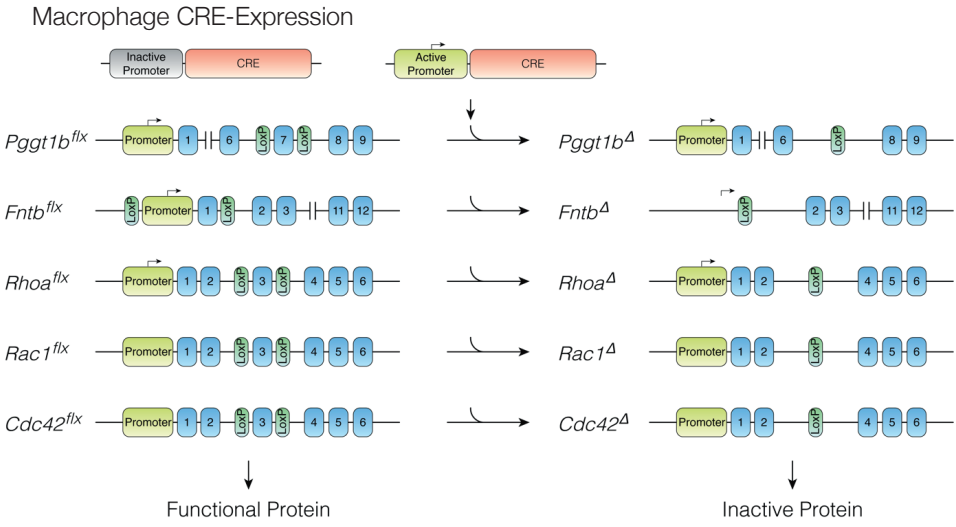

Figure 6. Inactivation of proteins in this thesis using the CRE/LoxP-system.

In paper II, *Cre* expression is under the control of the Lysozyme-M promoter (LysM-Cre). This promoter is active in myelomonocytic cells, including macrophages.

Statin Administration to Mice

In paper I, we studied the effects on heart function of long-term oral administration of statins in mice. Mice were given simvastatin or rosuvastatin supplemented chow with calculated doses of 40 mg/kg/day and 10 mg/kg/day per mouse, respectively. Treatment with statin-supplemented chow was initiated at 3-4 weeks of age until the age of 1 year, at which time the experiments were terminated. Control mice were fed the same chow without the statin supplementation.

The administered dose of simvastatin was based on previously published atherosclerosis studies[137], where commonly used doses of oral administration range from 10 to 100 mg/kg/day[138]. Importantly, these doses do not lower blood lipid concentrations in mice, which suggests that the observed effects of treatment are likely cholesterol-independent[139]. Due to differences in the pharmacokinetics of simvastatin and rosuvastatin, common recommended doses of rosuvastatin are 4 times lower than simvastatin. Thus, the dose of rosuvastatin administered to mice in this study was 10 mg/kg/day. The doses used in this study is high compared to doses used in the clinic; the maximum recommended dose of simvastatin in humans is 80 mg/day, which translated into approximately 1 mg/kg/day (depending on body weight). However, the metabolic rate in mice is much higher, and to achieve the same biological effects drug concentrations need to be increased[140].

Ethical Considerations

All animal experiments presented in this thesis were approved by the Research Animal Ethics Committee in Gothenburg, and great care was taken not to cause unnecessary suffering for the animals. Mice were kept in as good living environments as possible given the experimental procedures, and they were terminated when showing signs of suffering, as described in the humane endpoint included in our experimental ethics.

Sequencing DNA of DCM Patients

In the third paper, an observational study of DCM patients was conducted. The study used material from the Swedish Registry of Dilated Cardiomyopathy, into which patients with idiopathic DCM at all stages of care in cardiology departments of 7 Swedish hospitals were recruited, from May 1997 to August 2006.

The registry classified idiopathic DCM as left ventricular dilation with ejection fraction below 50%. Patients that suffered heart failure for any of the following reasons were excluded: ischemic heart disease, uncontrolled hypertension, significant valvular disease, significant systemic infection, excessive alcohol consumption, insulin-treated diabetes mellitus, endocrine disorders (including pheochromocytoma and acromegaly), systemic diseases, previous cancer treatment (including irradiation), tachycardia-induced cardiomyopathy, or other primary cardiomyopathy.

192 patients from the Swedish DCM registry with available blood samples were randomly selected to be part of our study. 17 were excluded due to familial relations to other participants or because of insufficient DNA quality.

All enrolled patients gave informed consent to participate in the study.

Main Findings

Paper I:

Long-term statin administration inhibits protein geranylgeranylation and causes cardiomyopathy in mice

Chronic statin treatment reduces myocardial function in mice

The positive effects associated with statin treatment in patients diagnosed with atherosclerosis and coronary heart disease are well established, and they have been shown to confer a range of positive side-effects[62]. Improved myocardial function has been proposed as one these pleiotropic effects[100, 101, 102]. However, clinical data is inconclusive[103, 104].

In this paper, we treated healthy wild-type mice with simvastatin and rosuvastatin from the time of weaning up until the age of 12 months. Statins were administered orally in the chow at doses averaging around 40 mkg/kg/day for simvastatin, and 10 mg/kg/day for rosuvastatin. At 12 months, the hearts of simvastatin-treated mice had a 10,5% reduction in ejection fraction and a tendency towards increased lumen size, both in systole and diastole, as compared to controls (Figure 1, A-D). Treated mice also had increased relative heart weight, interstitial fibrosis, and myocardial hypertrophy. No observable change in cholesterol or triglyceride levels suggests that the observed effect is independent on lipids. Furthermore, liver health markers and liver histology demonstrated that the liver was unaffected by simvastatin, suggesting that the given dose was non-toxic for the liver and still damaging to the heart.

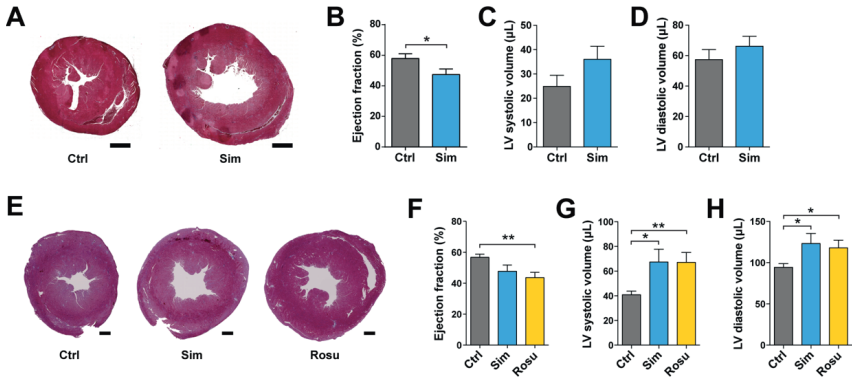

Figure 1. Statin treatment cause mild hypertrophic cardiomyopathy in wild-type mice. (**A**) Representative photomicrographs of Masson's Trichrome-stained horizontal heart cross-sections. (**B-D**) Ultrasound measurements of (**D**) ejection fraction, (**E**) left ventricle systolic volume, and (**F**) left ventricle diastolic volume (n = 8/group). (**E-H**) Second statin experiment. (**F-H**) (n = 8/group). Error bars presented as s.e.m. * $P < 0.05$, ** $P < 0.01$.

