ANNA OKSANEN

Proprotein Convertase Enzymes FURIN and PCSK7 in Immune Regulation

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ACADEMIC DISSERTATION
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"I need to write down my observations.
Even the tiniest ones;
they are the most important."

-Tove Jansson (1914-2001)

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LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original publications, which are referenced in the text by their Roman numerals (I-III):

I **Oksanen A**, Aittomäki S, Jankovic D, Ortutay Z, Pulkkinen K, Hämäläinen S, Rokka A, Corthals GL, Watford WT, Junttila I, O'Shea JJ, Pesu M. Proprotein Convertase FURIN Constrains Th2 Differentiation and Is Critical for Host Resistance against Toxoplasma gondii. *J Immunol* 2014;193(11)5470-9.

II Turpeinen H*, **Oksanen A*,** Kivinen V, Kukkurainen S, Uusimäki A, Rämet M, Parikka M, Hytönen VP, Nykter M, Pesu M. Proprotein Convertase Subtilisin/Kexin Type 7 (PCSK7) Is Essential for the Zebrafish Development and Bioavailability of Transforming Growth Factor β1a (TGFβ1a). *J Biol Chem* 2013;288(51)36610-23.

III Turpeinen H, Raitoharju E, **Oksanen A**, Oksala N, Levula M, Lyytikäinen LP, Järvinen O, Creemers JW, Kähönen M, Laaksonen R, Pelto-Huikko M, Lehtimäki T, Pesu M. Proprotein convertases in human atherosclerotic plaques: the overexpression of FURIN and its substrate cytokines BAFF and APRIL. *Atherosclerosis* 2011;219(2)799-806.

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ABBREVIATIONS

ADAM	a disintegrin and metalloproteinase
APC	antigen presenting cell
APRIL	tumor necrosis factor ligand superfamily member 13
BACH2	BTB and CNC homology 2
BAFF	tumor necrosis factor ligand superfamily member 13B
BCR	B cell receptor
BMP	bone morphogenetic protein
CD	cluster of differentiation
CHD	coronary heart disease
CD4cre-fur$^{f/f}$	T cell–specific FURIN conditional knockout
CRISPR	clustered regularly interspaced short palindromic repeats
DC	dendritic cell
DOCK2	dedicator of cytokinesis 2
dpf	day post-fertilization
ECM	extracellular matrix
ef1α	elongation factor 1α
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FACS	flow cytometry
FBS	fetal bovine serum
FoxP3	forkhead box P3
fur	fes/fps upstream region
GATA-3	GATA binding protein 3
GDF	growth differentiation factor
GWEA	genome-wide expression array
HBV	hepatitis B virus
HIV	human immunodeficiency virus
hpf	hours post-fertilization
HR	homologues recombination
HRP	horseradish peroxidase
hu	human

Ig	immunoglobulin
IFN	interferon
IL	interleukin
iTreg	inducible T regulatory cell
LAP	latency associated peptide
LDL	low-density lipoprotein
LDL-C	low-density lipoprotein cholesterol
LDLR	low density lipoprotein receptor
LITA	left internal thoracic artery
LPS	lipopolysaccharide
MHC	major histocompatibility molecule
MMP	matrix metalloproteinase
MO	morpholino
MBTPS1	membrane-bound transcription factor peptidase site 1
MS	mass spectrometry
mut	mutant
NHEJ	non-homologues end-joining
NK cell	natural killer cell
nTreg	natural T regulatory cell
OVA	ovalbumin
PACS-1	phosphofurin acidic cluster sorting protein 1
PAMP	pathogen-associated molecular pattern
PCSK	proprotein convertase subtilisin/kexin
pERK	phosphorylated extracellular signal-regulated kinase
PRR	pattern recognition receptor
Q-RT-PCR	quantitative real-time PCR
RA	rheumatoid arthritis
RAC	Ras-related C3 botulinum toxin substrate
RC	random control
RORɣt	RAR-related orphan receptor gamma
SDF-1α	stromal cell–derived factor 1α
SNP	single nucleotide polymorphism
STAg	soluble *Toxoplasma gondii* antigen
STAT	signal transducer and activator of transcription
T-bet	T-box expressed in T cells
TCR	T cell receptor
Tfh	follicular T cell
TGFβ	transforming growth factor β

TGN	trans-Golgi network
T. gondii	*Toxoplasma gondii*
Th	T helper cell
TLR	Toll-like receptor
TNF	tumor necrosis factor
Treg	regulatory T cell
VSMC	vascular smooth muscle cell
wt	wild type
zf	zebra fish

ABSTRACT

Our immune system is challenged daily by harmful pathogens and misbehaving cells of our own tissues. A powerful immune response is essential for overcoming these challenges. However, an uncontrolled reaction might expose the host to immune system disorders, including autoimmunity and allergies. Diseases such as rheumatoid arthritis, type 1 diabetes, and asthma are an increasingly heavy burden both for patients and society, and novel therapeutic and diagnostic interventions for preventing and treating immune system diseases are thus required. The regulation of the immune system is mediated by various cell types, secreted molecules and direct cell-to-cell contacts. Importantly, many biomolecules are initially synthesized as inactive proproteins, which then undergo a proteolytic maturation step. Proprotein convertase subtilisin/kexin (PCSK) enzymes catalyze the endoproteolytic activation of a variety of proproteins, including receptors, cytokines, and pathogenic agents. Although PCSKs have a critical role in cellular homeostasis, their immunoregulatory functions are still incompletely understood.

This study investigated the role of PCSKs in immune regulation by using various methods and model organisms. The first-characterized and most studied PCSK member FURIN was shown to be critical for the correct polarization of T helper cells as well as for the cell-mediated immune response against a prototypic intracellular pathogen, *Toxoplasma gondii* in a mouse model. Further, T cell specific FURIN interacting proteins were captured from human T cells resulting in the identification of the RAC/DOCK2 interaction complex, which links FURIN to the regulation of cytoskeleton dynamics in T cells. In addition, the role of the evolutionarily most ancient PCSK enzyme, PCSK7, was studied during vertebrate development and in the regulation of gene expression using a zebra fish model. Interestingly, contrary to its mammalian counterpart, the zebra fish PCSK7 was found to be essential for embryogenesis and for the expression of genes coding proteins of immune system. This study also demonstrated that both the human and zebra fish PCSK7s are capable of proteolytically processing pro-TGFβ1, which might indicate a sophisticated spatiotemporal regulation of the cytokine's bioavailability. Furthermore, a systematic approach was used to analyze the expression of PCSKs in human atherosclerotic vascular samples. FURIN was found

to be the major upregulated PCSK in the immune cells of atherosclerotic plaques, where its substrate cytokines BAFF and APRIL were also overexpressed. In addition, this study demonstrated, for the first time, that the expression of FURIN is induced also in macrophages in response to immunoactivation. This suggests that FURIN plays a regulatory role also in innate immunity.

Collectively, this study shows that PCSKs mediate both unique and redundant, and in most cases critical roles, in regulating the immune system. The utilization of two vertebrate model organisms and human patient samples demonstrated that PCSK mediated immunoregulatory functions are evolutionary conserved, but a deeper understanding of their cell-type specific spatiotemporal regulation is essential. This study provides new insights for the therapeutic targeting of PCSKs and their substrates suggesting novel ways to treat or diagnose immunological diseases.

TIIVISTELMÄ

Haitalliset taudinaiheuttajat ja elimistön omat epätarkoituksenmukaisesti käyttäytyvät solut haastavat immuunijärjestelmämme päivittäin. Voimakas immuunivaste on välttämätön näiden uhkien voittamiseksi, mutta toisaalta kontrolloimattomana se saattaa altistaa isäntänsä autoimmuunireaktioille tai muille immuunivasteen häiriötiloille. Esimerkiksi nivelreuma, tyypin 1 diabetes ja astma kuormittavat sekä kasvavaa potilasjoukkoa että yhteiskuntaa, minkä takia uusia diagnostisia ja terapeuttisia kohteita on välttämätöntä tutkia. Immuunijärjestelmää säädellään erilaisten solutyyppien, eritettävien sytokiinien ja suorien solukontaktien avulla. Monet biomolekyylit tuotetaan aluksi toimimattomina proproteiineina, jotka sitten aktivoidaan proteolyyttisesti. Proproteiinikonvertaasi subtilisiini/keksiini (PCSK) entsyymit katalysoivat endoproteolyyttisesti erilaisten proproteiinien, kuten reseptorien, sytokiinien ja patogeenisten proteiinien aktivoitumista. PCSK-entsyymeillä on tärkeä tehtävä solujen homeostaasissa, mutta niiden immuunivastetta säätelevät tehtävät tunnetaan vielä puutteellisesti.

Tässä väitöskirjassa tutkittiin PCSK-entsyymien toimintaa immuunivasteen säätelyssä erilaisia menetelmiä ja malliorganismeja hyödyntäen. Tutkimus osoitti, että ensimmäiseksi löydetty ja eniten tutkittu PCSK-entsyymi FURIN on hiirimallissa välttämätön auttaja-T-solujen erilaistumiselle sekä soluvälitteiselle immuunivasteelle solunsisäistä taudinaihettajaa, *Toxoplasma gondii* -parasiittia vastaan. Lisäksi työssä tarkasteltiin FURIN-vuorovaikutuksia ihmisen T-soluissa ja identifioitiin uusi RAC/DOCK2-vuorovaikutuskompleksi, joka yhdistää FURIN-entsyymin solutukirangan säätelyyn. Toisessa osatyössä tutkittiin evolutionaarisesti kaikkein vanhimman PCSK7-entsyymin toimintaa seeprakalan kehityksessä ja geenien säätelyssä. Päinvastoin kuin nisäkkäillä, seeprakaloilla PCSK7:n osoitettiin olevan välttämätön alkionkehitykselle ja immuunivasteen geenien ilmentymiselle. Työssä havaittiin lisäksi, että sekä ihmisen että seeprakalan PCSK7-entsyymit proteolyyttisesti prosessoivat TGFβ1-sytokiinin esimuotoa, mikä saattaa viitata tämän sytokiinin ajasta ja paikasta riippuvaan säätelytapaan. Kolmannessa osatyössä analysoitiin systemaattisesti PCSK-geenien ilmentymistä ihmisen ateroskleroottisissa kudosnäytteissä. Tutkimuksessa havaittiin, että FURIN ja sen substraattimolekyylit BAFF ja APRIL ovat yli-ilmentyneet ateroskleroottisen plakin immuunisoluissa.

Lisäksi osoitettiin tiettävästi ensimmäistä kertaa, että FURIN indusoituu makrofageissa vasteena immuuniaktivaatiolle, mikä viittaa mahdolliseen FURIN-välitteiseen säätelyyn myös synnynnäisessä immuunivasteessa.

Tämä väitöstutkimus osoitti, että PCSK-entsyymit ovat välttämättömiä immuunivasteen säätelyssä sekä ainutlaatuisten että päällekkäisten toimintojensa kautta. Kahden eläinmallin ja potilasaineiston avulla todettiin PCSK-välitteisen immuunisäätelyn olevan evolutionaarisesti konservoitunutta, joskin tarkempia tutkimuksia erilaisille solutyypeille ominaisista säätelytavoista tarvitaan. Tästä tutkimuksesta saadun uuden tiedon pohjalta voidaan jatkaa PCSK-entsyymeihin ja niiden substraattimolekyyleihin kohdentuvaa lääke- ja diagnostiikkakehittelyä erityisesti immunologisissa taudeissa.

1 INTRODUCTION

The immune system is a complex network of specialized cell types, tissues, and organs that all work together to protect us against various threats. An appropriate immune reaction is needed both for the elimination of harmful pathogens including bacteria, viruses, and multicellular parasites, and for protection against misbehaving malignant cells. The two branches of the immune system regulate each other in order to facilitate an appropriate response and timing. The innate immunity offers a fast but non-specific response, whereas the adaptive immunity can supplement this by mediating the highly specific, powerful and long-lasting clearance of pathogens. The aberrant activation or inappropriate behavior of any of these factors can result in immune-mediated pathologies such as immunodeficiency, autoimmunity, or allergy. The strict regulation of all immune cells is essential for avoiding these disorders, which constitute also a high burden for patients and the health care system.

Our knowledge of the mechanisms of immune regulation is still elusive, regardless of the active research into the topic. On the molecular level, immune cells are regulated through various mechanisms including the proteolytic maturation of physiologically important proteins such as enzymes, cytokines, and receptors. The discovery of the proteolytic activation of pro-insulin in 1967 opened up a new field of research, and decades later the proprotein convertase subtilisin/kexin (PCSK) enzyme family was discovered. The first mammalian PCSKs were identified 25 years ago, and ever since these enzymes have been shown to mediate essential functions in cellular homeostasis as well as in human health and disease.

Most of the previous studies on PCSKs have, however, been performed using *in vitro* assays and recombinant proteins, which have resulted in our current knowledge of the structural and biochemical properties of these enzymes. In order to understand the biological significance of and the redundancy among the PCSKs, we need versatile model systems and *in vivo* studies to answer remaining questions. In addition, the function of the PCSKs in immune cells and host responses have been poorly defined so far.

This study was aimed at exploring PCSK-mediated immune regulation using two model organisms and human patient samples. Elucidation of the molecular mechanisms behind the functions of PCSKs especially in the immune system would

improve our knowledge on the development of normal immune responses as well as pathologies. Due to the high interest in diagnostic and therapeutic interventions for targeting PCSKs, more information about their function in health and diseases is greatly needed.

2 REVIEW OF THE LITERATURE

2.1 The immune system

Humans and other organisms are exposed daily to various intra- and extracellular threats including pathogens, toxic substances and misbehaving cancerous cells. The protective immune system must recognize and distinguish various pathogens from each other, and importantly from the tissues of the organism. The front line of the immune defense is provided by the innate immunity, followed by the slower but more specific adaptive immunity (Murphy, 2011). The innate immunity is evolutionarily highly conserved and found also in plants and invertebrates, whereas the adaptive response arose much later in jawed fish (Flajnik & Kasahara, 2009). The two branches of immunity must co-operate closely to achieve appropriate responses, which makes strict regulation a key characteristic of this complex system (Iwasaki & Medzhitov, 2010).

The innate immunity provides fast but non-specific response against invading agents. In addition to specific cell types, innate mechanisms include physical and chemical barriers at the sites of pathogen entry including the skin, the gastrointestinal and respiratory tracts, and the eyes (Elias, 2007). The innate response also activates the complement cascade, which helps to identify and clear pathogens (Gasque, 2004). In general, macrophages, neutrophils, and dendritic cells (DCs) are specialized in conducting phagocytosis on pathogens and dead cells, whereas basophils, eosinophils, and mast cells serve through releasing mediators of inflammation such as cytotoxic proteins and enzymes that also play a crucial role in allergies. Natural killer (NK) cells are specialized in killing compromised host cells, including tumor cells and virus infected cells (Kvell et al., 2007; Murphy, 2011). The most recently characterized cell types of the innate immunity are innate lymphoid cells, which can mediate either cytotoxic or stimulatory effects on other cell types in order to maintain tissue homeostasis (Artis & Spits, 2015).

In innate immunity the recognition of pathogens is mediated through specific pattern recognition receptors (PRRs) presented on the surface or as part of intracellular compartments of an immune cell. The PRRs recognize common pathogenic patterns, which are found in various bacteria, viruses, and parasites, but

which are not present in the cells of the host organism. These characteristics are referred to as pathogen-associated molecular patterns (PAMPs) (Akira et al., 2006). A subgroup of PRRs, the Toll-like receptors (TLRs), participates in specific receptor-ligand pairs to mediate intracellular signaling. For instance TLR-4 is activated through a lipopolysaccharide (LPS) stimulus, whereas TLR-7 recognizes single-stranded RNA (Miller et al., 2005). Collectively, the innate immune response is fast and efficient but it lacks specific and sustained features.