Using 40 mg/kg/day is a relatively high dose compared to doses used in the clinic, and to see if the effects of 40 mg/kg/day could be recapitulated at lower doses, we performed the same experiment with 3 and 15 mg/kg/day. Treated mice showed a trend towards reduced ejection fraction and systolic left ventricle volume, and left ventricle diastolic volume was significantly increased. Relative heart weight was also increased in both groups, suggesting that the

cumulative effect of long-term statin treatment, even at lower doses, can be negative for heart function, and that it is not due to toxic side-effects.

Different statins have variable properties, and can therefore have different off-target effects. To test if the observed cardiomyopathy could be reproduced with another statin, we administered rosuvastatin to wild-type mice. Similar to simvastin treated mice, rosuvastatin-treated mice also exhibited reduced myocardial function, with a 23,1% reduced ejection fraction and increased systolic and diastolic left ventricle volume (Figure 1, E-H).

Active RHOA levels are increased in hearts of long-term simvastatin-treated mice

RHOA and RAC1 are known mediators of cardiac hypertrophy[27, 52, 53, 57], and they have been found to be accumulated in their active, GTP-bound, form when not prenylated[73, 74]. We therefore hypothesized that the cardiomyopathy observed after statin treatment could be caused by increased RHOA-GTP levels. Indeed, there was a ~3-fold increase in RHOA-GTP levels in simvastatin-treated hearts (40 mg/kg/day) compared to controls (Figure 2A) with a similar trend observed in lower simvastatin doses.

Treating cardiomyocyte cell lines with simvastatin, rosuvastatin also increased RHOA-GTP levels, and caused a morphology change. Both RHOA-GTP levels and the morphology change were rescued by the addition of GGPP, but not FPP, suggesting that the adverse effects of statin treatment was due to inhibited geranylgeranylation (Figure 2, C and D).

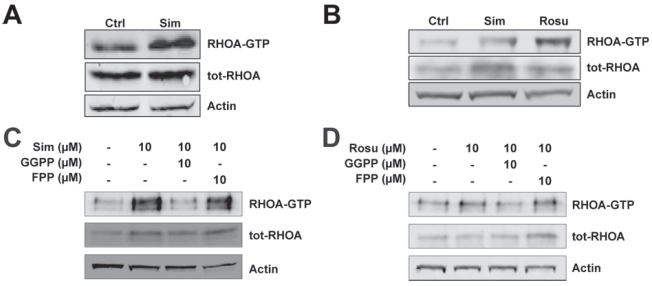

Figure 2. (**A, B**) Western blot of heart lysates showing levels of GTP-bound and total RHOA in (**A**) simvastatin treated mice and (**B**) simvastatin and rosuvastatin treated mice. (**C, D**) Western blots showing levels of GTP-bound and total RHOA in HL-1 cardiomyocytes after 24h (Sim) and 48h (Rosu) treatment. Actin was used as a loading control.

Cardiomyocyte-specific knockout of FTase and GGTase-I cause DCM in mice

We then hypothesized that GGTase-I is essential for normal heart function. To test this, we generated cardiomyocyte-specific *Pggt1b* knockout mice (cGGTko), to inactivate GGTase-I. In addition, to evaluate the effect of complete prenylation deficiency on heart function, we also generated cardiomyocyte-specific knockout of *Fntb* (cFTko), inactivating FTase in mouse hearts.

cGGTko, cFTko, and the double knockout cFTGGTko mice were born at expected Mendelian frequencies. At 3 months, cGGTko and cFTko mice had normal heart morphology, whereas cFTGGTko mice had developed severe dilated cardiomyopathy (Figure 3A), interstitial fibrosis, and cardiomyocyte hypertrophy, with no surviving mice at 4 months of age.

Echocardiography analysis of 7-moth-old cGGTko and cFTko mice showed decreased ejection fraction and increased left ventricle volume in both groups compared to controls (Figure 3, B-D). At 12 months both groups showed signs of DCM and had 40% and 20% mortality rate, respectively (Figure 3E). Hypertrophic muscle fibers were present in both cGGTko and cFTko,

however, only cGGTko mice had increased fibrosis. The gradual progression of reduced contractility and dilation is comparable to that of human DCM.

The cardiomyopathy that developed in cGGTko mice was more severe than in cFTko mice, suggesting that geranylgeranylation has a more important role in heart function.

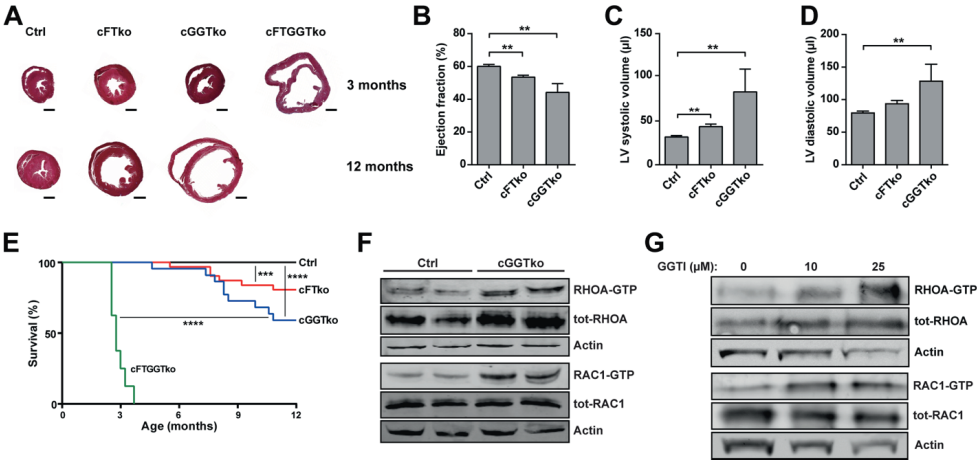

Figure 3. Cardiomyocyte-specific knockout of FTase and GGTase-I causes dilated cardiomyopathy in mice. (**A**) Photomicrographs of Masson's Trichrome stained horizontal heart sections. (**B-D**) Ultrasound measurements of (**B**) ejection fraction, (**C**) left ventricle systolic volume, and (**D**) left ventricle diastolic volume (n = 10-28/group). (**E**) Kaplan-Meier survival curve (n = 8-63/group). (**F**) Western blots of heart lysates showing levels of GTP-bound and total RHOA and RAC1. (**G**) Western blots of HL-1 cell line lysates showing levels of GTP-bound and total RHOA and RAC1 after 24 h GGTI treatment. Actin was used as a loading control. Error bars presented as s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

GGTase-I deficiency hyperactivates RHOA and RAC1 in mouse hearts

Similar to statin-treated mice, RHOA-GTP levels were higher in cGGTko hearts. Furthermore, RAC1-GTP levels were also increased (Figure 3F), which supports previous studies that found that RHOA and RAC1 activities were increased in *Pggt1b* knockout macrophages[73, 74], both of which are known mediators of cardiac hypertrophy. Treating a cardiomyocyte cell line with a GGTase-I inhibitor also increased RHOA and RAC1 activity (Figure 3G). Together, these data show that inhibited geranylgeranylation in the heart hyperactivates RHOA and RAC1, and suggest that the increased RHOA activity in statin-treated mice and cells is due to inhibited geranylgeranylation.

Knockout of RHOA, but not RAC1, improves myocardial function in GGTase-I deficient mice

Increased activity of RHOA and RAC1 has been shown to be important mediators of DCM[27, 53, 57]. We therefore hypothesized that RHOA or RAC1 mediates the DCM in cGGTko mice. To test this hypothesis, we bred cGGTko mice with mice harboring knockout alleles of RHOA or RAC1, to generate conditionally homozygous and heterozygous knockouts of *Rhoa* (cGGTko:*Rhoa*$^{fl/fl}$ and cGGTko:*Rhoa*$^{fl/+}$) and *Rac1* (cGGTko:*Rac1*$^{fl/fl}$ and cGGTko:*Rac1*$^{fl/+}$) in a GGTase-I deficient background.

cGGTko:*Rhoa*$^{fl/+}$ and cGGTko:*Rac1*$^{fl/+}$ mice developed DCM similar to cGGTko mice, which shows that a 50% decrease of RHOA or RAC1 activity is not sufficient to rescue the DCM. In cGGTko:*Rhoa*$^{fl/fl}$ mice, however, heart morphology, cardiomyocyte hypertrophy, and myocardial fibrosis was normalized (Figure 4, A-C), and all mice were alive at the end of the experiment. In contrast, cGGTko:*Rac1*$^{fl/fl}$ mice developed a more severe DCM compared to cGGTko mice. This suggests that DCM mediated by GGTase-I deficiency is in turn mediated by RHOA activity.

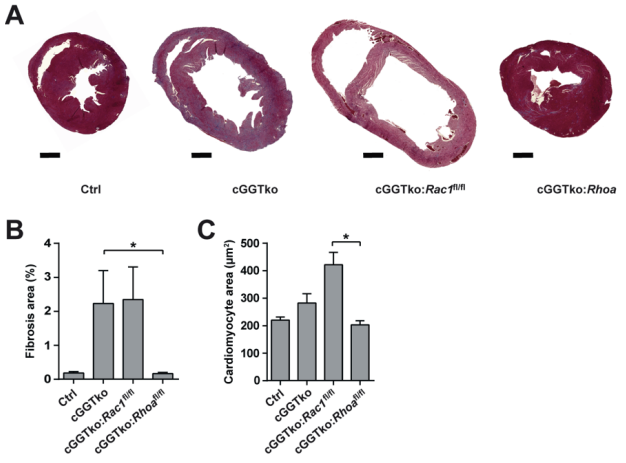

Figure 4. Knockout of RHOA, but not RAC1, prevents cardiomyopathy of cardiomyocyte-specific GGTase-I deficient mice. (**A**) Photomicrographs of Masson's Trichrome stained horizontal heart sections. (**B**) Quantification of fibrosis (n = 3-10/group). (**C**) Quantification of cardiomyocyte cross-sectional area (n = 3-10/group). Error bars presented as s.e.m. * $P < 0.05$.

Paper II:

Protein prenylation restrains innate immunity by inhibiting Rac1 effector interactions

RAC1 mediates erosive arthritis in GGTase-I deficient mice

Previous publications have shown that GGTase-I deficiency in macrophages enables excessive pro-inflammatory signaling and causes erosive arthritis in mouse joints, and, surprisingly, increased RHO-GTPase activity. We therefore hypothesized that RAC1, RHOA, or CDC42 mediate the inflammation caused by GGTase-I deficient mice.

To test this hypothesis, we generated GGTase-I deficient mice (*Pggt1bΔ/Δ*) with macrophage-specific heterozygous knockout of either *Rac1* (*Rac1Δ/+Pggt1bΔ/Δ*), *Rhoa* (*RhoaΔ/+Pggt1bΔ/Δ*), or *Cdc42* (*Cdc42Δ/+Pggt1bΔ/Δ*). Histological analysis showed that one allele knockout of *Rac1* significantly reduced synovitis and erosion in mouse joints at the age of 12 weeks, normalizing the disease phenotype (Figure 1, A-C).

Furthermore, the increased pro-inflammatory signaling in response to LPS stimulation of primary GGTase-I deficient macrophages was markedly reduced in *Rac1Δ/+Pggt1bΔ/Δ* cells. *Rac1* inactivation reduced caspase-I mediated maturation and secretion of the pro-inflammatory cytokine IL-1β by 90-100%, and the secretion levels of the pro-inflammatory cytokines TNF and IL-6 were normalized (Figure 1D). Inactivation of *Rhoa* and *Cdc42* did not have an effect on cytokine secretion.