Adaptive immunity, also known as acquired immunity, has evolved to provide specific and even lifelong protection. Innate immune cells can destroy many pathogens and hinder others, but fine-tuned and long-lasting adaptive responses are often needed to finally resolve the infection (Murphy, 2011). Adaptive immune cells can also proliferate into memory cells, which provide a fast and powerful response if a particular pathogen is encountered again in the future. This immunological memory is also utilized when the immune protection is acquired through vaccination (Bevan, 2011; Pulendran & Ahmed, 2011).

After encountering an antigen in the periphery of the body, an antigen presenting cell (APC) such as a DC becomes activated and migrates into the lymph nodes to present the antigen to T lymphocytes (Joffre et al., 2009). The following activation and proliferation leads to humoral and cell-mediated responses directed by two lymphocyte subtypes, B cells and T cells, respectively. Although their function results in different immune responses, these cells share various critical features needed for successful adaptive responses. Importantly, efficient and highly specific antigen recognition arises from the clonal expansion of selected lymphocytes based on their antigen receptors. Lymphocytes originate from hematopoietic stem cells in the bone marrow, and undergo somatic gene recombination during maturation to be able to represent a huge repertoire of antigen recognizing receptors (Kondo et al., 2001). To avoid non-functional or self-reactive receptors, lymphocytes undergo both positive and negative selection during their maturation (von Boehmer & Melchers, 2010). Naïve B cells can recognize intact micro-organisms, proteins as well as other molecular structures through the B cell receptor (BCR), whereas the T cell receptor (TCR) binds only processed peptides bound to the major histocompatibility (MHC) molecules presented specifically by the APCs (MHC-II), or by any cell type (MHC-I) (Miles et al., 2015). Once activated through an antigen receptor, lymphocytes proliferate and differentiate into effector cells that can mediate the elimination of the pathogen through various mechanisms.

Activated B cells can differentiate into antibody producing plasma cells or long-live memory cells. During proliferation, BCR is further modified to achieve its full

binding capacity and an appropriate response towards a specific antigen. These events include somatic hypermutations to increase binding capacity, and class switch recombination to customize the antibody's effector function. Antibodies are soluble forms of BCR that can be secreted into the extracellular space to recognize pathogens and target them for phagocytosis, for example (Pieper et al., 2013). The two main subsets of T cells, cluster of differentiation 4 positive T helper (CD4+ Th) cells and CD8+ cytotoxic T cells, maturate in the thymus and get activated through TCR signaling. CD4+ Th cells recognize antigens bind to MHC-II presented by the APCs, whereas CD8+ cytotoxic T cells bound antigen-MHC-I complexes presented by any cell type (Miles et al., 2015). Cytotoxic T cells can directly kill compromised cells such as tumor or virus-infected cells, whereas Th cells provide positive and negative regulation for both innate and adaptive immune cells. CD4+ Th cells can further differentiate into distinct effector subpopulations and thus regulate a variety of functions including antibody secretion and class switch recombination on B cells, the activation of cytotoxic cells, cytokine secretion from innate immune cells, as well as the suppression of inflammation (Chang et al., 2014). Collectively, Th cells regulate all the functions of the immune system and thus provide an important target for the treatment of immune pathologies.

The capability of the immune system to achieve powerful, tissue-damaging responses in the body comes with major risks of reacting against the wrong stimuli. On the other hand, disabled responses can leave the body vulnerable to common pathogens and malignant cells. If the effector and suppressor functions are unbalanced, the body might encounter severe immunopathologies (Goodnow et al., 2005; Murphy, 2011). Autoimmunity arises when the immunological tolerance against self-antigens fails and immune responses are targeted against the body's own cells and tissues. An autoimmune reaction can be a systemic response with antibodies not specific to a particular tissue, such as systemic lupus erythematous (SLE) (Grammatikos & Tsokos, 2012), or a local response targeting a specific organ, such as type 1 diabetes (Knip & Siljander, 2008). Both genetic and environmental factors have been linked to the high prevalence of autoimmune diseases in developed countries. However, efficient and safe therapeutic interventions targeted to hinder self-reactiveness are still to be discovered (Davidson & Diamond, 2001; Feldmann & Steinman, 2005). Further, allergy and asthma arise from hypersensitive immune responses against normally harmless substances from the environment (Holgate & Polosa, 2008), whereas primary or acquired immunodefiencies refer to conditions where the immune system is compromised or entirely absent resulting in

opportunistic infections or cancer susceptibility (Cunningham-Rundles & Ponda, 2005).

2.2 CD4+ T helper cells

CD4+ Th cells orchestrate both innate and adaptive immune responses by guiding the function and differentiation of other cell types through cytokine secretion or direct cell-to-cell contacts. Effector functions include processes such as macrophage activation, B cell support, and cytotoxic CD8+ T cell guidance. In contrast, regulatory T cells (Treg), the suppressive lineage of the Th cells, are critical for the attenuation of inflammation and the maintenance of immune tolerance (Murphy, 2011).

2.2.1 T helper cell differentiation

The successful activation of a Th cell requires at least two distinct signals from an antigen presenting cell. An antigen bound to the MHC-II molecule is recognized by the TCR on the surface of a naïve T cell. Simultaneously, a TCR associated co-receptor CD4 binds to the MHC-II molecule, following CD3-mediated signaling into the cell. In addition to TCR activation, a T cell needs a second signal from a co-stimulatory receptor, such as CD28 or CD40L, which binds to its counterpart presented on the surface of the antigen presenting cell (Brownlie & Zamoyska, 2013). Following activation in a particular cytokine environment, a Th cell may differentiate into one of the Th subsets, as defined by a particular pattern of gene expression, cytokine production, and resulting biological function. Several Th subsets have been characterized so far, and four of them, namely Th1, Th2, Th17, and Treg, have been studied in great detail. Also additional subsets, including Th9, Th22, and follicular T cells (Tfh), have been described but it is still unclear how these populations arise and whether they actually overlap with the more traditional subsets (Yamane & Paul, 2013; Zhu et al., 2010).

From a typical point of view, the differentiation process for a Th cell consists of two steps: a TCR-driven induction and a cytokine-driven polarization phase. Each Th subset utilizes a different set of master transcription factors, including cytokine induced members of the signal transducer and activator of transcription (STAT) family, and produces key cytokines to enhance the commitment of Th cell lineage

and the simultaneous suppression of others (Yamane & Paul, 2013). Naïve Th cells differentiate into Th1 cells through the activation of STAT4 and the upregulation of T-box expressed in T cells (T-bet) in the presence of the cytokines interleukin 12 (IL-12) and interferon gamma (IFNɣ) (Szabo et al., 2003). Instead for Th2 cells the commitment driving cytokines are IL-2 and IL-4, acting together with STAT5, STAT6, and GATA binding protein 3 (GATA-3) (Yamane & Paul, 2013). In response to induction, Th1 cells produce mainly IFNɣ, whereas Th2 cells are characterized by the secretion of IL-4, IL-5, and IL-13 (Zhu et al., 2010).

In addition to internal factors and the cytokine environment, also the strength of the antigen signal plays a critical role during the initiation phase of Th polarization. In general, strong TCR signaling enhances Th1 commitment, whereas weaker signals lead to the Th2 direction (Corse et al., 2011). At low concentrations antigens induce only a weak and transient phosphorylation of extracellular signal-regulated kinase (ERK), which leads to IL-4 independent early induction via GATA-3, IL-2 and STAT5, and finally to IL-4 dependent polarization via IL-4 and STAT6. In comparison, a high concentration of a cognate peptide leads to suppressed GATA-3 expression and prolonged ERK activation, and favors the mechanisms of Th1 differentiation (Yamane & Paul, 2013).

Transforming growth factor beta 1 (TGFβ1) mediates a suppressive function against Th1 and Th2 commitment, and induces Th17 and inducible T regulatory cells (iTreg) differentiation. In contrast, IFNɣ and IL-4 repress IL-17 induction indicating the importance of both positive and negative Th differentiation signals (de Jong et al., 2010; Harrington et al., 2005). In addition to TGFβ1, pro-inflammatory cytokines IL-6 and IL-23 are needed for Th17 differentiation, which drives the activation of STAT3 and the induction of RAR-related orphan receptor gamma (RORɣt) (Li et al., 2007; Zúñiga et al., 2013). The dose-dependence of TGFβ1 has also been reported to shape Th17 commitment both in mice and humans, whereas human memory Th cells have been demonstrated to adapt to the Th17 phenotype easier than the naïve Th cells, which indicates a difference in Th17 polarization between mice and humans (de Jong et al., 2010; Hatton, 2011). Furthermore, TGFβ1 also drives the proliferation of iTregs in the presence of IL-2, resulting in the activation of STAT5 and upregulation of forkhead box P3 (FoxP3) (Fontenot et al., 2005; Josefowicz et al., 2012). Th17 cells produce IL-17, IL-21, and IL-22 (Hatton, 2011; Zúñiga et al., 2013), whereas iTregs are the source of immunosuppressive TGFβ1 and in some cases IL-10 (Maynard et al., 2007; Zhou et al., 2009). In addition, thymus-derived natural Treg (nTreg) cells represent another suppressive T cell population, but in contrast to iTregs, FoxP3 induction in nTregs

is not dependent on TGFβ1 (Oh & Li, 2013). Figure 1 summarizes the general characters of the main Th subsets discussed here.

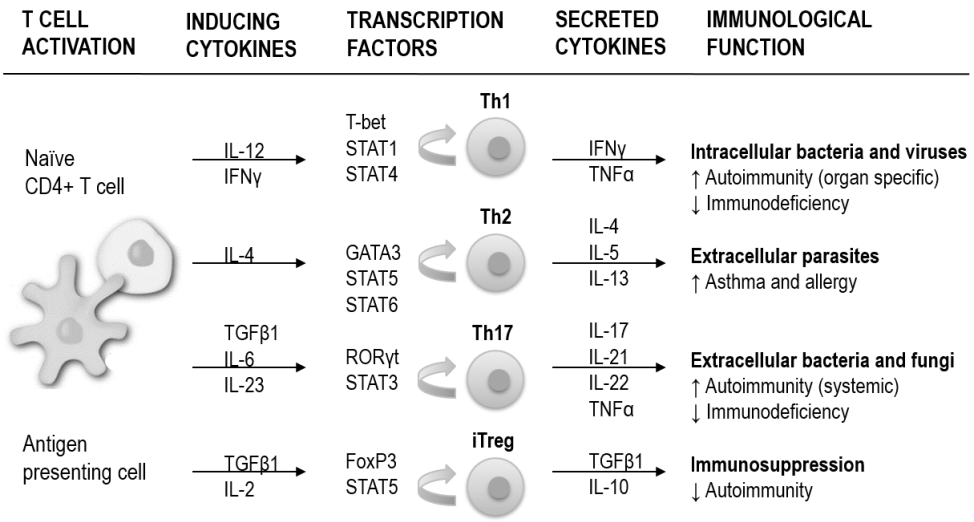

Figure 1. CD4+ T cell differentiation and immunological function. A naïve CD4+ T cell is activated by an antigen presenting cell via TCR stimulation and costimulatory signal. Depending on the cytokine environment, an activated T cell can polarize into a specific Th cell subset. Each subset express certain transcription factors, which can be used also for cell identification purposes. A differentiated Th cell secretes typical cytokines to mediate its immunological function, which can be either stimulatory or suppressive. Gain- or loss-of function changes in T cell biology can lead to pathological consequences which are indicated by arrows. The figure is based on Bluestone et al., 2009; O'Shea & Paul, 2010; and Zhu et al., 2010.

Although intensive study has revealed a lot about the signature transcription factors and cytokines for different Th subsets, it is now know that the cell identity and lineage commitment are also shaped by dynamic epigenetic landscapes. The epigenetic information includes histone tail modifications, DNA methylation, noncoding RNAs and a variety of chromatin interactions (Kanno et al., 2012). The chromatin state of the signature cytokine genes seems to point towards lineage commitment, whereas dynamic epigenetic states of master transcription factor genes argue more for plasticity (Wei et al., 2009). Epigenetic analyses have also revealed new insights into the function of the classical transcription inducers such as the STATs. A study by Wei and colleagues demonstrated that STATs can also mediate a suppressive function in T cell polarization, as is demonstrated by STAT4 mediated repression of STAT6 targets in Th1 cells (Wei et al., 2010).

Further, genome-wide studies have revealed lineage specific enhancer regions that guide early polarization events in human T cells (Hawkins et al., 2013). Recently, also distal superenhancer regions, which map the key regulatory nodes, were shown to be associated with T cell identity in mice (Vahedi et al., 2015). Importantly, the study showed that BTB and CNC homology 2 (BACH2), a negative regulator of effector T cell differentiation, possesses the strongest superenhancer in all effector T cell subsets studied. Interestingly, these global epigenetic studies argue that distal regulatory elements harbor a significant amount of single nucleotide polymorphisms (SNPs), which are associated with various immunological diseases including type 1 diabetes and rheumatoid arthritis (Hawkins et al., 2013; Vahedi et al., 2015).

Based on numerous high-throughput studies, Th cells seem to be more flexible in changing their phenotype than was initially thought (Baxter & Jordan, 2013). A polarized Th cell can express more than one transcription factor simultaneously, and cytokine secretion has also been demonstrated to be context-dependent and to adapt flexibly to the inflammation environment (O'Shea & Paul, 2010). A mouse study demonstrated that IL-4+ memory Th2 cells can participate in IFNγ secretion in response to a viral challenge (Lohning et al., 2008), and a recent study with human T cells demonstrated that antigen primed, highly heterogeneous CD4+ memory T cell responses arise from the selective expansion of suitable T cell clones that can adapt flexible phenotypes depending on the inflammation environment (Becattini et al., 2015). Interestingly, effector T cells may also acquire a suppressive regulatory function during prolonged chronic inflammation (O'Garra & Vieira, 2007), or vice versa (Zhou et al., 2009). Further, the anti-inflammatory IL-10 cytokine is secreted from many immune cell types, and its main function is to silence excessive immune responses, especially Th1 responses (O'Garra & Vieira, 2007). Thus it is interesting that some immunological challenges such as a *Toxoplasma gondii (T. gondii)* infection are characterized by IFNγ and IL-10 producing Th1 cells that are essential both for the elimination of the pathogen but also for the attenuation of the inflammation that could be harmful for the host (Jankovic et al., 2007).

In summary, based on our current knowledge it seems that the tissue microenvironment and epigenetic factors play a critical role in determining T cell identity and immunological function. An activated Th cell that has the potency to adjust its effector function during the course of an infection could be more useful for the host response than a terminally differentiated Th cell (Kanno et al., 2012). Importantly, an aging organism with a lower number of naïve T cells has also the advantage of a plastic memory T cell repertoire that can flexibly adapt to various phenotypes. Finally, it is essential to take T cell plasticity into account when

designing future therapeutic interventions. For example, administration of modified Tregs that could turn into pro-inflammatory Th17 cells would have significant consequences for the treatment of autoimmune disorders (O'Shea & Paul, 2010).

2.2.2 T helper cell function

After the differentiation phase has been completed, a polarized Th cell begins to mediate its immunological function. Dysregulated Th responses may lead to various diseases including autoimmunity and allergies, which has been demonstrated widely both in human patients and transgenic animals models. Th1 cells activate cell-mediated responses by secreting mainly IFNγ, which then activates macrophages and cytotoxic CD8+ T cells to act against intracellular bacteria and viruses as well as tumor cells (Szabo et al., 2003). An overactive or misstargeted Th1 response plays a role in various autoimmune diseases including type 1 diabetes (Roep & Peakman, 2011) and multiple sclerosis, as well as in delayed-type hypersensitivity reactions (Raphael et al., 2014). In comparison, a defective Th1 response may lead to the accelerated proliferation of tumor cells (Schreiber et al., 2011), and a susceptibility to various pathogens, such as *Mycobacterium tuberculosis* (Salgame, 2005) or *T. gondii* (Denkers & Gazzinelli, 1998). *T. gondii* is an obligate intracellular parasite with a wide geographic distribution and the ability to infect almost all nucleated cell types in many species, which makes it a commonly used infection model in immunological research. The parasite establishes itself within the brain and skeletal muscle tissues of its host generating cysts in the chronic phase, which can be lethal for immunocompromised patients (Luetjen et al., 2006; Suzuki, 2002). Collectively, adoptive immunotherapies with naturally occurring or genetically engineered T cells targeted against self-tissues may offer a powerful tool for treating autoimmune diseases and cancer (Restifo et al., 2012).