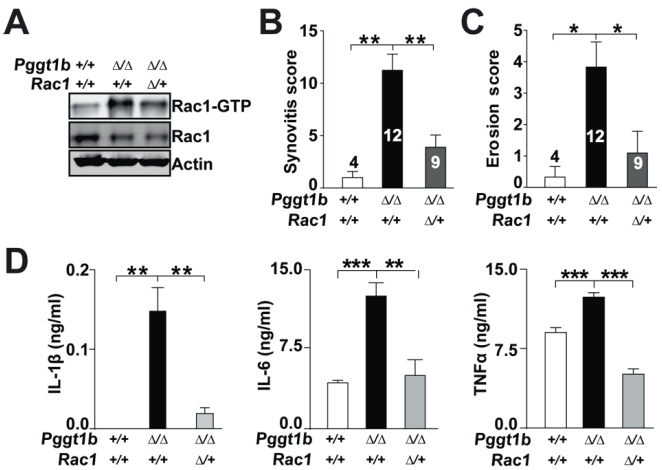

Figure 1. *RAC1* haploinsufficiency rescues arthritis and inflammatory signaling in *Pggt1b*$^{\Delta/\Delta}$ mice. (**A**) Western blots showing steady-state levels of GTP-bound and total RAC1 in primary bone marrow (BM) macrophages isolated from *Pggt1b*$^{\Delta/+}$, *Pggt1b*$^{\Delta/\Delta}$, and littermate *Rac1*$^{\Delta/+}$*Pggt1b*$^{\Delta/\Delta}$ mice. Actin was used as a loading control. (**B, C**) Synovitis and erosion score in joints of 12-week-old mice (*n = 4-9/genotype*). (**D**) Cytokine concentrations, 8 hours after LPS (10 ng/ml) stimulation, in medium of BM macrophages isolated from mice (*n = 3-4/genotype*). Error bars presented as s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

These results suggest that RAC1 mediates the development of rheumatoid arthritis in GGTase-I deficient mice.

IQGAP1 binds non-prenylated RAC1 and mediates erosive arthritis

In *Pggt1b*Δ/Δ macrophages RAC1 accumulated in its active, GTP-bound, form. However, RAC1 total protein levels were reduced (Figure 1A), and gene expression levels were unchanged, which suggests that blocking prenylation of RAC1 encourages the GDP to GTP exchange but also decreases protein stability.

Ubiquitin enrichment of macrophage lysates followed by blotting for RAC1 revealed that non-prenylated RAC1 had increased levels of ligated ubiquitin (Figure 2A), indicating an increased ubiquitin-mediated proteasomal degradation rate which could explain the reduction in total RAC1 levels. This was corroborated by proteasomal inhibitors, which increased total RAC1 levels in macrophages.

To determine the mechanism behind non-prenylated RAC1 accumulation in its GTP-bound form and increased ubiquitination levels, which ultimately lead to excessive inflammatory signaling and arthritis, we performed immunoprecipitation of RAC1 followed by mass-spectrometry. This revealed a list of 717 proteins with significantly changed RAC1 interaction. The protein with the largest interaction change was Ras GTPase-activating-like protein 1 (IQGAP1), which is a protein that is known to stabilize RAC1 in its GTP-bound form. The IQGAP1-RAC1 interaction was increased 2-3 fold in *Pggt1b*Δ/Δ macrophages compared to controls (Figure 2B).

To investigate the role of IQGAP1 in the disease phenotype in GGTase-I deficient mice, we generated *Iqgap1-/-* mice on a *Pggt1b*Δ/Δ background. Knockout of *Iqgap1* reduced synovitis and erosion levels by 60-70% and normalized cytokine secretion by LPS-stimulated macrophages. Importantly, *Iqgap1* knockout restored RAC1-GTP accumulation and ubiquitination, and thereby increased total RAC1 levels to that of controls (Figure 2, C-F).

These results indicate that IQGAP1 is essential for the increased activity of RAC1, and therefore the inflammatory signaling, in *Pggt1b*Δ/Δ macrophages.

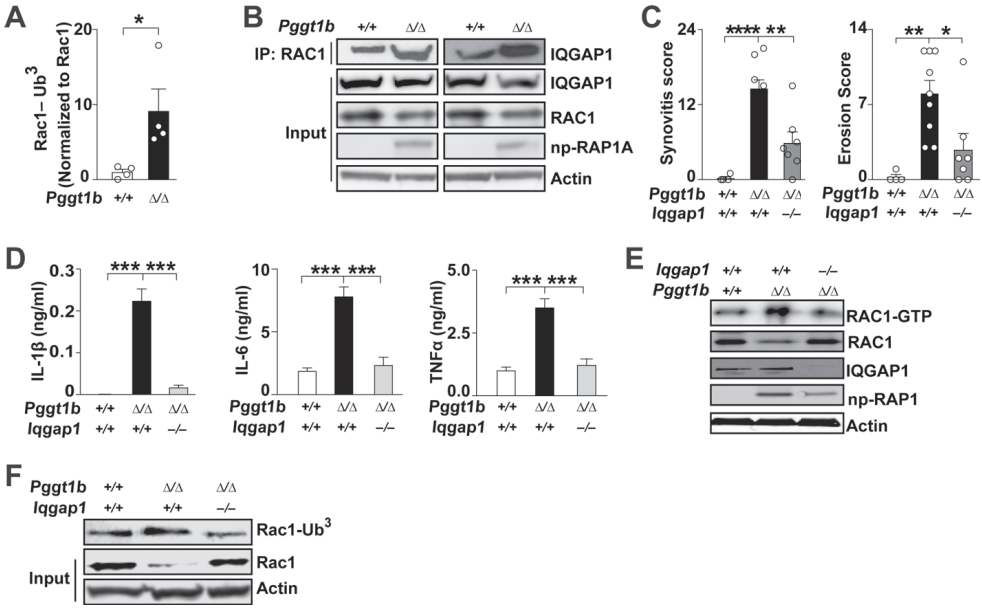

Figure 2. Quantification of Immunoprecipitation (IP) of Ubiquitin (Ub) followed by western blots for RAC1. (*n* = 4/genotype). (**B**) Immunoprecipitation (IP) of RAC1 in BM macrophage lysates followed by western blots for IQGAP1. Direct western blots were performed on the same lysates (Input) to quantify total IQGAP1 and RAC1 levels. Non-prenylated RAP1A was used as marker of GGTase-I-deficient cells; Actin was used as a loading control. (**C**) Synovitis and erosion scores in joints of 12-week-old mice (*n = 4-9/genotype*). (**D**) Cytokine concentrations, 8 hours after LPS stimulation, in medium of BM macrophages isolated mice (*n* = 3/genotype). (**E**) Western blots showing levels of GTP-bound RAC1, total RAC1, and IQGAP1 in macrophages isolated from mice. (**F**) Immunoprecipitation (IP) of Ubiquitin (Ub) followed by western blots for RAC1. Direct western blots were performed on the same lysates (Input) to quantify total levels of RAC1 and the loading control Actin. Error bars represent s.e.m. * P < 0.05, ** P < 0.01, *** P < 0.001.

TIAM1 binds non-prenylated RAC1 and stimulates RAC1 activity

IQGAP1 does not have any GEF of GAP activity and can therefore not stimulate the GDP to GTP exchange of RAC1 itself[36]. We hence hypothesized that an increased interaction with a GEF, or a decreased interaction with a GAP, explains the accumulation of RAC1 in its GTP-bound form. Immunoprecipitation of various known GEFs revealed a 2-6 fold increased interaction between non-prenylated RAC1 and T-cell lymphoma and metastasis 1 (TIAM1) compared to controls (Figure 3A). Inhibiting TIAM1 expression in primary *Pggt1b*Δ/Δ macrophages with siRNAs not only reduced RAC1-GTP levels, but also increased total RAC1 levels and reduced IL-1β production (Figure 3, B and C). We also found that IQGAP1 and TIAM1 interact, and that this interaction is increased in GGTase-I deficient cells (Figure 3D).

We conclude from this evidence that non-prenylated RAC1 accumulates in its GTP-bound form due to increased interaction with TIAM1 and IQGAP1 and that these interactions drive the inflammatory phenotypes of GGTase-I deficient macrophages both *in vivo* and *in vitro*. The finding that RHO GTPases are hyperactivated in a *Pggt1b*Δ/Δ setting also questions the viability of prenylation inhibitors for the treatment of the disease.

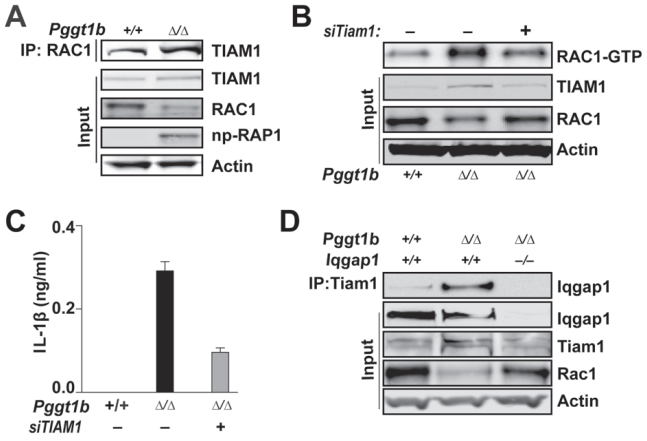

Figure 3. Tiam1 binds non-prenylated Rac1 and supports GTP loading and Il-1β production. (**A**) Immunoprecipitation (IP) of RAC1 in macrophage lysates followed by western blots for TIAM1. Direct western blots were performed on the same lysates (Input) to quantify total TIAM1 and RAC1 levels. Actin was the loading control. (**B**) Western blot showing levels of GTP-bound Rac1 in lysates of BM macrophages pre-incubated for 24 h with scrambled or *Tiam1*-targeted siRNAs. Direct western blots were performed on the same lysates (Input) to quantify total TIAM1 and RAC1 levels. (**C**) Concentration of Il-1β, 8 hours after LPS stimulation, in medium of BM macrophages pre-incubated for 24 h with scrambled or *Tiam1*-targeted siRNAs (n = 2 genotype). (**D**) IP of TIAM1 followed by western blot for IQGAP1. Direct western blots were performed on the same lysates (Input) to quantify total IQGAP1, TIAM1, and Actin levels. Error bars represent s.e.m. $* P < 0.05$.

Statins mimics the effects of GGTase-I deficiency

Since statins have been proposed to reduce prenylation[23, 62] we wanted to investigate whether statin treatment could induce similar cellular phenotypes as GGTase-I deficiency. Treating three different macrophage cell lines with either Atorvastatin, Rosuvastatin, or Simvastatin increased RAC1-GTP levels and reduced total RAC1 levels, just as in GGTase-I deficient cells. Furthermore, statin treatment was accompanied by increased production of IL-1β, IL-6, and TNF, which was normalized in most cases by co-incubating statins with GGPP, verifying that the observed effect was due to inhibited prenylation. Moreover, inhibiting IQGAP1, either by genetic deletion in primary macrophages or by siRNAs, in conjunction with statin treatment also normalized cytokine production, suggesting that statins mimic the effects of GGTase-I deficiency (Figure 4D).

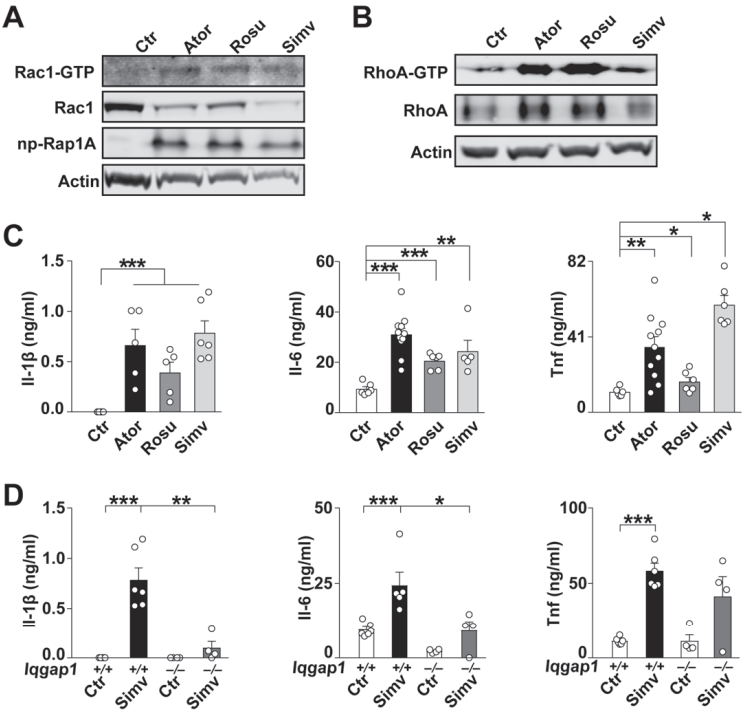

Figure 4. Statins increase Rac1-GTP and cytokine production in a GGPP- and Iqgap1-dependent fashion. (**A**) Western blots showing levels of GTP-bound and total RAC1 in lysates of RAW 264.7 macrophages incubated for 3 weeks with Atorvastatin (5 μM), Rosuvastatin (2.5 μM), and Simvastatin (1 μM). Np-RAP1A was used as a marker of GGTase-I-deficient cells and Actin as a loading control. (**B**) Western blots showing levels of GTP-bound and total RHOA in the same cells as in **A**. (**C**) Il-1β, Il-6, and TNF concentration, 8 hours after LPS treatment, in medium of primary mouse macrophages (n = 3 mice) incubated for 60 h with Atorvastatin (10 μM), Rosuvastatin (10 μM), and Simvastatin (5 μM). (**D**) Cytokine concentration in medium of LPS-stimulated *Iqgap1*$^{+/+}$ and *Iqgap1*$^{-/-}$ macrophages incubated with Simvastatin (5 μM) for 60 h. Error bars represent s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Paper III:

Massive parallel sequencing questions the pathogenic role of missense variants in dilated cardiomyopathy

Genetic variants in DCM patients

Up to 35% of DCM diagnoses are attributed to pathological alterations in the genome[114] and sequencing studies have demonstrated that disease is caused by a complex pattern of mutations. Disease-causing variants are often present in a large number of genes, and are associated with various degrees of DCM penetrance[141]. Further complicating the situation is the fact that, although two or more putative disease-causing variants have been reported in a large subset of patients in a DCM cohort, rare variants have also been reported in healthy individuals[122, 123, 142]. The large number of genetic alterations in healthy individuals complicates the diagnosis of DCM by genetic sequencing, as it is difficult to separate benign from disease-causing mutations, and emphasizes the importance of studying genotype-phenotype correlations.