Th2 cells secrete the pro-inflammatory cytokines IL-4, IL-5, IL-13, and IL-33 to activate B-cell mediated humoral responses including antibody class-switching to immunoglobulin E (IgE). Th2 cells activate also eosinophils, basophils, and mast cells, and are crucial in immunity against helminths (Hirahara et al., 2011; Murphy, 2011). Overactive Th2 responses lead easily to allergy and asthma, as well as autoimmune diseases mediated through humoral immune responses (Gould & Sutton, 2008; Raphael et al., 2014), whereas a defective function may generate insufficient immunity against extracellular pathogens such as helminths (Jankovic et

al., 2006). Therapeutic hindering of Th2 cells is thus considered a potential treatment for the aforementioned diseases (Holgate & Polosa, 2008).

Further, Th17 cells protect the body against extracellular bacteria and fungi through IL-17, IL-21, and IL-22 secretion (Zúñiga et al., 2013). Overactive Th17 cells and especially IL-17 secretion have been linked to various autoimmune disorders including rheumatoid arthritis and systemic lupus erythematous (Raphael et al., 2014). Importantly, the unstable phenotype of the Th17 cells argues that inflammatory conditions are also affected by plasticity between different T cell subsets (Raphael et al., 2014; Zúñiga et al., 2013). Defective Th17 function may lead to a lack of immune protection on the skin and mucosal surfaces (Zúñiga et al., 2013), and mutations in the gene coding for human STAT3 are linked to impaired Th17 polarization and hyper-IgE syndrome (Milner et al., 2008). Some of the more recently discovered Th effector subsets are important for the defense against intestinal helminths (Th9), for the contribution to the skin immunity (Th22), or for the stimulation of germinal center B cells (Tfh). Defects in the functions of these subsets may lead to atopy and allergy (Th9), diseases like rheumatoid arthritis and psoriasis (Th22), or systemic lupus erythematosus (Tfh) (Jiang & Dong, 2013; Raphael et al., 2014). But it remains to be determined whether these Th subsets and their physiological functions are independent or derived from other populations.

Thymus derived nTregs and peripherally induced iTregs serve as vital mediators of suppressive immune regulation and the prevention of autoimmunity. Most of the self-reactive T cells are deleted by the central tolerance already during the development phase in the thymus, followed by the peripheral tolerance that can target autoreactive T cells in peripheral tissues (Oh & Li, 2013). The main function of the Tregs is to attenuate inflammation by suppressing pro-inflammatory immune cells, particularly self-reactive T cells, and to hinder excessive immune responses against harmless non-self-antigens, such as commensal bacteria and pollen. The master transcription factor of Tregs, FoxP3 induces anti-inflammatory TGFβ1 and IL-10 secretion, but Treg guided suppression is also mediated through direct cell-to-cell contacts (Josefowicz et al., 2012; Wing & Sakaguchi, 2010). Both human and mice with loss-of-function mutations in the FoxP3 gene suffer from a fatal, T-cell dependent immune disorder characterized by multi-organ defects (Fontenot et al., 2005). Additionally, immune disorders including type 1 diabetes, asthma and multiple sclerosis are linked to impaired Treg functions (Raphael et al., 2014), whereas overactive peripheral tolerance is often induced in the tumor microenvironment resulting in the defective clearance of tumor cells (Nishikawa & Sakaguchi, 2014). In light of this, autoimmune diseases can be seen as an alteration

in the balance between functional Tregs and self-reactive effector cells, in which genetic risk and environmental factors play a significant role. Therefore, modifications to the Treg and effector Th cell balance offer various ways of controlling the immune system (Wing & Sakaguchi, 2010).

In addition to acute responses, all Th populations contribute also to the immunological memory. Long-term memory of a particular antigen offers a way to respond rapidly and robustly upon antigen re-exposure. Memory T cells are not a single cell type but rather a heterogeneous population characterized by antigen-independent persistence and self-renewal (Becattini et al., 2015; Chang et al., 2014). Despite more than 20 years of intensive investigation into T cell differentiation and function, the full extent of the different subsets and the regulatory pathways are still incompletely understood. Details on how effector, regulatory, and memory T cells are generated and further regulated could offer numerous therapeutic and diagnostic solutions for major health problems.

2.3 Biology of the proprotein convertase (PCSK) enzymes

The diversity of the proteins encoded by the genome of an organism can be increased remarkably through various post-translational modifications. Limited proteolysis is a general alteration during the maturation process of many proteins, and it is mediated by specific enzymes, proteases. This biochemical phenomenon was first demonstrated in 1967 with the discovery of the proteolytic activation of pro-insulin (Steiner et al., 1967). Since then, almost 600 human proteases have been identified that conduct proteolysis by diverse biochemical mechanisms (Turk et al., 2012). The rapid development of new technologies and *in vivo* models has led to the identification of physiological substrates, and has directly linked proteolytic action to many diseases, which makes proteases also valuable targets for therapeutic applications. One important group of proteases, the proprotein convertase subtilisin/kexin (PCSK) enzymes conduct the endoproteolytic cleavage of cognate target proteins in diverse subcellular locations. In general, targeted endoproteolysis leads to the activation, inactivation or altered biological function of other enzymes, cytokines and pathogenic proteins (Artenstein & Opal, 2011; Seidah & Prat, 2012).

2.3.1 The PCSK family

The nine members of the PCSK family are phylogenetically ancient proteases and highly similar to the bacterial subtilisin and the yeast kexin, which originally led to the identification of their eukaryotic homologs (Seidah, 2011a). The first seven members (PCSK1-2, FURIN, PCSK4-7) recognize single or paired basic amino acids [(R/K)Xn(R/K)R], where R is arginine, K lysine, X any amino acid other than cysteine, and n represents the number 0, 2, 4, or 6 (Artenstein & Opal, 2011). The human gene *fur* (fes/fps upstream region) encoding PCSK3 is officially referred to as FURIN, and for clarity all the other members are referred to here as PCSK1-7 (Seidah, 2011a). The highly conserved catalytic triad from the first seven PCSKs consists of a cluster of negatively charged amino acids, which mediate the preference for the basic amino acids in the target site (Henrich et al., 2003). However, some variation in the preference for a cleavage sequence has been demonstrated (Duckert et al., 2004). The newest PCSK members, membrane-bound transcription factor peptidase site 1 (MBTPS1 or PCSK8), and PCSK9 have a different domain structure and target sequence compared to the seven core PCSK enzymes. PCSK8 does not require basic amino acids in the cleavage site [RX(L/V/I)X, where L is leucine, V valine, and I isoleucine] (Patra et al., 2007), whereas PCSK9 has been reported to have only an autocatalytic activity: it converts itself into an active, secreted form (Lambert et al., 2012).

The expression of the PCSKs is transcriptionally regulated through various signaling pathways, and many substrate molecules demonstrate coordinated expression together with their activating convertases (Turpeinen et al., 2011). For instance, the expression of FURIN is induced by its own substrate molecule TGFβ1 (Blanchette et al., 1997; Blanchette et al., 2001), and several other cytokines also upregulate its expression in a cell type specific manner (Chen et al., 2008; Laprise et al., 2002; Lund et al., 2004). In addition, expression correlation analyses have revealed tissue-specific PCSK-PCSK enzyme pairs, which could point to redundant proteolytic function (Turpeinen et al., 2011). Interestingly, in addition to endogenous sterol receptor binding proteins, the expression of the PCSK9 gene is induced by statin drugs (Awan et al., 2014).

In addition to transcriptional regulation, PCSKs are also targets for various post-translational modifications before becoming enzymatically fully active. Zymogens, the inactive precursors of PCSKs, are first modified in the endoplasmic reticulum (ER) before a final, pH and calcium dependent maturation step either in the trans-Golgi network (TGN) or on the cell surface. Pro-domains of the inactive

PCSKs help to facilitate protein folding and inhibit the activity of the catalytic triad before the final subcellular location has been reached (Seidah & Prat, 2012). Further, the intracellular trafficking of at least FURIN is critically regulated through phosphofurin acidic cluster sorting protein 1 (PACS-1), which guides FURIN from the TGN to further subcellular destinations (Crump et al., 2001).

2.3.2 Tissue expression and subcellular localization of PCSKs

Although the seven core PCSKs share common cleavage site preferences, they do differ greatly in their tissue expression profiles and subcellular localization. Each PCSK has a specific pH preference, which further regulates the site of proteolytic action (Artenstein & Opal, 2011; Thomas, 2002). Importantly, PCSK1 and PCSK2 are active only in secretory granules, whereas other core PCSKs function constitutively. Due to their restricted cellular localization in the secretory granules, PCSK1-2 are mainly found in neural and endocrine tissues (Seidah & Prat, 2012), and in addition they are also expressed in macrophages (Refaie et al., 2012). The ubiquitously expressed FURIN, PCSK6, and PCSK7 recycle between trans-Golgi network, the cell membrane, and endosomes as membrane-bound proteins (Artenstein & Opal, 2011). This dynamic trafficking and cycling between different subcellular compartments is highly regulated and involves the PACS-1 sorting protein at the TGN (Crump et al., 2001). The expression of PCSK1-2, FURIN, PCSK5, and PCSK7 in immune cells is covered in detail in section 2.4.2.

PCSK4 is found to be expressed only in germinal cells, and it is thought to be localized on the cell surface (Seidah, 2011a). In comparison, PCSK5 is alternatively spliced to produce a membrane bound (PCSK5B) and a secreted form (PCSK5A) of the protease. The balance between these two forms depends on the cell type: most of the membrane bound PCSK5B is expressed in the kidney and in the small intestine (Seidah, 2011a). It is also possible that some of the other core PCSKs are secreted into the extracellular space, including macrophage expressed FURIN (Meissner et al., 2013). The ubiquitously expressed PCSK8 is found in the cis- and medial-Golgi, endosomes and lysozymes, but not on the cell membrane (Pullikotil et al., 2007). In the cells of liver, kidney and small intestine PCSK9 is directly secreted into the extracellular space (Seidah, 2011b). A schematic illustration of the diverse subcellular localizations of the action of PCSKs is shown in Figure 2, and other characteristics of the enzymes are highlighted in Table 1.

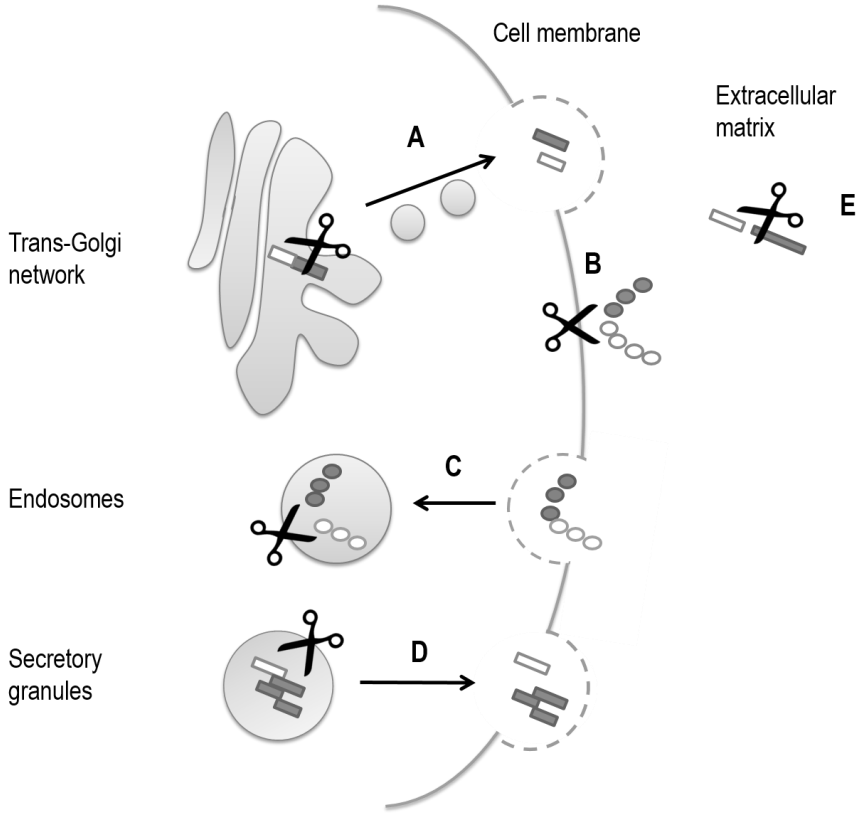

Figure 2. Subcellular processing compartments for PCSKs. Based on various *in vitro* assays and immunohistochemical analyses, the substrate processing of the PCSKs is thought to occur in at least four subcellular compartments. The trans-Golgi network (TGN) is the main processing site for FURIN, PCSK5, PCSK6, and PCSK7 (A). FURIN, PCSK5, and PCSK6 can be transported from TGN to the cell membrane, where they can conduct the processing of extracellular targets such as viral glycoproteins (B). PCSK7 can achieve the cell membrane both from the TGN but also directly from the endoplasmic reticulum (ER). FURIN, PCSK5, and PCSK7 can be recycled from the cell membrane via an endocytic pathway, and these mildly acidic early endosomes are the activation site for many toxins and pathogenic proteins (C). Apart from the constitutive secretion pathway, PCSK1 and PCSK2 are sorted from the TGN directly to secretory granules, where they wait signals for the regulated secretion of hormones and cytokines (D). Some of the PCSKs can also be secreted from the cell as full-length or truncated forms, and they can conduct proteolysis in the extracellular matrix (ECM) (E). The information in the figure is adapted from Artenstein & Opal, 2011; Seidah & Prat, 2012; Seidah et al., 2013; and Thomas, 2002.

2.3.3 Substrates of PCSKs

PCSKs process a wide panel of substrates varying from endogenous hormones (insulin, glucagon), cytokines (TGFβ, BAFF), receptors (insulin receptor), serum proteins (von Willebrand factor), adhesion proteins (integrins), ECM components (aggrecan) and other enzymes (MMPs) to pathogenic proteins originating from bacteria (diphtheria/anthrax toxin) or viruses (HIV, Ebola). Some examples of these key substrates for each of the PCSKs are presented in Table 1. Hence, PCSKs serve as important regulators of cellular homeostasis and infection biology (Artenstein & Opal, 2011; Turpeinen et al., 2013). Interestingly, proteolytic action can also lead to the inactivation of a target protein, an example of this is the inactivation of PCSK9 by FURIN cleavage in hepatocytes (Essalmani et al., 2011). In addition to its catalytic activity at least FURIN can also function as a non-proteolytic chaperone facilitating matrix metalloproteinase 28 (MMP-28) secretion (Pavlaki et al., 2011).

The constitutively active PCSKs, namely FURIN, PCSK5, PCSK6, and PCSK7 share numerous substrates at least based on *in vitro* assays (Nelsen & Christian, 2009; Remacle et al., 2008). However, the concept of PCSK redundancy should also take into account the cellular context: timing of expression, subcellular localization, and the interacting partners of individual PCSKs and their substrates. These fundamental questions can be assessed only *in vivo* (Seidah & Prat, 2012).

Table 1. Tissue distribution, subcellular localization, and substrates of PCSKs. The information is from Artenstein & Opal, 2011; Seidah & Prat, 2012; and Thomas, 2002.