To investigate the range of genetic variants in DCM, we sequenced 41 genes that have previously had putative disease-causing variants reported, for germ-line variants in a Swedish idiopathic DCM cohort of 176 patients. Variants to be included in the analysis had to meet two criteria: first, they had to be protein-altering, and two, they had to have an allele frequency of less than 0,04% in two different reference populations (1000 Genomes and ESP6500). In 79 of the 179 patients, 102 different variants were found. These included 79 missense variants, 12 stopgain variants, 7 frameshift indels, 3 in-frame indels, and 1 splicing variant. Eighteen of these variants had been described before and 84 were novel findings.

Rare protein-altering variants are associated with poor DCM prognosis

Harboring at least one variant correlated with disease debut at a significantly lower age (Figure 1A). Patients with at least one variant also had decreased transplant-free survival (Figure 1B), overall survival, and ejection fraction at diagnosis. The data also suggests that the development of DCM in this group was more aggressive; transplant-free survival calculated from time of symptom debut was also reduced. A total of 17 patients (10%) had two variants, 6 (3%) had 3 variants, and 1 (0,6%) had 4 variants, however, the prognoses of these patients were similar to those that only had one variant.

To test whether the trend in transplant-free survival was mainly driven by mutations in a subset of the sequenced genes, a stepwise model simplification approach was employed. The model described transplant-free survival and included 13 genes used as individual additive explanatory variables. Iterating to remove the least significant gene in each step, to find the strongest correlation explaining the transplant-free survival, found that the strongest model was explained by three genes: *LMNA*, *MYH7*, and *TTN* (Figure 1C). This suggests that variants in these three genes are associated with a more aggressive DCM, with variants in *LMNA* causing the worst prognoses.

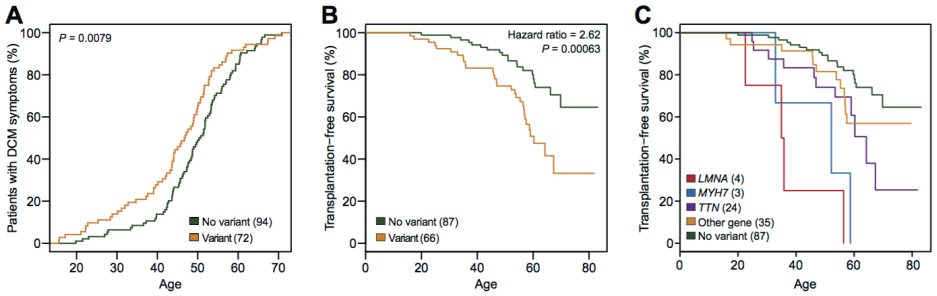

Figure 1. Detection of genetic variants is associated with more severe DCM. (**A**) Cumulative distribution of age at first symptom leading to a DCM diagnosis, for patients with a candidate variant compared to those without. (**B–C**) Kaplan–Meier plot showing transplant-free survival for patients with any candidate variant (**B**) or candidate variants in selected genes (**C**), compared to patients without such variants. P (LMNA vs no variant) = 6×10^{-9}; P (MYH7 vs no variant) = 9×10^{-5}, P (TTN vs no variant) = 0.01; P (other gene vs no variant) = 0.09; Log-rank test.

Frequency of missense variants in DCM-related genes is similar in healthy reference cohorts and DCM patients

Next, we compared the frequency of rare protein-altering variants in our DCM cohort to a reference cohort of 503 healthy individuals (European ancestry cohort of the 1000 Genomes project, 1000G Europeans). As expected, the number of truncating variants was significantly higher in DCM patients, and the number and distribution of synonymous variants were similar in the two groups, suggesting that the two groups are comparable. However, the prevalence of non-synonymous missense variants was similar in DCM patients and in the reference cohort (Figure 2A). An extended analysis comparing the allele frequency of these variants in our DCM cohort to the general population, as estimated by the ExAC database, revealed that the frequency of missense variants was not higher in DCM patients (Figure 2B).

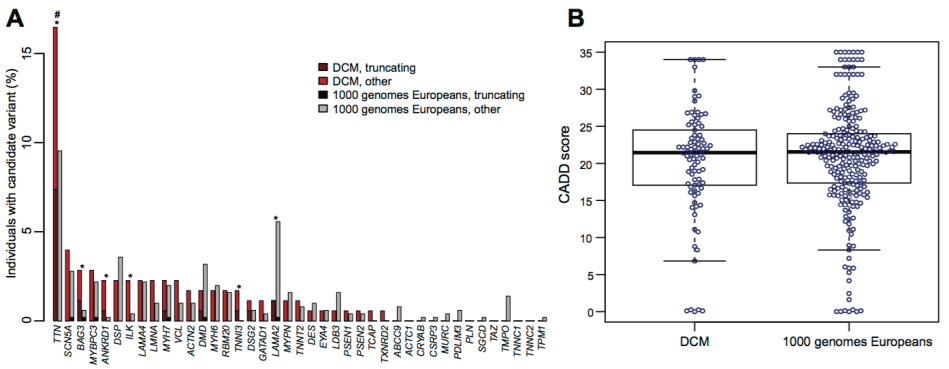

Figure 2. The prevalence and predicted severity of missense variants are similar in DCM patients and healthy reference individuals. (**A**) Percentage of DCM patients and healthy reference individuals (1000G Europeans) harboring rare truncating variants (stopgain or frameshift) or other rare protein-altering variants (missense, splice site and in-frame indels) in DCM-associated genes. Fisher's exact test was used to test for differences in variant prevalence: *P = 0.01–0.05, false discovery rate-adjusted P not significant (counting all rare protein-altering variants). #P = 2×10^{-8}, false discovery rate-adjusted P = 7×10^{-7} (truncating variants only). (**B**) CADD score distribution for rare missense variants in DCM patients and healthy ref- erence individuals. P = 0.50, t-test.