	Tissue distribution	Subcellular localization	Substrate examples
PCSK1	Neuroendocrine, immune cells	Secretory granules	insulin, growth hormone releasing hormone
PCSK2	Neuroendocrine	Secretory granules	β-endorphin, insulin, glucagon
FURIN	Ubiquitous	TGN, cell surface, endosomes	growth factors/cytokines (TGFβ), receptors (insulin receptor), adhesion molecules (integrins), metalloproteinases (MMP14), bacterial toxins (anthrax), viral glycoproteins (HIV, Ebola)
PCSK4	Germinal	Not described, cell surface?	insulin-like growth factor 2
PCSK5	Widespread (e.g. adrenal cortex, intestine, kidney, ovary)	TGN, cell surface, endosomes, secretory granules, ECM	growth differentiation factor 11, *in vitro* overlap with FURIN, PCSK6-7
PCSK6	Widespread: (e.g. muscle, heart, intestine, kidney)	TGN, cell surface, ECM	growth factors (Nodal), metalloproteinases (ADAM-TS4), viral glycoproteins (HIV), *in vitro* overlap with FURIN, PCSK5, PCSK7
PCSK7	Ubiquitous	TGN, cell surface, endosomes	growth factors (BMPs), receptors (transferrin), *in vitro* overlap with FURIN, PCSK5-6
PCSK8	Ubiquitous	TGN, cell surface, endosomes	transcription factors (CREBs), viral glycoproteins (Lassa)
PCSK9	Liver, intestine, kidney, central nervous system	TGN, cell surface, endosomes, extracellular	only autoactivation, binds to low density lipoprotein receptor (LDLR)

Immunologically one of the most interesting substrate groups for PCSKs are the members of the TGFβ superfamily. This evolutionarily conserved signaling system arose before the separation of arthropods and vertebrates, which argues for its critical role in an organism's homeostasis (Newfeld et al., 1999). TGFβ itself mediates pleiotropic effects on development, homeostasis, cancer, and immunology mainly through SMAD transcription factor signaling. Mammals encode three homologous isoforms of TGFβ1-3 with similar *in vivo* properties, and of these TGFβ1 is the predominant form in the immune system (Govinden & Bhoola, 2003; Oh & Li, 2013). Various cell types have TGFβ receptors on their surface, and this cytokine positively and negatively regulates many other growth factors in a context-dependent manner. To increase its regulatory potency, the mature TGFβ may also form heterodimers with other TGFβ family members including bone morphogenetic proteins (BMPs) and growth differentiation factors (GDFs) (Li et al., 2006; Travis & Sheppard, 2014). Initially, TGFβ is encoded as an N-terminal propeptide (latency associated peptide, LAP) associated with the bioactive TGFβ. At the Golgi network, LAP and TGFβ are separated by a PCSK cleavage mediated mainly by FURIN (Dubois et al., 2001). After this, LAP remains non-covalently associated with TGFβ inhibiting its function, and this complex can bind additional proteins prior to secretion and integrin activation (Annes et al., 2003). Both the PCSK-mediated cleavage and the release of LAP from bioactive TGFβ are important steps in the regulation of TGFβ activity and numerous signaling pathways (Li et al., 2006; Travis & Sheppard, 2014).

2.3.4 Interaction studies on PCSKs

The identification of novel interactions for PCSKs can help to identify new substrates, but also important regulatory molecules. Traditionally, PCSKs have been linked to specific substrates through *in vitro* assays using recombinant proteins (Basak et al., 2011). Recently, novel PCSK substrate molecules and interaction candidates have been identified also by a variety of high throughput methods. The interactions can be identified by computational methods combining both cleavage site preferences as well as structural, temporal, and spatial parameters. Using a multistep *in silico* pipeline, Shiryaev and colleagues were able to identify almost 500 potential FURIN targets in the human proteome (Shiryaev et al., 2013). Combining bioinformatics with the new sequencing technologies will help us to discover new PCSK interactions and their potential association with diseases (Tian et al., 2012).

As was discussed earlier, also expression correlation studies offer a useful method for discovering new interactions including coordinated expression of PCSK-PCSK pairs (Turpeinen et al., 2011). However, although computational analyses offer a great tool for predicting protein interactions, all of the findings must be further verified in biological systems (Remacle et al., 2008; Turpeinen et al., 2011).

In addition to computational analyses, affinity chromatography purification combined with proteomics offers an eligible method for capturing cell-type specific protein complexes under physiological conditions (Mann et al., 2001; Walther & Mann, 2010). For instance, single-step Strep-tag affinity purification combined with mass spectrometric (MS) peptide identification has proved to be a successful technology for capturing functional protein complexes from mammalian cells (Junttila et al., 2005). Keeping in mind that high-throughput interaction methods result often in high rates of noise and false positives, they should only be used to scale down the number of potential interactions whose biological relevance should then be studied *in vivo* (Ethier et al., 2006; Orchard et al., 2007).

2.4 PCSKs in health and disease

In vitro experiments have yielded a vast amount of information on the biochemical properties and substrates of PCSKs, but a complete understanding of the non-redundant functions *in vivo* is still lacking. Genetically altered animals and humans with gain- or loss-of-function mutations in the PCSK gene regions indicate *in vivo* specificity in substrate processing (Scamuffa et al., 2006; Turpeinen et al., 2013). By now, it is known that PCSKs are critical for proper embryogenesis and development, cellular homeostasis, and immune responses. Defects in PCSK activity can lead to various pathologies such as cancer, lipid disorders, and neurodegenerative diseases (Seidah et al., 2013). Because the PCSKs proteolytically activate also bacterial toxins such as anthrax and diphtheria, or coat proteins from viruses including human immunodeficiency virus (HIV) and Ebola, they can be seen as potential targets for clinical interventions also in combatting infectious diseases or bioterrorism (Artenstein & Opal, 2011; Shiryaev et al., 2007).

2.4.1 Animal models and human patients

Over the past two decades both germline and conditional knockout animal models have been used successfully to investigate the biological significance of each of the PCSKs (Scamuffa et al., 2006). Recent developments in genome-wide technologies have also increased the information on the genetics of PCSKs in humans (Turpeinen et al., 2013). Table 2 summarizes the phenotypes of germinal cell PCSK knockout mice and studies on PCSK genetics in humans.

A germline deletion of the mouse PCSK1 or PCSK2 results in severe growth retardations and hormonal disturbances in for example glucose metabolism (Furuta et al., 2001; Zhu et al., 2002). These two enzymes have high redundancy and their simultaneous deletion results in a lethal phenotype (Seidah & Prat, 2012). In humans, three different mutations and various SNPs have been found in the PCSK1 gene region, all of which link its function to monogenic obesity, altered glucose metabolism, and even infertility (Farooqi et al., 2007; Jackson et al., 1997; Jackson et al., 2003). Likewise, genetic variations in the human PCSK2 have been linked to type 2 diabetes (Jonsson et al., 2012).

FURIN is the most investigated PCSK, thus also the available information on its function is extensive but not truly disclosed. A germline deletion of the mouse *furin* is embryonic lethal due to defects in ventral closure and axial rotation (Roebroek et al., 1998). However, heterozygote mice lacking one of the *furin* alleles have no developmental defects, which might indicate that only a small amount of active FURIN is enough to mediate its critical functions (Roebroek et al., 1998). Several tissue-specific knockout mouse models have been used to bypass the early lethality of the *furin* germline knockout, and they have revealed both redundant but also cell-type specific roles for FURIN. To this end, the interferon inducible Mx1-Cre transgene mice crossed with mice bearing the endogenous *furin* gene flanked by two loxP sites have been used to demonstrate unique and redundant functions of FURIN. In FURIN deficient hepatocytes, some of the analyzed FURIN substrates were processed completely (insulin receptor), whereas others were processed only partially (albumin, vitronectin). These results demonstrate that there is a partial redundancy between FURIN and other PCSKs in the liver (Roebroek et al., 2004). In contrast, FURIN was shown to be critical for proper insulin secretion from pancreatic β-cells (Louagie et al., 2008), and newborn mice with *furin* deficiency in their endothelial cells died prenatally due to cardiac malfunction (Kim et al., 2012). Importantly, T cell expressed FURIN was shown to be critical for proper T cell function and peripheral tolerance (Pesu et al., 2008), which will be discussed in more

detail in section 2.4.2. Mutations in the *fur* gene itself have not been reported in humans, but a single nucleotide polymorphism on its promoter region affects the outcome of a hepatitis B virus infection (Lei et al., 2009). In addition, FURIN has been nominated as a genetic risk factor for hypertension in several independent large-scale studies (Ganesh et al., 2013; International Consortium for Blood Pressure Genome-Wide Association Studies, 2011).

PCSK4 has been shown to be active only in germinal cells, thus a defect in its function is linked to infertility and gestation problems (Gyamera-Acheampong et al., 2006; Mbikay et al., 1997). Due to its restricted expression, no human phenotypes have been reported so far. PCSK5 deficiency in mice is lethal (Essalmani et al., 2006), probably due to the lack of mature growth differentiation factor 11 (GDF11) (McPherron et al., 1999). Polymorphisms in the human PCSK5 gene region have strong association with neural disorders, such as Alzheimer disease (Furney et al., 2011). In addition, PCSK5 could regulate lipid metabolism (Iatan et al., 2009), and have a protective role in intestinal cancers (Sun et al., 2009). Further, newborn PCSK6 knockout mice are viable, although 25% of them die embryonically (Constam & Robertson, 2000). Knockout mice have defects in bone morphogenesis and left/right axis formation, and consistently two human SNPs on PCSK6 gene region associate likewise with the severity of osteoarthritis, and left handedness together with dyslexia (Malfait et al., 2012; Scerri et al., 2011).

The evolutionarily most ancient PCSK7 is essential for *Xenopus* embryogenesis (Senturker et al., 2012), but its unique role in mammalian development has been harder to interpret. First, it was described that PCSK7 knockout mice are viable and healthy (Villeneuve et al., 2002), but a recent study demonstrated that knockout mice suffer from learning and memory associated disorders (Wetsel et al., 2013). Based on these results, it is possible that the level of redundancy between the PCSKs is different in lower vertebrates. In humans, polymorphisms in the PCSK7 gene region associate with the amount of a soluble transferrin receptor that regulates iron metabolism (Oexle et al., 2011), and cardiovascular events including triglyceride and LDL levels (Middelberg et al., 2011). However, the biological relevance of these associations remains to be determined.

The newest PCSK family members, PCSK8 and PCSK9 are tightly linked to lipid metabolism. A germinal cell knockout of the mouse PCSK8 is lethal (Mitchell et al., 2001), and a conditional targeting of liver cells leads to significantly decreased cholesterol levels in mice (Yang et al., 2001). Although PCSK9 processes only itself, its essential function on the degradation of the low-density lipoprotein receptor (LDLR) has made it a target for active research. PCSK9 knockout mice suffer from

hypocholesterolemia (Rashid et al., 2005), and transgenic animals overexpressing PCSK9 have significantly higher levels of low-density lipoprotein cholesterol (LDL-C) than do wild type controls (Zaid et al., 2008). Interestingly, both loss- and gain-of-function mutations on PCSK9 have been reported in humans, and the phenotypes match perfectly with mice (Abifadel et al., 2009). In the future, polymorphisms in the PCSK9 gene region might reveal new ways of regulating lipid metabolism and provide strategies for its therapeutic targeting (Seidah, 2011a).

Table 2. Phenotype of the germinal cell PCSK knockout mice, human patient mutations, and genetic associations with human traits and diseases. These studies are also reviewed in Artenstein & Opal, 2011; Seidah & Prat, 2012; Thomas, 2002; and Turpeinen et al., 2013.

	Germinal cell knockout mice	Human patient mutations	Human genetic association
PCSK1	increased mortality, dwarfism, hyperproinsulenemia *(Zhu et al., 2002)*	early-onset obesity, impaired glucose homeostasis, diarrhea *(Farooqi et al., 2007; Jackson et al., 1997)*	obesity, proinsulin levels, glucose homeostasis *(Jackson et al., 1997; Jackson et al., 2003)*
PCSK2	growth retardation, chronic hypoglycemia *(Furuta et al., 2001)*	not determined	chronic kidney disease, type 2 diabetes, myocardial infarction *(Jonsson et al., 2012)*
FURIN	embryonic lethality (day 10.5), impaired axial rotation, no heart looping *(Roebroek et al., 1998)*	not determined	blood pressure *(Ganesh et al., 2013),* hypertension *(International Consortium, 2011),* HBV infection outcome *(Lei et al., 2009)*
PCSK4	decreased fertility *(Gyamera- Acheampong et al., 2006; Mbikay et al., 1997)*	not determined	not determined
PCSK5	embryonic lethality (at birth), impaired anteroposterior patterning *(Essalmani et al., 2006)*	not determined	neurodegenerative disorders *(Furney et al., 2011)*, high-density lipoprotein levels *(Iatan et al., 2009)*
PCSK6	25% embryonic lethality (day 15.5), cardiac malformation, central nervous system defects *(Constam & Robertson, 2000)*	not determined	handedness in dyslexia *(Scerri et al., 2011)*, blood pressure, osteoarthritis *(Malfait et al., 2012)*
PCSK7	loss of anxiety, disabled learning, novelty seeking phenotype *(Wetsel et al., 2013)*	not determined	iron homeostasis *(Oexle et al., 2011)*, lipid metabolism *(Middelberg et al., 2011)*
PCSK8	embryonic lethality (day 3.5), no epiblast formation *(Mitchell et al., 2001)*	not determined	cholesterol and lipid metabolism *(de Windt et al., 2007)*
PCSK9	increased hepatic cholesterol uptake *(Rashid et al., 2005)*	gain-of-function: hypercholesterolemia, loss-of-function: hypocholesterolemia *(Abifadel et al., 2009)*	cholesterol and lipid metabolism *(Abifadel et al., 2009)*

2.4.2 PCSKs in regulating the immune system

PCSKs participate in the regulation of the immune system by directly controlling the function of immune cells, as well as by processing integral parts of a variety of bacteria and viruses (Figure 3). Apart from pathogen processing, a specific immunological function has been demonstrated for PCSK1, PCSK2, FURIN, PCSK5, and PCSK7.

PCSK1 and PCSK2 are known for their essential role in neuroendocrinological processes, but they are also highly expressed in myeloid cells (LaMendola et al., 1997; Vindrola et al., 1994) and immune organs, including the spleen, thymus and lymphatic ganglia *in vivo* (Lansac et al., 2006). The ubiquitously expressed FURIN has been detected in various immunological cell types and tissues (Dubois et al., 2001), as has PCSK7, which was initially identified in an aggressive lymphoma, where the *pcsk7* gene region harbored a chromosome translocation breakpoint (Meerabux et al., 1996). The following characterization demonstrated that PCSK7 is highly expressed in the colon and lymphoid tissues (Seidah et al., 1996). Moreover, the expression of FURIN and PCSK7 have been shown to be distributed similarly in rat thymus and lymph nodes (Lansac et al., 2006), which might argue for redundant functions between these two PCSKs also in an immunological context.

A more detailed profiling of PCSKs expressed in CD4+ T cell has demonstrated that FURIN and PCSK7 are the major convertases in T cell biology (de Zoeten et al., 2009; Pesu et al., 2006), although also low levels of PCSK1 (de Zoeten et al., 2009) and PCSK5 (Elhage et al., 2015) expression were detected. Interestingly, differentiated T cell subsets demonstrate distinct PCSK profiles, since FURIN is preferentially expressed in Th1 cells (Pesu et al., 2006), whereas PCSK7 expression is highly induced in activated Tregs (de Zoeten et al., 2009; Elhage et al., 2015). FURIN has been characterized as an IL-12 target gene both in mice (Lund et al., 2004) and humans (Pesu et al., 2006), and its expression has been shown to be induced in an IL-12/STAT-4 dependent manner as well as through TGFβ1, whereas its inhibition leads to decreased IFNɣ production (Lund et al., 2004; Pesu et al., 2006; Thieu et al., 2008). A recent study also demonstrated that the genomic locus of *furin* harbors a strong superenhancer region particularly in Th1 cells, which suggest a critical role for FURIN in determining T cell identity (Vahedi et al., 2015).

Further, T cell specific FURIN knockout mice develop an age-related systemic autoimmune disease, which is characterized by hyperactivated CD4+ and CD8+ T cells, proinflammatory cytokine production, autoantibody secretion, and lack of Treg-mediated peripheral tolerance (Pesu et al., 2008). Further, FURIN deficient T

cells are resistant to Treg mediated suppression, and secrete less of the anti-inflammatory TGFβ1 cytokine than wild type cells. However, a comparison of the FURIN knockout T cells with TGFβ1 deficient T cells and wild type controls reveals that in addition to regulating the bioavailability of TGFβ1, FURIN also mediates additional functions in T cell biology (Li et al., 2007; Pesu et al., 2008).

In addition to activating secreted molecules in T cells, PCSKs are also linked to the regulation of Treg biology through their potential role in processing the FoxP3 transcription factor (de Zoeten et al., 2009; Elhage et al., 2015). *In vitro* cleavage experiments have revealed that PCSK1 and PCSK7 derived from mouse T cells were able to process the C-terminus of FoxP3, and T cells overexpressing different isoforms of FoxP3 mediated distinct responses characterized by changes in gene expression and a potency to prevent experimental colitis in mouse (de Zoeten et al., 2009). A subsequent study on human Tregs revealed that also human FoxP3 can be processed proteolytically. However, in contrast to a previous mouse study, the C-terminally processed FoxP3 failed to mediate a suppressive function *in vitro* (Elhage et al., 2015). Whether FoxP3 is processed by the PCSKs *in vivo,* and what is the biological significance of these different isoforms remains to be determined.

In macrophages, a pathogen challenge through lipopolysaccharide (LPS) administration induces the expression of PCSK1 and PCSK2, and activates the secretion of antimicrobial peptides from secretory granules (Gagnon et al., 2013; Lansac et al., 2006). A more detailed study by Refaie and colleagues (2012) using PCSK1 knockout mice revealed that this protease specifically attenuates pro-inflammatory cytokine secretion from macrophages, and the lack of PCSK1 predisposed the mice to a LPS-induced septic shock. In contrast, the survival of PCSK2 or PCSK7 knockout mice challenged with a similar LPS dosage did not differ from wild type controls. Interestingly, the PCSK1 deficiency also caused a global hyperactivation of the proinflammatory Th1 pathway, which indicates that the lack of PCSK1 also affects the CD4+ T cell response through the stimulation of cells of the innate immune response (Refaie et al., 2012). In myeloid cells, also adipocytokine induced FURIN expression has been shown to be essential for the transformation of monocytes into macrophages, which finally drives macrophage chemotaxis (Kappert et al., 2013). Of note, the expression levels or the function of PCSKs in B cells have not been directly addressed so far.

As was discussed earlier, FURIN and to lesser extend other PCSKs are essential for the initial activation of many bacterial toxins and viral glycoproteins (Artenstein & Opal, 2011). Without the proteolytic maturation step, these pathogens fail to infect host cells, and the organism is rescued from potentially lethal consequences

(Thomas, 2002). Interestingly, FURIN, PCSK5, and PCSK7 have been directly linked also to the regulation of antigen presentation or recognition in a few studies, which demonstrate both the redundant and unique aspects of their function.

FURIN can directly participate in antigen presentation on MHC-I molecules through processing peptides at the TGN, which bypasses the conventional peptide loading process in the ER. This FURIN-mediated antigen processing pathway may cover up to 30% of the surface MHC-I complexes, which demonstrates the importance of this alternative route (Medina et al., 2009). In contrast, a study by Leonhardt and colleagues (2010) showed that PCSK7 but not FURIN can rescue unstable MHC-I molecules if the peptide loading complex fails to generate stable structures in the conventional ER route. PCSK7 deficient cells demonstrated delayed, reduced and unstable surface expression of the MHC-I complexes, which argues that also PCSK7 is important for regulating antigen presentation (Leonhardt et al., 2010). An expression correlation study has also revealed a strong association between human PCSK7 and MHC-class genes, which might propose a non-redundant function for PCSK7 as a regulator of antigen presentation and immunity (Turpeinen et al., 2011). Finally, the localization and activity of the human Toll-like Receptor 7 (TLR-7) has been shown to be regulated through PCSK-mediated proteolysis (Hipp et al., 2013). This study concluded that FURIN, PCSK5 and PCSK7 can mediate TLR-7 activation, which is critical for its function in the recognition of single-stranded viral RNA in endosomes.

In addition to *in vitro* studies and PCSK deficient mouse models, the immunomodulatory function of these enzymes has also been demonstrated for two autoimmune pathologies. In rheumatoid arthritis (RA), the systemic administration of FURIN has been proposed to have a protective role, although controversially in the same study, the local elevation of FURIN activity associates with inflammation both in human RA patients and in experimental mice (Lin et al., 2012). This study suggests that a systemic increase in FURIN levels could rebalance the Th1/Th2 ratio and enhance the suppressive function of Tregs, which could finally lead to reduced inflammation in a collagen induced arthritis mouse model. However, the mechanism behind the extracellular action of FURIN remains to be determined.

In multiple sclerosis, which is a T cell mediated autoimmune disease affecting the central nervous system, FURIN and PCSK2 have been shown to regulate the initial inflammatory steps (Shiryaev et al., 2009). This study suggests that the inflammatory proteolytic pathway mediated by FURIN, PCSK2 and their target MMPs could initiate a cascade that may then lead to the passage of autoreactive T cells into nervous tissues resulting finally in a chronic disease stage. Collectively,

multifaceted functions of the PCSKs in the immune system make them promising therapeutic targets, although a more detailed understanding on their unique and redundant functions is needed.

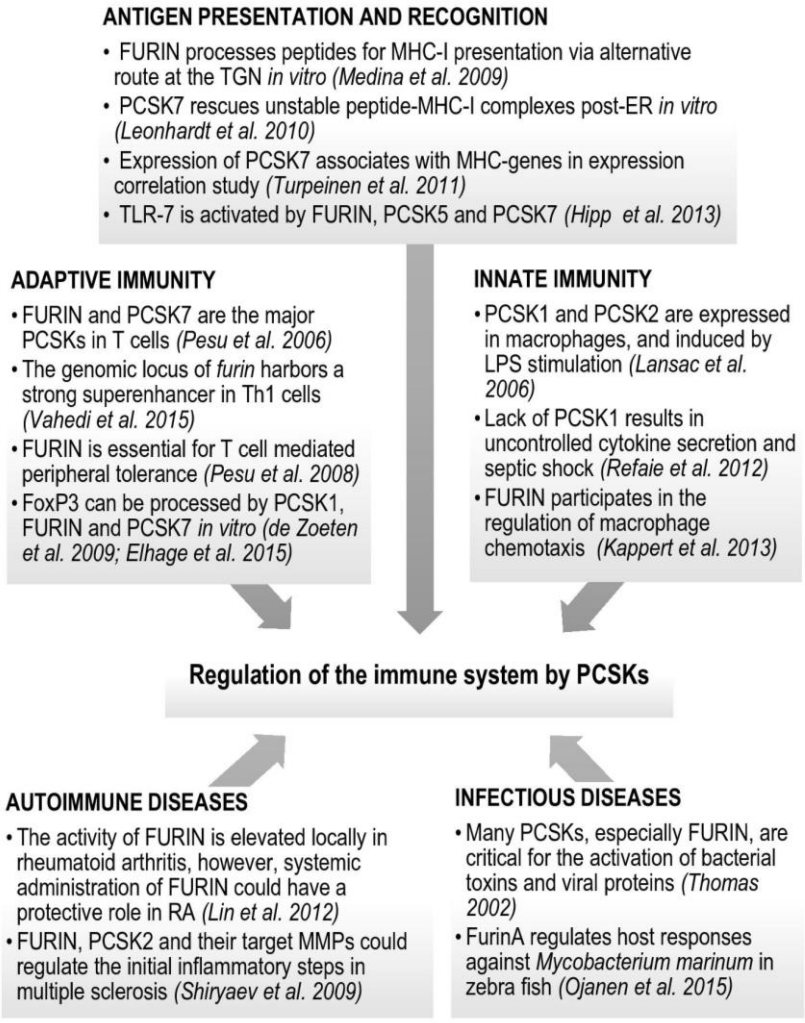

Figure 3. PCSKs have multifaceted roles in the regulation of the immune system.

2.4.3 PCSKs in the pathogenesis of atherosclerosis

Atherosclerosis, which is the chronic inflammation of the arterial wall, is typically characterized by extensive local inflammation and a dysfunction in lipid metabolism. Atherosclerosis of the coronary arteries, which supply blood to the heart, is the primary reason for coronary heart disease (CHD) and the leading cause of mortality in developed countries (Libby et al., 2011). Interestingly, many substrate molecules of the PCSKs mediate a critical role in the pathogenesis of this highly endemic disease (Stawowy et al., 2005b). The initial step of atherosclerosis includes the activation of an endothelial monolayer, which attracts immune cells of the blood, mainly monocytes, to attach and migrate into the vascular intima. In the tissue, monocytes mature into macrophages, which uptake lipids and transform into foam cells (Libby, 2006). Next, vascular smooth muscle cells (VSMCs) migrate from the vascular media into the intima, and overproduce proteins typical for the ECM. Inside a lesion many cell types undergo cell death, and generate a lipid containing necrotic core. Advanced plaques contain also cholesterol crystals and microvessels, and generally cause clinical manifestations ranging from tissue ischemia to a risk of developing thrombosis (Galkina & Ley, 2009). In addition to macrophages, lesion specific CD4+ T cells have a key regulatory function in the plaques, mediated mainly through a powerful pro-atherosclerotic Th1 response (Libby et al., 2011; Matsuura et al., 2014).

Several growth factors, chemokines, adhesion molecules and ECM proteins are activated through PCSK-mediated proteolysis. Thus, every stage of atherogenesis involves multiple targets of PCSKs varying from cell-contact integrins to ECM degrading MMPs suggesting a central role for PCSKs in the regulation of atherosclerosis (Stawowy & Fleck, 2005; Stawowy & Kappert, 2011). For instance, FURIN and PCSK5 are linked with an MMP activation cascade in blood mononuclear cells, and immunohistochemically colocalized with their substrate molecules in atherosclerotic plaques (Stawowy et al., 2005a; Stawowy et al., 2005b). Further, in atherogenesis the migration of vascular smooth muscle cells from the vascular media to the intima is dependent on integrin cleavage, which has been shown to be mediated partially by a FURIN-like protease (Kappert et al., 2010). In addition, FURIN can also participate in the regulation of macrophage migration (Kappert et al., 2013). Recently, a large-scale transcriptome profiling study and the immunohistochemical staining of human atherosclerotic lesions revealed that PCSK6 expression is upregulated in the vascular smooth muscle cells and fibrous cap of the symptomatic carotid plaques compared to stable lesions (Perisic et al.,

2013). In addition, PCSK8 and PCSK9 play a critical role in vascular biology by systematically regulating lipid metabolism (Lambert et al., 2012; Seidah, 2011b). Collectively, targeting PCSKs or their substrate molecules could provide novel ways to treat atherosclerosis.

2.4.4 Therapeutic potential of targeting PCSKs

The main challenges of the therapeutic targeting of PCSKs lie in designing drugs that are specific for the target enzyme, and do not interfere with the critical roles several PCSKs have in cellular homeostasis. Targeting PCSK activity can include small-molecule inhibitors, neutralizing antibodies, chemically synthesized PCSK prodomains, and antisense RNAs (Seidah & Prat, 2012; Tian & Jianhua, 2010). Inside the cells, PCSKs are also inhibited by endogenous molecules including the FURIN-binding serpin proteinase inhibitor 8 (Kappert et al., 2013; Leblond et al., 2006). Currently, both the inhibition and systemic administration of PCSKs have been proposed as therapeutic means for treating various pathologies (Komiyama et al., 2009; Lin et al., 2012).

In different cancers, the inhibition of FURIN or PCSK6 could offer a way to diminish tumor growth and invasiveness, and several *in vivo* models and a few clinical studies already support this idea (Bassi et al., 2001; D'Anjou et al., 2011; Scamuffa et al., 2008; Senzer et al., 2012). Since many bacterial toxins and viral glycoproteins are substrates of FURIN and, to a lesser extent, of other PCSKs, their inhibition could hold an answer for inhibiting infections and fighting bioterrorism (Shiryaev et al., 2007). PCSK1-2 targeting is under investigation for the treatment of diabetes (Vivoli et al., 2012), and the germ cell specific PCSK4 offers an interesting target for a male contraceptive (Seidah & Prat, 2012). Further, inhibition of PCSK8 could be a way of treating disorders in lipid metabolism. However, because of the essential role of PCSK8 in cellular homeostasis, this might also result in numerous side effects (Artenstein & Opal, 2011). At the moment, the target that holds most promise is PCSK9 and its interaction with the LDL receptor in the treatment of hypercholesterolemia. As was discussed earlier, mice and humans deficient in PCSK9 have hypocholesterolemia; blocking the interaction between PCSK9 and the LDL receptor in patients has been shown to mimic this situation. Thus, multiple drugs that inhibit PCSK9 are under extensive clinical testing (Norata et al., 2014).

In addition to their therapeutic potency, PCSKs could also be used as diagnostic tools. At least FURIN and PCSK5 have been shown to be secreted into the

extracellular space (Essalmani et al., 2006; Meissner et al., 2013), and the analysis of PCSK levels in the serum could offer new methods for diagnosing acute and chronic pathologies. Recently, it was demonstrated that chronic typhoid carriers have elevated serum FURIN levels (Kumar et al., 2014). Extensive interactome studies and redundancy profiling will help to understand the biology of the PCSKs in more depth, and possibly help use them as therapeutic targets in the future.

2.5 Studying the function of PCSKs in zebra fish

The zebra fish, *Danio rerio*, has emerged as a powerful vertebrate model for addressing various questions in biomedical research. Originally, this small tropical aquarium fish was used for developmental research, but during the last two decades its use has extended into studies including genetics, neurobiology, toxicology, and immunology (Sullivan & Kim, 2008). Numerous advantages and an increasing amount of available tools make zebra fish an intriguing model organism for biomedical research (Meeker & Trede, 2008).

Compared to the mouse, which is historically the most common vertebrate model organism in biomedical research, the breeding and maintenance of zebra fish are more cost-effective, since one pair can produce up to 300 new progeny per week with minor space requirements. In addition, the external fertilization and rapid development result in translucent and easily manipulative zebra fish embryos, where most of the organs are fully developed by day 5 post-fertilization (dpf). However, difficulties in cell culture methods as well as a lack of suitable antibodies have attenuated the development of common applications that are used frequently with other model organisms (Meeker & Trede, 2008; Sullivan & Kim, 2008).

Recently, the annotated zebra fish genome was published by Howe and colleagues (2013). The zebra fish genome has ~26,000 protein coding genes, significantly more than the human genome with ~20,000 genes, and this difference has most probably arisen during the teleost-specific whole-genome duplication. A comparison between these two genomes shows that 70% of the human genes have at least one orthologue in the zebra fish genome (Howe et al., 2013). Importantly, the identification of human orthologues in the zebra fish genome is essential to accelerate genetic studies on human diseases.

Also the PCSK family is found to be highly conserved between zebra fish and humans. Interestingly, the *furin* gene has undergone a gene duplication in the zebra fish genome, and is thus present as two homologues (*furina* and *furinb),* which

correspond to one mammalian gene (Freeman et al., 2007; Walker et al., 2006). Previously, PCSK1-2 (Morash et al., 2009), FURIN (Walker et al., 2006), PCSK5 (Chitramuthu et al., 2010), PCSK8 (Schlombs et al., 2003), and PCSK9 (Poirier et al., 2006) have been investigated in zebra fish. The dysfunction of both FurinA and PCSK8 lead to defects in skeletal and cartilage formation, whereas PCSK5 and PCSK9 are involved in neurological development. Further, zebra fish FurinA has been linked to fin development (Carney et al., 2010) and immune responses, where its deficiency induces the expression of *pcsk1* and *pcsk2* (Ojanen et al., 2015). Although these studies argue for an important role for PCSKs in zebra fish biology, the function of the remaining PCSKs and their redundancy in zebra fish remain to be characterized.