Missense variant pathogenicity scores and distributions were similar in DCM patients and healthy reference cohorts

It is possible that, while variant frequency in DCM patients was similar to healthy cohorts, missense variants observed in DCM patients have more damaging effects on the translated proteins. To test this, we compared computationally predicted pathogenicity scores of the two datasets. Surprisingly, the pathogenicity scores of the detected rare variants in our DCM cohort had a very similar distribution to that of rare variants in the 1000G Europeans dataset, as predicted by four different methods.

We now hypothesized that, while the pathogenicity scores of the detected missense variants were similar to those found in reference cohorts, the locations of the mutations might be different, and may therefore have a more damaging impact. We therefore compared the location of our detected variants, in the genes with highest variant prevalence, and other previously reported DCM-associated variants (in the same genes) and cohorts to the 1000 Genomes reference cohort. We found that the variant location distributions across the genes were similar in DCM and healthy reference cohorts, with the exception of the *LMNA* gene, where variants were detected mainly in the intermediate filament region in DCM cohorts, which was not the case in healthy cohorts (Figure 3).

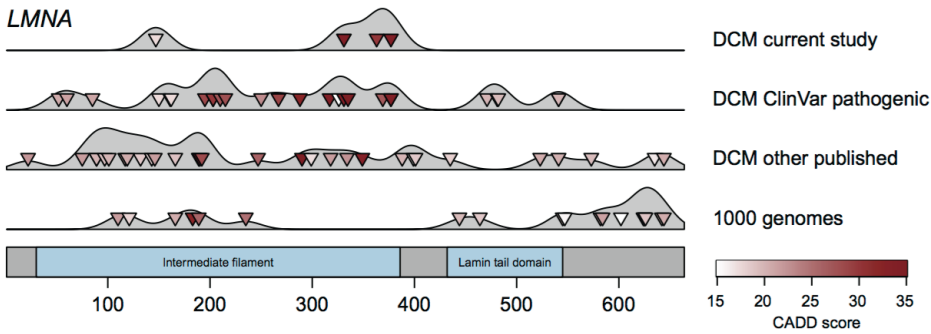

Figure 3. Missense variants in the intermediate filament region of LMNA are associated with DCM. Triangles show locations of missense variants along the LMNA protein sequence (Ensembl ID ENSP00000357283, 664 amino acids) and grey curves indicate variant density. Triangle colour indicates CADD pathogenicity score. Protein domains from the Pfam database are indicated in blue. Note that this figure shows all missense variants irrespective of allele frequency, in contrast to the other analyses in this study, where only rare variants were considered.

These findings further demonstrate the complex genetic nature of DCM and the resulting difficulty in separating benign from disease-causing variants, and question the importance of missense variants in the development of the disease.

Discussion and Future Perspectives

Paper I

In paper I, we show that long-term statin administration causes cardiomyopathy in mice, and question the hypothesis that statins improve disease symptoms in patients diagnosed with hypertrophic and dilated cardiomyopathy. The data suggests that the statin-induced cardiomyopathy is mediated by increased RHOA activity, a known mediator of cardiac hypertrophy, caused by reduced geranylgeranylation in the heart. These are valuable findings in light of the increasing indications for statin treatment, and the increasing use of statins in clinics, even for patients at lower risk of developing cardiovascular disease.

It is still well documented, however, that statins confer benefits in reducing the risk of myocardial infarction and stroke, and improve overall survival in patients with increased cholesterol levels and atherosclerosis. In this paper, we focus primarily on heart function; the blood lipid levels in the mice are normal, and they are otherwise healthy. Thus, a possible explanation for the discrepancy between our findings and clinical studies may be that the potentially negative direct effect on heart function, as found in our mice, is counteracted by the beneficial effects in humans at risk of developing atherosclerosis. For example, statins are known to reduce inflammation and increase vascular function in treated patients, which have positive effects on heart function. There are studies, however, that have demonstrated unchanged or even reduced myocardial function after statin treatment. Two large randomized controlled trials of statins in ischemic heart disease patients showed no demonstrable benefit on heart function, and the explanation may lie in the counteraction of reduced myocardial function to the positive effects of reduced cholesterol levels. A small study using strain imaging to evaluate heart function in cholesterol-matched patients has even shown a negative effect. However, while our data suggests some caution in the use of statins this is still a pre-clinical study, and the data should be considered carefully.

In this paper, we also show that GGTase-I and FTase deficiency cause dilated cardiomyopathy in mice, with GGTase-I deficiency exhibiting a more severe form, which is likely explained by the fact that GGTase-I has many more substrates than FTase, thus having a greater impact. In these experiments we also found increased RHO protein activity, and both RHOA and RAC1 accumulated in their active form when non-prenylated. This is contrary to the current *CAAX* protein dogma, and it could be argued that while GTP-loading is increased, RHO proteins are mislocalized and are unable to signal properly. However, knocking out RHOA clearly rescued the cardiomyopathy, demonstrating that RHOA was still able to signal downstream and cause disease.

The data suggests that inhibiting prenylation in general, and geranylgeranylation in particular, is detrimental to normal heart function, which should be considered in the use and development of prenylation inhibitors. And the hyperactivation of RHO proteins highlights the developing concept that prenylation may restrain RHO GTPase activity.

This paper also highlights the need for long-term studies when studying heart function in mice. Simvastatin, cGGTko, and cFTko all exhibited slow-onset cardiomyopathy, with normal heart function at 3-4 months of age and clear signs of disease at 12 months. Cardiomyopathy normally starts as a compensatory mechanism to an abnormal stress that becomes pathological, which is not a quick process.

Paper II

In paper II, we provide a mechanistic explanation for the development of rheumatoid arthritis in GGTase-I deficient macrophages in mice, and challenge the dogma that protein prenylation inhibits RHO GTPase signaling. Our data show that inhibiting prenylation in macrophages

increases the affinity of RAC1 to TIAM1 and IQGAP1, leading to an accumulation in its active, GTP-bound, form, triggering excessive pro-inflammatory signaling and cytokine production, and thus ultimately causing severe rheumatoid arthritis in mice.

GGTase-I has at least 60 substrates for prenylation, among them RAC1, RHOA, and CDC42, all three of which had increased GTP-bound levels in GGTase-I deficient macrophages. However, it only required the inactivation of one RAC1 allele to normalize the pro-inflammatory cytokine production in macrophages. This suggests that RAC1 is the primary mediator of inflammatory signaling in GGTase-I deficient macrophages.