Immunological questions have also been addressed in the zebra fish, since its immune system resembles that of mammals (Goody et al., 2014; Oosterhof et al., 2014; van der Sar et al., 2004). All jawed vertebrates have both the innate and adaptive immune systems. However, the adaptive responses in zebra fish are fully functional only after 4-6 weeks (Meeker & Trede, 2008). Most if not all of the human immune cell types are present in the zebra fish, but they mature partially in different organs. Importantly, zebra fish T cell development resembles that of mammals, including similarities in gene expression profiles and thymus maturation (Lam et al., 2002; Traver et al., 2003). A study on zebra fish *tbet*, *foxp3* and *stat6* demonstrated that immunomodulatory signals induce the expression of T cell transcription factors as well as many cytokine genes resembling the response of the mammalian immune system (Mitra et al., 2010). Inflammatory proteins as well as pathogen recognition receptors are also highly homologous to the mammalian ones, including an immune regulatory function of TGFβ1 in teleost fish (Hardie et al., 1998; Rombout et al., 2014). A recent study on the zebra fish immune response against *Candida albicans* revealed that TGFβ signaling is one of the major pathways that shape the course of both the primary and secondary infection (Lin et al., 2014).

Various infections and immune system disorders have been reconstructed in zebra fish revealing new evolutionarily conserved aspects on immune regulation, including a previously discussed role of FurinA in the regulation of the host response against *Mycobacterium marinum* (Ojanen et al., 2015) as well as numerous viral infections (Sullivan & Kim, 2008). Also autoimmune pathologies such as Crohn's disease have been successfully studied in zebra fish (Marjoram et al., 2015). However, it is important to keep in mind that many cytokines and chemokines have undergone a gene duplication in the zebra fish genome, and it still remains unclear how much

functional redundancy they actually share with their mammalian counterparts (Sullivan & Kim, 2008).

Several methods for zebra fish genome targeting have been established, including large genetic screens based on random point mutagenesis (Amsterdam & Hopkins, 2006). Further, individual zebra fish genes can be silenced using antisense morpholinos (MO), which are chemically modified short oligomers that bind efficiently to transcribed mRNAs. By targeting the MO to bind directly to the translation start codon or splicing site, translation or splicing can be inhibited, respectively (Bill et al., 2009). MOs are injected directly into fertilized eggs and the silencing effect can last up to 10 days, which allows specific studies on zebra fish development (Meeker & Trede, 2008). However, problems with specificity, off-target binding and neural toxicity limit the use of this technology (Eisen & Smith, 2008). Further, conventional knockout technologies have been unsuitable for generating stable, specifically targeted mutant zebra fish lines. New site-specific genome editing technologies including the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 system could thus offer an alternative method for future zebra fish genome targeting applications (Hwang et al., 2013).

3 AIMS OF THE STUDY

The proprotein convertase (PCSK) enzymes play a crucial role in human health and disease, including the regulation of the immune system. The *in vitro* activity of PCSKs has been widely studied. However, little is known about their non-redundant, cell type-specific functions. The purpose of this study was to investigate the PCSK family in cellular homeostasis and specifically in the regulation of the immune system. Further knowledge on the function of PCSKs will deepen our understanding of the immune system and its disorders.

The detailed objectives of the study were:

1) To study the role of the proprotein convertase FURIN in T helper cell differentiation and to identify novel FURIN interacting proteins in T cells

2) To characterize the function of PCSK7 in the development of zebra fish and in the regulation of gene expression

3) To investigate the PCSK family in human atherosclerosis and immune cell activation

4 MATERIALS AND METHODS

4.1 Experimental animals (I-II)

T cell–specific FURIN conditional knockout (CD4cre-fur$^{f/f}$) mice in the C57BL/6 background have been described earlier (Pesu et al., 2008; Roebroek et al., 2004). CD4cre-fur$^{f/f}$–OTII mice were generated by crossing CD4cre-fur$^{f/f}$ and fur$^{f/f}$ littermate controls with mice bearing OTII-TCR. IL-12p40 knockout animals were purchased from Taconic Biosciences. All mice were housed under pathogen-free standard conditions. Detailed protocols for the *T. gondii* infection and antigen specific polarization assay are described in original publication I.

Zebra fish embryos and adult fishes were obtained through natural crosses of individuals of the wild-type AB strain, and maintained under standardized conditions in a flow-through system at 28.5°C. A detailed protocol for the morpolino injections is described in original publication II.

4.2 Human subjects (III)

Vascular plaque samples were obtained during open vascular procedures as a part of the Tampere Vascular Study from the Division of Vascular Surgery and Heart Center, Tampere University Hospital, Finland (Oksala et al., 2009). Control samples were obtained during coronary artery bypass grafting from the left internal thoracic arteries (LITA). Serum samples for cytokine measurements were collected from 16 patients (>50% stenosis in ≥2 arteries), and from 16 sex-, body mass index- and age-matched healthy controls without stenosis in any of the coronary arteries. The classification of the samples was based on the recommendation of the American Heart Association. The demographic data of the patients is presented in original publication III.

4.3 Antibodies (I-III)

Table 3. Antibodies used in the study. Abbreviation: HRP-horseradish peroxidase, * a kind gift from Prof. John Creemers, KU Leuven, Belgium.

NAME	DETAILS	MANUFACTURER	USED IN STUDY
Primary antibodies			
anti-actin	mouse monoclonal, clone C4	Millipore	III
anti-DAPI	nuclear counterstain	Life Technologies	I
anti-ERK	rabbit polyclonal	Cell Signaling Technologies	I
anti-pERK	rabbit polyclonal	Cell Signaling Technologies	I
anti-FLAG	mouse monoclonal, clone M2	Sigma-Aldrich	I
anti-FURIN	rabbit polyclonal	* Prof. Creemers	I
anti-FURIN	mouse monoclonal, clone MON-152	Alexic Biochemicals	I, III
anti-MYC	mouse monoclonal, clone 9E10	Sigma-Aldrich	I, II
anti-Strep	mouse monoclonal	IBA	I
anti-V5	mouse monoclonal	Invitrogen	I
Secondary antibodies			
anti-mouse HRP	HRP-conjugated	R&D Systems	I-III
anti-rabbit HRP	HRP-conjugated	R&D Systems	I
anti-rabbit HRP-biotin	HRP-biotin conjugated	R&D Systems	I
anti-mouse Alexa 488	fluorecent	Life Technologies	I
anti-rabbit Texas Red	fluorecent	Life Technologies	I
anti-mouse IgG2b	isotype control	Sigma-Aldrich	I
anti-mouse IgG1	isotype control	Sigma-Aldrich	I
FACS antibodies			
anti-CD4	rat, clone RM4-5	eBioscience	I
anti-CD8	rat, clone 53-6.7	eBioscience	I
anti-CD62L	rat, clone MEL-14	eBioscience	I
anti-Gata3	rat, clone TWAJ	eBioscience	I
anti–IL-4Rα	rat, clone M1	BD Biosciences	I
anti-Phalloidin	*Amanida Phalloides*, clone P5282	Sigma-Aldrich	I
anti-STAT6 (pY641)	mouse, clone J71-773.58.11	BD Biosciences	I
anti-Tbet	mouse, clone eBio4B10	eBioscience	I

4.4 Plasmid constructs and cloning (I, II)

For publication I, a site-directed-mutagenesis kit was used to introduce an aspartic acid to alanine (D153A) mutation to the catalytic triad of the wild type human FURIN cDNA in pSVL-huFURIN (ATCC) according to the manufacturer's instructions (QuickChange Mutagenesis, Stratagene). This resulted in a previously described inactive FURIN enzyme (Creemers et al., 1993). The cDNAs of wild type and D153A mutant FURIN were subcloned into pcDNA3.1-StrepIII (a kind gift from Prof. Jukka Westermarck, Turku Centre for Biotechnology, Finland). Other plasmids used in study I were pcDNA-RAC1-V5, pCI-DOCK2-FLAG (kind gifts from Prof. Yoshinori Fukui, Kyushu University, Fukuoka, Japan), and pcDNA3.1-huFURIN-Myc-His (huFURIN subcloned from pSVL-huFURIN to pcDNA3.1-Myc-His, Invitrogen).

For publication II, the sequence encoding the zebrafish *tgfβ1a* gene was amplified from the wild-type AB zebrafish cDNA (7 dpf) by PCR (forward oligo, 5'-GGAGAATTCGCCATGAGGTTGGTTTGCTTGGTGCTG; and reverse oligo, 5'-CATGGTGGTGAGGAACTGCAAGTGCAGTGGTACCGGA). The insert was subcloned into the pcDNA3.1-Myc-His plasmid (Invitrogen) and sequenced. Other plasmids used in this study included human TGFβ1 (ATCC) subcloned into pcDNA3.1-Myc-His (Invitrogen), pSVL-huFURIN (ATCC), pcDNA3-huPCSK7-FLAG (a gift from Prof. J. Creemers, KU Leuven, Belgium), pME18S-FL3-zfFurinA, pME18S-FL3-zfFurinB, and pCMV-SPORT6.1-zfPCSK7 (last three from imaGenes).

4.5 Cell culture, transfection, and generation of stable cell lines (I-II)

Human Jurkat E6-1 T cells (ATCC: TIB-152) were cultured in RPMI 1640 medium (Lonza), human HeLa cells (ATCC: CCL2) in DMEM medium (Lonza), and FURIN-deficient hamster RPE.40 cells (a kind gift from Prof. J. Creemers, KU Leuven, Belgium) in Ham's F-12 medium (Lonza), all supplemented with 10% fetal bovine serum (FBS), L-glutamine, and antibiotics.

In study I, Jurkat E6-1 T cells were electroporated (1025 μF, 260 V) with pcDNA3.1-huFURINwt-StrepIII, pcDNA3.1-huFURIN-D153Amut-StrepIII, or empty pcDNA3.1-StrepIII as a control. Each transfection consisted of 20 μg of plasmid DNA and $8x10^6$ cells. The amount of the Geneticin antibiotic (Calbiochem) was titrated, and an optimal concentration (600 μg/ml) was added to the cell cultures

three days post-transfection. cDNA-expressing cell lines were selected with clonal dilution, and the constant expression of the recombinant fusion protein was evaluated by Western blot analysis.

HeLa cells were transiently transfected with pcDNA-RAC1-V5, pCI-DOCK2-FLAG, and pcDNA3.1-huFURIN-Myc-His using FuGENE® 6 Transfection Reagent (Promega) according to the manufacturer's instructions. Cells were split a day before the transfection to a 6-multiwell plate (300,000 cells/well), and transfected with total of 0.5-1 μg plasmid DNA. Protein interaction studies were performed 48 hours post-transfection.

In study II, pcDNA3.1-huTGFβ1-Myc-His, pSVL-huFURIN, pcDNA3-huPCSK7-FLAG, pcDNA3.1-zfTGFβ1a-Myc-His, pME18S-FL3-zfFurinA, pME18S-FL3-zfFurinB, or pCMV-SPORT6.1-zfPCSK7 were transfected into RPE.40 cells using FuGENE® 6 Transfection Reagent (Promega) according to the manufacturer's instructions. A day before the cells were split to a 6-multiwell plate (150,000 cells/well), and transfected with a total of 750 ng plasmid DNA per well. Protein lysates and the culture medium were collected 48 hours post-transfection.

4.6 Cell purification, activation, and flow cytometry (I, III)

For mouse polyclonal T cell activation studies in publication I, naïve T cells were isolated from the spleen and lymph nodes of CD4cre-fur$^{f/f}$ and littermate control mice with the CD4+CD62L+ T Cell Isolation Kit (Miltenyi) according to the manufacturer's instructions. Cells were activated for 72 h with plate-bound anti-CD3 and -CD28 (10+10 μg/ml) antibodies in serum-free X-VIVO 20 medium (Lonza). IL-12 (10 ng/ml), TGFβ1 (0.5 ng/ml), or anti–IL-4 (2 μg/ml) were added to the cell cultures during the activation. Cell culture medium was collected for cytokine measurements, and RNA was extracted for quantitative real-time PCR (Q-RT-PCR) experiments.

The expression of IL-4Rα on the cell surface was analyzed in cells isolated from mouse spleens and lymph nodes by staining the cells with anti–IL-4Rα (BD Biosciences), anti-CD4, and anti-CD62L (eBioscience) antibodies, and analyzed by flow cytometry. For the detection of tyrosine phospho-STAT6, freshly isolated CD4+CD62L+ T cells were stimulated with IL-4 (1 or 10 ng/ml) for 15 min, following fixation with 4% paraformaldehyde and permeabilization with cold 90% methanol overnight. The staining was performed in 0.5% Triton X-100/0.1% BSA/PBS with an anti-pSTAT6 Ab (BD Biosciences).

To study transcription factor expression in CD4+ and CD8+ T cells, thymocytes from 12-week old CD4cre-fur$^{f/f}$ and littermate mice were isolated and stained with anti-CD4, anti-CD8, anti-T-bet and anti-GATA-3 antibodies (eBioscience). For the detection of phosphorylated ERK, the CD4+ T Isolation Kit (L3T4; Miltenyi) was used to isolate CD4+ T cells from the spleen and lymph nodes. Cells were stimulated with soluble anti-CD3 (10 μg/ml) for 5–60 min, and protein lysates were used for immunodetection. All flow cytometry (FACS) analyses in study I were performed using a FACSCalibur or a FACSCanto instrument (BD Biosciences), and data were analyzed with the FlowJo software (Tree Star).

In publication III, human CD14+ monocytes, CD4+ Th cells, and CD19+ B cells were isolated from the peripheral blood of healthy donors with magnetic beads according to the manufacturer's instructions (Miltenyi Biotec). Cells were cultured in complete RPMI 1640 medium containing 10% FBS. Monocytes were activated for 24 hours with LPS (1 μg/ml, Sigma–Aldrich), T cells for 24 hours with plate-bound anti-CD3 and anti-CD28 (5 μg/ml each, eBiocience), and B cells for 24 hours with the Toll-like receptor 7/8-ligand resiquimod (R-848, 2,5 μg/ml, Sigma), which was followed by RNA extraction.

4.7 Biochemical analysis of proteins (I-III)

4.7.1 Cell lysis and Western blotting (I-III)

In study I, the constant expression of recombinant wild type and mutant FURIN in Jurkat T cell clones was verified by Western blot analysis (anti-FURIN MON-152, and HRP-conjugated anti-mouse secondary antibody). Cells were lysed in buffer containing 50 mM Tris pH 7.5, 10% glycerol, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 50 mM NaF, and the Complete Mini –protease inhibitor cocktail (Roche Applied Science).

In study II, cell culture supernatants were collected 48 hours post-transfection, and RPE.40 cells were lysed in Triton X-100 lysis buffer (20 mM Tris-HCl pH 8.0, 300 mM NaCl, 20% glycerol, 0.1% Triton X-100, 1 mM EDTA, 50 mM NaF, 1 mM tris(2-carboxyethyl)phosphine hydrochloride) supplemented with protease inhibitors (Complete Mini, Roche Applied Science). Immunodetection was performed using an anti-Myc primary antibody and a HRP-conjugated anti-mouse secondary antibody.

In study III, human tissue samples from an atherosclerotic plaque and an adjacent macroscopically healthy part from the same artery were pulverized in liquid nitrogen and lysed in a buffer containing 0,9% NaCl, 20 mM Tris pH 7.0, 1% Triton X-100, and 1 mM phenylmethylsulphonyl fluoride (PMSF). The protocol was adapted from (Cromheeke et al., 1999). The lysates were used to detect FURIN (MON-152) and actin with a HRP-conjugated anti-mouse secondary antibody.

For all immunodetections, equal amounts of proteins were separated by SDS-PAGE, and immunoblotting was performed using specific primary antibodies and HRP-conjugated secondary antibodies. The visualization was done using the ECL™ Western blotting detection kit (GE Healthcare) and the AGFA CP1000 imaging system. Signal intensities were analyzed using the ImageJ software (Schneider et al., 2012).

4.7.2 Strep-tag affinity purification and mass spectrometry (I)

Cell membrane fractions from Jurkat T cell lines were isolated with the Mem-PER Eukaryotic Membrane Protein Extraction Kit (Thermo Scientific). Briefly, cells were lysed and proteins solubilized according to the kit's instructions followed by the separation of hydrophobic proteins. Next, FURIN and associated proteins were affinity purified from lysates through Strep-tag columns (IBA) according to the manufacturer's instructions. During protein lysis and affinity purification, 2 mM calcium chloride was added to the buffers to enhance FURIN's activity (Molloy et al., 1992).

The presence and purity of recombinant FURIN in the elutes were verified with anti-FURIN (MON-152) and anti-Strep primary antibodies by immunodetection. Eluted proteins were separated by one-dimensional SDS-PAGE gel (Miniprotean precast gel, Bio-Rad) and visualized by silver staining according to a MS compatible protocol from Turku Centre for Biotechnology, Finland (available online 20.1.2015: http://www.btk.fi/proteomics/services/protocols/).

Target bands were cut out from the silver-stained gels, and after enzymatic protein digestion and extraction, peptides were identified by MS (collaboration with Proteomics Facility, Turku Centre for Biotechnology, Finland). The analysis was performed by liquid chromatography/electrospray ionization–tandem MS on a nanoflow HPLC system coupled online to an Orbitrap Velos MS instrument. Database searches were performed by Mascot (version 2.2.6) against the SwissProt (UniProt) protein sequence database (version 2010_09). The Scaffold 3 software

(Proteome Software, USA) was used to further analyze identified proteins. The data was filtered through validation parameters (i.e., molecular weight match with the gel band, minimum 2 unique peptides identified, and a minimum of ~10% sequence coverage) based on discussions at the Proteomics Facility (Turku Centre for Biotechnology, Finland).

4.7.3 Co-immunoprecipitation and co-immunofluorescence (I)

For co-immunoprecipitation, HeLa cells were transiently transfected with pCI-DOCK2-FLAG and pcDNA3.1-huFURIN-MYC-His. Cells were lysed (lysis buffer: 50 mM Tris pH 7.5, 10% glycerol, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 50 mM NaF, 1 mM TCEP, and Complete Mini protease inhibitors from Roche) and precleared with protein G-Sepharose 4 FastFlow beads (GE Healthcare). Anti-FLAG was used to capture DOCK2 and anti-Myc for FURIN, in parallel with antibody isotype and resin controls. Protein elutes were separated with SDS-PAGE and transferred to a nitrocellulose membrane. The immunodetection was performed using anti-FLAG or anti-Myc primary antibodies, and an anti-mouse HRP-conjugated secondary antibody. Visualization was done using the ECL Western blotting detection kit (GE Healthcare) and the AGFA CP1000 imaging system.

To study the co-localization of tagged fusion proteins inside the cell, HeLa cells were transiently transfected with pcDNA-RAC1-V5 and pcDNA3.1-huFURIN-Myc-His. Three days post-transfection, cells were fixed on coverslips with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and stained with rabbit anti-FURIN and mouse anti-V5 for RAC. The nucleus was stained with DAPI, and specific protein expression was visualized with anti-rabbit Texas Red or anti-mouse Alexa 488, using an ApoTome microscope and the AxioVision software (Zeiss).

4.8 F-actin polymerization assay (I)

Stable FURIN wild type and control Jurkat E6-1 T cell lines were starved in RPMI 1640 medium supplemented with 1% FBS and stimulated with 250 ng/ml of stromal cell–derived factor-1α (SDF-1α; Peprotech) for 0–120 s. Cells were immediately fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100, stained for polymerized F-actin (Phalloidin-FITC), and analyzed with flow cytometry.

4.9 Cytokine measurements (I, III)

For study I, equal amounts of mouse T cells were cultured in serum-free X-Vivo 20 medium (Lonza), and cytokines were quantified with a cytometric bead array Th1/2/17 kit (BD Biosciences) or IL-4 ELISA (Peprotech) according to the manufacturer's instructions. In study III, an ELISA measurement of serum cytokine levels in atherosclerotic patients and healthy controls was performed according to the manufacturer's instructions [R&D Systems: Quantikine Human BAFF/BLys (DBLYS0) and human APRIL/TNFSF13 (DY884)].

4.10 Quantitative real-time PCR, Q-RT-PCR (I-III)

In publication I, total RNA from mouse T cells was isolated with the RNeasy kit (Qiagen) and reverse transcribed into cDNA with the iScript kit (BioRad). Gene expression levels were analyzed using the SsoFast EvaGreen supermix and normalized to the ribosomal *18s* gene. In publication II, the expression of the zebra fish *tgfβ1a* mRNA was measured from *pcsk7* exon3 + p53 and random control (RC) morphant embryos at 48 hours post-fertilization (hpf). The housekeeping gene elongation factor 1α (*ef1α*) was utilized for normalization, otherwise the protocol followed the one described above. Primers were designed using the PrimerQuest software (Integrated DNA Technologies, Inc.), and the primer sequences are presented in Table 4.

In publication III, total RNA was isolated with the RNAeasy kit (Qiagen) from human CD14+ monocytes, CD4+ T cells, and CD19+ B cells and the mRNA expression of *fur* and housekeeping gene *b2m* were quantified with Q-RT-PCR using primers and probes from Applied Biosystems (Hs00159829_ml). All Q-RT-PCR analyses (I-III) were performed in the CFX96 apparatus together with the CFX Manager Software (BioRad), and relative mRNA expression values were calculated with the delta-delta cycle threshold (ΔΔCt) method.

Table 4. Primers used in Q-RT-PCR analyses (I,II).

Gene	Sequence (5'-3'), FOR-forward, REV-reverse	Amplicon (bp)
Study I		
tbet (mouse)	FOR: TCAACCAGCACCAGACAGAG	110
	REV: AAACATCCTGTAATGGCTTGTG	
gata3 (mouse)	FOR: TTATCAAGCCCAAGCGAAG	75
	REV: TGGTGGTGGTCTGACAGTTC	
il4ra (mouse)	FOR: GACTGGATCTGGGAGCATCA	102
	REV: CAGTCCACAGCGCTATCCAG	
18s (mouse)	FOR: GTGATCCCTGAGAAGTTCCAG	143
	REV: TCGATGTCTGCTTTCCTCAAC	
Study II		
ef1a (zebra fish)	FOR: CTGGAGGCCAGCTCAAACAT	174
	REV: ATCAAGAAGAGTAGTACCGCTAGCATTAC	
tgfβ1a (zebra fish)	FOR: CAGGATGAGGATGAGGACTA	132
	REV: CAGCCGGTAGTCTGGAATA	
stat4 (zebra fish)	FOR: CTCCAGCTTCGAGATCCACT	272
	REV: CTTTGGGCTGTCAGGGTGAA	

4.11 Statistical analyses (I-III)

The nonparametric Mann–Whitney U test was used to determine statistical significance in mouse experiments (I) and atherosclerotic expression analyses (III). The Student's t-test was used in cell line experiments (I) and zebra fish gene expression studies by Q-RT-PCR (II). Survival after a *T. gondii* infection was analyzed using the log-rank (Mantel-Cox) test (I). In all analyses p values <0.05 were considered statistically significant.

4.12 Ethical considerations (I-III)

All experiments in mice were conducted in accordance with the guidelines of the National Animal Experiment Board (Finland) or the National Institutes of Health

Animal Care and Use Committee (National Institutes of Health, Bethesda, MD, USA).

All zebra fish studies were performed in accordance with the Finnish Laboratory Animal Welfare Act 62/2006, the Laboratory Animal Welfare Ordinance 36/2006, and Authorization LSLH-2007-7254/Ym-23 by the national Animal Experiment Board (Finland).

The tissue collection and study of human vascular samples were approved by the Ethics Committee of Tampere University Hospital (Finland), and the clinical investigation followed the principles of the Helsinki declaration.

5 SUMMARY OF THE RESULTS

5.1 FURIN is a central regulator of T cell-mediated immunity in mouse (I)

Previous studies have described FURIN as an essential regulator of T cell biology both in mouse and human (Lund et al., 2004; Pesu et al., 2006; Pesu et al., 2008), however, its detailed function has remained unknown. In order to better understand the multifaceted role of FURIN in T cells, we studied its function utilizing a pathogen challenge, T cell differentiation assays, and finally FURIN specific protein interaction studies in T cells.

5.1.1 FURIN is critical for host resistance against Toxoplasma gondii

T. gondii offers a useful infection model for assessing the role of a gene in cell-mediated immunology, because a strong Th1-mediated immune response is essential for the clearance of the pathogen (Denkers & Gazzinelli, 1998; Gaddi & Yap, 2007). In the acute phase of the infection, the proinflammatory IL-12 has a major role in activating macrophages to produce early IFNγ and tumor necrosis factor (TNF) (Gazzinelli et al., 1994; Reis e Sousa et al., 1997). Since FURIN is regulated in an IL-12 –dependent fashion *ex vivo* (Pesu et al., 2006), we first assessed whether *furin* expression could be induced in splenic CD4+ T cells by a *T. gondii* stimulus *in vivo*. By analyzing *furin* mRNA levels in the splenic CD4+ T cells from wild type and IL-12 deficient mice infected with the *T. gondii* ME-49 strain, we demonstrated that *furin* expression is induced and regulated by IL-12 also during a parasite infection *in vivo* (I, Fig. 1A). To further study the role of FURIN in the host response, we infected CD4cre-fur$^{f/f}$ and littermate control mice with *T. gondii*. There were no significant differences between the groups during the early IL-12-dependent phase of the infection, including mouse survival and serum cytokine levels (I, Fig. 1B and Suppl. Fig. 1). However, in six weeks all of the infected CD4cre-fur$^{f/f}$ mice succumbed to the infection (I, Fig. 1B). The chronic phase of the *T. gondii* infection is generally characterized by parasite specific T cells, which secrete high levels of IFNγ and TNF

needed to prevent reactivation of the brain cysts. Also the immunosuppressive IL-10 plays an important role in the control of excessive immune responses, thus the lethality of the CD4cre-fur$^{f/f}$ mice could be explained by a defective immune response or alternatively by a massive inflammatory response. For this reason, we analyzed the brain cyst counts in moribund animals and found significantly more intracranial cysts in CD4cre-fur$^{f/f}$ mice compared to wild type controls suggesting a defect particularly in protective host responses (I, Fig. 1C).

Further, the splenocytes from infected animals were restimulated *ex vivo* with a soluble *T. gondii* antigen (STAg), which revealed further specific details on the function of FURIN. A flow cytometric analysis disclosed that the antigen specific Th1 and Th1-IL-10+ cell populations as well as the levels of secreted IFNɣ, TNF, and IL-10 were diminished in splenocytes derived from CD4cre-fur$^{f/f}$ mice indicating an essential role for FURIN in T cell –mediated host resistance against *T. gondii* (I, Fig. 1D-E, Suppl. Fig. 2).

5.1.2 Th1/Th2 balance is affected by FURIN deficiency

Next we studied T cell proliferation in the absence of FURIN to find out if this could explain the defective Th1 response in a *T. gondii* infection. CD4cre-fur$^{f/f}$ mice were crossed with TCR transgenic (OTII) mice, and naïve CD4+ T cells were stimulated in neutral culture conditions with varying concentrations of ovalbumin (OVA), a cognate antigen peptide. In wild type cells, a weak TCR stimulus leads to Th2 polarization and IL-4 production, whereas a stronger activation induces Th1 differentiation and IFNɣ secretion (Yamane et al., 2005). However, FURIN deficient T cells failed to respond correctly to an increasing TCR stimulus, and showed an intrinsic predominant Th2 phenotype at all OVA concentrations (I, Fig. 2). To find out if this was a result of altered TCR signaling or cytokine responses, we studied FURIN deficient T cells in more detail.

To reveal the role of individual cytokines in FURIN regulated T cell polarization, we used serum-free culture conditions to exclude all the sources of extracellular cytokines, including the FURIN substrate TGFβ1, which is present in the serum (Ghoreschi et al., 2010). The analysis of IL-4 production after a strong polyclonal stimulation by ELISA verified the previous finding of excessive IL-4 secretion as early as 24 hours after the TCR stimulation in FURIN deficient T cells (I, Fig. 3A). By using Q-RT-PCR analysis we detected an increased *gata3/tbet* transcription factor ratio in the absence of FURIN, which illustrates the polarization

defect also at the level of transcription regulation (I, Fig 3B). Further, we studied the responsiveness of FURIN deficient CD4+ Th cells to the IL-12 and TGFβ1 cytokines that drive Th1 and Th17/Treg differentiation, respectively (I, Fig. 3B-C). In response to an IL-12 stimulation, both wild type and FURIN deficient T cells showed increased IFNɣ secretion, but IL-12 failed to downregulate Th2 dominance (i.e. *gata3* expression, and IL-4/IL-13 production) in FURIN deficient CD4+ Th cells. Earlier it has been shown that FURIN deficient T cells respond to extracellular TGFβ1 by upregulating *foxp3* (Pesu et al., 2008). Although exogenous TGFβ1 was sufficient to decrease IL-4 secretion, FURIN deficient T cells failed to downregulate *gata3* expression and IL-13 secretion. In conclusion, a serum-free polarization experiment revealed that naïve FURIN deficient T cells were intrinsically Th2 biased, but could still respond to IL-12 and TGFβ1 stimuli.

5.1.3 FURIN inhibits IL-4Rα surface expression on naïve Th cells

During Th2 polarization, TCR activation leads to early IL-2/STAT5 mediated *gata3* transcription, and later to the activation of the IL-4/STAT6 pathway, which ultimately result in the production of IL-4, IL-5, IL-13, and IL-33 (Liao et al., 2008; Zhu et al., 2010). As was mentioned, the strength of the TCR stimulus can affect the level of ERK phosphorylation and further T cell polarization. However, we did not find signs of diminished ERK activation in response to an anti-CD3 stimulation in FURIN deficient T cells by a Western blot analysis (I, Fig. 4A). An investigation of thymic GATA-3 and T-bet levels by flow cytometry did not disclose any developmental differences between FURIN deficient T cells and wild type controls (I, Suppl. Fig. 3), leading to the hypothesis that FURIN could regulate the Th2 amplification phase through the IL-4/STAT6 pathway rather than by affecting thymocyte development or inhibiting initial TCR activation. To directly test this we inhibited the IL-4/STAT6 pathway with a neutralizing anti-IL-4 antibody on naïve T cell cultures and measured *gata3* mRNA expression levels by Q-RT-PCR after 72 hours of stimulation. Notable, the skewed Th2 phenotype of FURIN deficient T cells was returned to wild type levels by blocking IL-4 in cell cultures *in vitro* (I, Fig. 4B).

Next, we analyzed the mRNA expression and cell surface protein levels of IL-4 receptor alpha subunit (IL-4Rα), which mediates the IL-4 cytokine signal into the cell. On the mRNA level we did not observe any differences in *il4ra* expression (I, Fig. 4C), whereas by quantifying the expression of the cell surface receptor, we

observed elevated levels of IL-4Rα on naïve CD4+CD62L+ FURIN deficient T cells (I, Fig. 4D). The increase in surface expression resulted in a sensitized IL-4 response as was shown by higher levels of STAT6 phosphorylation in FURIN deficient T cells in response to a 15-minute IL-4 stimulus (I, Fig. 4E). Collectively, FURIN was shown to regulate the IL-4/STAT6 pathway by inhibiting the surface expression of the IL-4Rα protein in naïve CD4+ T cells, and thus control the Th1/Th2 balance.