Protein prenylation is thought to be essential for proper membrane localization and effector interactions of RHO GTPases, enabling them to become activated and propagate cellular signaling. In contrast, our results suggest that membrane localization of RAC1 is not affected by prenylation; the membrane/cytosol ratio of RAC1 in HEK cells harboring a mutated, non-prenylatable *CAAX* box, is unchanged. This also supports a previous discovery that found the membrane/cytosol ratio unchanged in GGTase-I deficient macrophages.

Not only is RAC1 localization unchanged when non-prenylated, it also accumulates in its GTP-bound form, and is able to signal downstream. The accumulation of RAC1 in its GTP-bound form is most likely explained by its increased interaction with TIAM1 and IQGAP1. siRNA against *Tiam1* restored RAC1-GTP levels and reduced cytokine production. The scaffolding protein IQGAP1, that has been reported to stabilize RAC1 in its GTP-bound form, had an increased association with non-prenylated RAC1, and inactivating it restored cytokine production and RAC1-GTP levels in cells, as well as reduced synovitis and erosion scores in mouse joints. IQGAP1 inactivation also restored GTP-levels of RHOA and CDC42 demonstrating its importance for RHO protein regulation in general.

Interestingly, while RAC1-GTP levels were increased in GGTase-I deficient macrophages, total RAC1 levels were reduced. First of all, this means that the RAC1 activity increase is not a general increase in the amount of RAC1 levels, and also that non-prenylated RAC1 more is unstable. We found that the reduction in RAC1 levels was caused by increased proteasomal degradation mediated by the increased association with IQGAP1. Inactivation of IQGAP1 also showed that it differentially regulates the RHO proteins, while the reduced RAC1 levels were restored, so were total RHOA and CDC42 levels, which were increased in GGTase-I deficient macrophages.

These findings lead us to conclude that IQGAP1 preferentially binds to non-prenylated RAC1, exposing it to ubiquitin-mediated proteasomal degradation, and makes it available for TIAM1-catalyzed GDP to GTP exchange, and stabilizes it in its GTP-bound form. IQGAP1 is therefore central to the pro-inflammatory cytokine production in GGTase-I deficient macrophages and rheumatoid arthritis in GGTase-I deficient mice. This also raises the possibility that targeting IQGAP1 in the treatment of auto-inflammatory disorders might be a viable strategy instead of targeting RAC1, as our study demonstrated that IQGAP1 deletion is not detrimental to mouse development, but RAC1 is. This is especially interesting in diseases such as mevalonate kinase deficiency, where reduced prenylation has been reported.

Our findings also provide new insight into the role of RAC1 and prenylation in innate immunity. RAC1 signaled through several pathways to elicit the inflammatory phenotype of GGTase-I deficiency. First, it increased ROS levels and p38 phosphorylation, triggering the transcription of IL-6, TNF, MMP13, which are previously established signaling events. Secondly, it activated caspase-I, which cleaves the progenitor of IL-1b, pro-IL-1b, enabling the secretion of mature IL-1b. This implies that RAC1 acts as a second signal for IL-1b maturation and secretion, initiating maturation after transcription triggered by extracellular signaling. Prenylation seems to restrain this process, as blocking it triggers the release of pro-inflammatory signaling.

Furthermore, statin treatment demonstrated similar effects to that of GGTase-I deficient macrophages. Statins work as dual prenylation inhibitors and in response to LPS stimulation,

macrophages exhibited increased RAC1-GTP levels and induced pro-inflammatory cytokine production. This is an interesting finding, since statins are normally attributed anti-inflammatory properties. The explanation for this may lay in the fact that other immune cells are affected by statin treatment in the body, for example T-cells.

Finally, in this paper we challenge the idea that prenylation is required for RHO protein signaling, and conclude that geranylgeranylation restrains RAC1 activation and pro-inflammatory signaling and the resulting immune response.

Paper III

In paper III, we show that rare protein-altering variants in genes associated with DCM correlate with early onset of disease and worse prognosis. However, we demonstrate that the prevalence and distribution of missense variants in DCM patients is similar in healthy individuals, confounding the interpretation of genetic testing in the clinic.

This study analyzes exons in a large number of DCM-associated genes, instead of already known variants, in pre-selected cohorts of DCM patients and healthy individuals. This offers the opportunity for a more unbiased correlation between genotype and phenotype in comparison to most other studies, which compare patients with known disease-causing variants in familial DCM to patients with idiopathic DCM.

We employed several different algorithms to evaluate the pathogenicity of detected mutations. These algorithms predict the impact of the detected mutation on the protein product (if it is predicted to be transcribed) and generate a pathogenicity score with the support of databases. These variants are then evaluated based on their prevalence in reference populations, where low variant prevalence is generally considered to indicate high pathogenicity. Most studies focus on detected variants only in the analyzed DCM cohorts and do not consider variants that may be found in healthy individuals, thus risking overestimating their role in disease development. Further, recent sequencing studies have shown that many rare protein-altering variants are, as can be expected due to normal genetic variation, present in healthy reference populations. Our study shows that the prevalence of rare missense variants is similar in DCM patients and reference cohorts. Furthermore, the location of a variant within a gene affects the impact that it has on the protein function, and we found that distribution of missense variants within genes were similar in DCM and healthy individuals, with the exception of *LMNA*. This has implications for interpreting sequencing results, as most of the analyzed missense variants in the healthy reference cohort likely do not cause DCM.

These findings also question the usefulness of variant pathogenicity predicting algorithms in DCM sequencing studies. The pathogenicity scores of missense variants in DCM patients and healthy individuals, according to the tools used, were similar in our study. And although these tools are constantly improving as new data is acquired, our study may warrant caution in interpreting their results.

Variants in the *LMNA* gene correlated with the worst prognosis in our cohort of DCM patients. Furthermore, we show that in a region of the intermediate filament region of *LMNA*, missense variants are only present in DCM cohorts, and not in the 1000 Genomes reference cohort. This information might be helpful in interpreting sequencing results in clinics.

Interestingly, despite the prevalence, location, and pathogenicity score similarities between DCM patients and reference individuals, the detected missense variants still correlated with earlier onset disease and reduced survival. This may be explained by the fact that in the group of DCM patients with missense variants, variants in some genes (such as *LMNA*) may be causative and confer a worse prognosis and therefore contribute to the negative correlation, whereas most other variants are incidental findings and have no effect on disease progression. Taken together, the

data presented in this study further demonstrates the complex genetic nature of DCM and the difficulties in interpreting sequencing results, and to overcome these difficulties, further experimental studies will be of great importance.

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Christian, hustomte #1

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