5.1.4 RAC/DOCK2 is a novel FURIN interacting complex in T cells

By exploring FURIN interaction partners in T cells, we sought to find key molecules involved in the regulation of T cell biology, including Th differentiation. First, we cloned FURIN - Strep-tag fusion constructs encompassing human wild type or enzymatically inactive FURIN, and generated Jurkat T cell lines stably expressing FURIN - Strep-tag fusion proteins or an empty control plasmid. Next, we captured FURIN interactomes from cell membrane lysates using Strep-tag affinity purification, and analyzed them on silver stained gels. Finally, we identified 12 candidate proteins from a 20 kDa band, which had a higher intensity in the wild type FURIN containing elutes than in inactive mutant FURIN elutes (I, Fig. 5A-C). After automated peptide database searches, we compared the molecular weight, sequence coverage, and the number of identified peptides of each of the candidates. Finally, Ras-related C3 botulinum toxin substrate 1-3 (RAC1-3) was specifically identified in elutes containing wild type FURIN.

RACs are small GTPases activated by dedicator of cytokinesis 2 (DOCK2), and together they play an important role in T cell biology. Interestingly, RAC can directly promote Th1 lineage commitment by upregulating IFNɣ (Li et al., 2000), and together with DOCK2 it serves as a critical regulator of IL-4Rα trafficking in T cells (Fukui et al., 2001; Tanaka et al., 2007). To verify our MS finding, we co-expressed tagged fusion proteins of FURIN, RAC1, and DOCK2 in HeLa cells. FURIN and RAC1 were found to co-localize (I, Fig. 5D), whereas FURIN and DOCK2 were captured together by co-immunoprecipitation (I, Fig. 5E). Since DOCK2 mediated RAC activation leads to changes in the T cell cytoskeleton, we wanted to investigate whether FURIN could promote the polymerization of F-actin. To this end, Jurkat T cells stably overexpressing wild type FURIN or empty vector were stimulated with the chemotactic SDF-1α cytokine for 15-120 seconds, and polymerized F-actin was stained and analyzed by flow cytometry. The cells overexpressing wild type FURIN

showed increased F-actin polymerization both at steady state and in response to SDF-1α (I, Fig. 5F). Based on our interaction studies, we thus propose that FURIN interacts with the RAC/DOCK2 complex and regulates the polymerization of F-actin, which has been earlier linked to the downregulation of IL-4Rα on the cell surface and the inhibition of Th2 polarization *in vivo* (Croker et al., 2002; Li et al., 2000; Tanaka et al., 2007). Collectively, our results indicate that FURIN expression is critical for the host response against *T. gondii*, and FURIN regulates the Th balance by inhibiting Th2 hyperpolarization. Potential mechanisms include the RAC/DOCK2 mediated down-regulation of the IL-4Rα protein on the cell surface.

5.2 PCSK7 is essential for zebra fish development and TGFβ1a regulation (II)

The phenotypic characterization of the evolutionarily most ancient PCSK enzyme, PCSK7, in knockout mice has been partially incomplete (Constam et al., 1996; Villeneuve et al., 2002; Wetsel et al., 2013). However, in lower vertebrates such as *Xenopus,* PCSK7 has been shown to be indispensable (Senturker et al., 2012). To address this conundrum further, we used a zebra fish model to assess how PCSK7 regulates vertebrate development and gene expression during embryogenesis. First, we searched for human PCSK orthologues in the zebra fish genome, and found matches for all but two PCSK family members (PCSK4 and PCSK6), whereas FURIN and PCSK5 were represented as two isoforms. Next, we used Q-RT-PCR to characterize PCSK gene expression levels during zebra fish development, and in various adult tissues. PCSKs showed variable expression patterns both during development and in a tissue specific manner, *pcsk7* being expressed highly in the brain, eye, and female gonads (II, Fig. 1). Based on homology modeling we concluded that the human and zebra fish PCSK7s showed remarkable structural similarities, but also some electrostatic differences that may affect their substrate binding capacity (II, Fig. 2-3). A previous analysis of the two FURIN isoforms demonstrated that especially FurinA is essential for zebra fish development and survival (Walker et al., 2006), which we could also verify using morpholino (MO) technology. Next, we wanted to inhibit the activity of PCSK7 in developing zebra fish embryos.

5.2.1 Embryogenesis and immune system gene expression are affected by PCSK7 silencing

We silenced PCSK7 in developing zebra fish by targeting exon 3 and exon 8 in PCSK7 with MO injections. The MOs were designed to result in the erratic splicing of the *pcsk7* pre-mRNA either by deleting (exon 3) or truncating (exon 8) nucleotides from the *pcsk7* mRNA (II, Fig. 6). All PCSK7 deficient embryos succumbed by 7 dpf showing abnormal development of the brain, eye, heart, and tail (II, Fig. 4, 7). We also observed an abnormal number of otoliths, inner ear bones, in PCSK7 deficient embryos (II, Fig. 8). The experiment was controlled with random controls (RC) and p53 MO injections, since p53 has been claimed to mediate off-target neural toxicity in MO experiments (Bedell et al., 2011; Eisen & Smith, 2008). The PCSK7+p53-MO injections mimicked the phenotype and survival of the PCSK7-MO injected embryos, whereas RC-MO did not disturb the development of the zebra fish. In addition, the *in vitro* transcribed *pcsk7* mRNA rescued the severity of the phenotype partially (II, Fig. 5), demonstrating the specificity of our MOs in targeting PCSK7.

Next, we analyzed the genome-wide mRNA expression pattern in PCSK7 deficient embryos at the gastrula stage (6 hpf), and later (24 hpf) when the gene expression stays relatively unchanged in wild type zebra fish (Mathavan et al., 2005). No changes in the expression levels of the PCSK enzymes were found. However, the expression of *pcsk7* itself was significantly reduced at 24 hpf, which might result from the cellular deletion of the MO targeted, defectively spliced pre-mRNA molecules. In addition, various gene categories were enriched at 6 hpf (development and transcriptional regulation), and at 24 hpf (metabolism) arguing for a critical PCSK7 dependence during development. Upregulated genes included the DNA-binding proteins paired box 2a (*pax2a*) and muscle segment homeobox C (*msxc*), as well as the extracellular follistatin a (*fsta*), whereas the otolith development related RNA-binding fox-1 homolog (*fox1*) was downregulated (II, Fig. 9).

Further, factors related to the immune system, including many cytokine signaling genes, were highly enriched at both time points. The downregulation of *stat4, tgfβ1a*, and colony stimulating factor 1 receptor a (*csfra*) links PCSK7 to the regulation of the innate and adaptive immunity. As was mentioned, FURIN has been shown to be a key regulator of mouse and human Th1 and Treg cells, in which STAT4 and TGFβ1 also play a critical role, respectively (Pesu et al., 2006; Pesu et al., 2008). Further, we verified the specific down-regulation of the *stat4 and tgfβ1a* genes also by Q-RT-PCR analysis in PCSK7 deficient embryos at 6 hpf.

5.2.2 PCSK7 regulates the expression and proteolytic cleavage of TGFβ1a

The discovery that the expression of *tgfβ1a* is downregulated in PCSK7 deficient zebra fish prompted us to study the role of PCSK7 in TGFβ1a biology in more detail. In addition to 6 hpf, we found *tgfβ1a* to be significantly downregulated also at the 48 hpf time point in PCSK7 silenced zebra fish embryos (II, Fig.10B). Based on mammalian studies, it seems that FURIN is the main activator of pro-TGFβ1, and mature TGFβ1 can directly upregulate FURIN expression (Blanchette et al., 1997; Dubois et al., 2001; Pesu et al., 2008). Also PCSK7 can proteolytically activate many mammalian TGFβ superfamily cytokines including bone morphogenetic protein 4 (BMP4) (Nelsen & Christian, 2009), and its expression has been shown to correlate with human *tgfβ1* (Turpeinen et al., 2011). However, the role of PCSK7 in pro-TGFβ1 maturation is incompletely understood.

To this end, we amplified pro-TGFβ1a from zebra fish cDNA, and subcloned it into a Myc-His expression vector. We verified the construct by sequencing, and co-expressed pro-TGFβ1a together with vectors encoding the zebra fish (zf) PCSKs FurinA, FurinB, or PCSK7 in FURIN-deficient hamster RPE.40 cells. For comparison, we also co-expressed human pro-TGFβ1-Myc-His together with human FURIN or PCSK7 encoding vectors in RPE.40 cells. The pro-TGFβ1 in the cell lysates and the secreted mature TGFβ1 from the supernatants were detected by Western blot using an anti-Myc antibody (II, Fig. 10A). To compare the efficiency of each PCSK in processing its substrate, we quantified the intensity of the pro-form and mature TGFβ1 bands, and defined the relative processing ratio. In line with previous result, human FURIN was highly effective in pro-TGFβ1 processing (a 6.4-fold increase compared to a transfection with TGFβ1 only), but also human PCSK7 cleaved the precursor effectively (3.4-fold). Interestingly, in addition to zf-FurinA (6.9-fold), also the zf-PCSK7 (2.3-fold) was active in pro-TGFβ1a processing, whereas zf-FurinB (1.5-fold) was inefficient in processing the pro-TGFβ1a in this *in vitro* experiment. Finally, we demonstrated that silencing TGFβ1a in developing zebra fish with MO injections results in phenotype similar to the PCSK7 inhibition characterized by organ abnormalities and an incorrect number of otoliths (II, Fig. 10C-D). To our knowledge, this is the first demonstration of the ability of zf-PCSK7 to regulate TGFβ1a expression and bioavailability in zebra fish. Collectively, we showed that PCSK7 is critical for zebra fish embryogenesis and immune gene expression, and that this phenotype can be at least partially explained by defects in TGFβ1a regulation.

5.3 FURIN is upregulated in human atherosclerotic plaques (III)

Atherosclerosis is a complex chronic disease characterized by endothelial injury, inflammation, and dysregulated lipid metabolism (Libby et al., 2011). PCSKs play a critical role in atherogenesis because many of the pro-atherosclerotic proteins are synthesized as inactive proproteins. Earlier, PCSKs have been linked to lipid metabolism and atherosclerotic inflammation (Abifadel et al., 2009; Perisic et al., 2013; Stawowy & Fleck, 2005), but a systemic analysis of the entire protease family and their substrate molecules has been lacking.

We used a genome-wide expression array (GWEA) to analyze the mRNA expression of the PCSK family members and their substrate molecules in atherosclerotic patients (III, Table 1-2). We analyzed vascular plaques from femoral and carotid arteries as well as abdominal aortas, whereas LITAs were used as controls to represent a non-atherosclerotic artery region. Interestingly, FURIN was the only PCSK to be significantly overexpressed in all of the atherosclerotic samples (>1.4-fold upregulation), whereas PCSK5 and PCSK8 were markedly downregulated (III, Fig. 1). We also analyzed the expression of 31 established PCSK substrate genes and found that most of them were upregulated in atherosclerotic samples (III, Table 2). These substrates included matrix metalloproteinases (MMP1, MMP11, MMP14, ADAM8, ADAM10, ADAMTS4) and two chemokines (CXCL9, CXCL10), which have been shown to influence tissue remodeling and inflammation in atherosclerotic plaques, respectively (Oksala et al., 2009; Stawowy et al., 2005b; Zernecke et al., 2008). In addition, we identified two highly related TNF family cytokines to be significantly upregulated in all of the atherosclerotic plaques, namely tumor necrosis factor ligand superfamily member 13 (APRIL/TNFSF13) and 13B (BAFF/TNFSF13B). These B cell activating cytokines, which are substrates of FURIN, have not been linked to atherosclerotic inflammation earlier.

Next, we verified the GWEA results by a Q-RT-PCR analysis and concluded that FURIN (up to 77-fold), and BAFF and APRIL (both up to 3-fold) were significantly overexpressed in atherosclerotic plaques, carotic artheries being the most prominent site (III, Fig. 1). By immunohistochemical staining we demonstrated the co-localization of FURIN, BAFF, and APRIL in plaque CD68+ macrophages (III, Fig. 2). In addition, CD3+ T cells and CD20+ B cells showed high levels of FURIN expression, whereas endothelial or smooth muscle cells were not stained by the FURIN antibody (III, Fig. 2). Finally, we wanted to investigate whether FURIN protein levels were higher in inflammatory plaques than in healthier vascular regions by Western blot analysis. Due to obvious ethical reasons, we were not able to use

arteries from healthy donors, instead we compared the femoral artery plaque regions to a macroscopically healthy vascular sample from the same patient. Also these data support our finding that FURIN is locally upregulated in the atherosclerotic plaque region (III, Suppl. Fig. 2).

5.3.1 Expression of FURIN is induced upon immune cell activation

The immunohistochemical staining, which shows that FURIN co-localizes with immune cells in plaques, prompted us to study the expression of FURIN in various immune cell populations in more detail. To this end, we isolated human CD14+ monocytes, CD4+ T cells, and CD19+ B cells from the peripheral blood collected from healthy donors. These cells were treated with typical immune response activators, and FURIN expression was measured by Q-RT-PCR (III, Fig. 2D). As was demonstrated earlier (Pesu et al., 2006; Pesu et al., 2008), FURIN was induced by anti-CD3/CD28 TCR activation in CD4+ T cells (7-fold upregulation). Interestingly, we found that TLR-4-mediated LPS activation in CD14+ monocytes also induced FURIN mRNA expression (11-fold upregulation). This novel finding suggests that FURIN is induced also in innate immune cells in response to activation. In contrast, FURIN induction was not demonstrated in a human CD19+ B cell population in response to stimulation with a TLR-7/8 agonist. Further, a SNP in the promoter region of FURIN found in a Chinese population has been shown to affect the expression of FURIN and the outcome of a hepatitis B infection (Lei et al., 2009). However, that particular SNP or other SNPs that were selected from the FURIN locus did not associate with upregulated FURIN expression in our artery sample set (III, Suppl. Table 1).

5.3.2 BAFF and APRIL levels are not elevated in atherosclerotic patient serum

Expression analyses and immunohistochemical findings demonstrated that FURIN and its substrate cytokines BAFF and APRIL are upregulated in atherosclerotic plaques (III, Table 2, Fig. 1-2). Previously, the serum levels of BAFF and APRIL have been linked to immunological pathologies such as systemic lupus erythematosus or autoimmune hepatitis (Matsushita et al., 2008; Migita et al., 2007; Migita et al., 2010). To investigate whether elevated BAFF/APRIL levels could also be diagnosed from atherosclerotic patient serum, we conducted ELISA measurements from severe atherosclerotic patients and healthy controls. We could

not observe any significant differences between the groups (III, Suppl. Fig. 1), which argues for a more local role for these cytokines in atherogenesis. In conclusion, we demonstrated that FURIN is the most prominent PCSK to be upregulated in atherosclerotic plaques, and its expression is induced upon immunological activation both in CD4+ T cells and in macrophages. Finally, the FURIN substrate cytokines BAFF and APRIL were identified as novel factors in local atherosclerotic plaque inflammation.

6 DISCUSSION

The immune system has evolved to protect us against external threats as well as misbehaving cells. In order to achieve and quench powerful immune responses the system must be under strict regulation. Overactive immune cells lead easily to autoimmune reactions, whereas insufficient responses might expose the organism to otherwise harmless pathogens. PCSKs mediate the endoproteolysis of inactive proproteins, and these proteases critically regulate cellular homeostasis and development of various diseases. However, the role of PCSKs in immunity has been incompletely understood. In the present study, we demonstrated that the most studied PCSK enzyme FURIN is critical for maintaining the Th1/Th2 balance and the mouse host response against intracellular parasitic infections (I). Further, we used zebra fish to study the role of the most ancient of these enzymes, PCSK7, in development, immune system gene regulation and TGFβ1a cytokine bioavailability (II). In addition, patient samples were used to investigate the PCSK family in human atherosclerotic plaques and immune cell activation (III). Altogether, the novel findings described in these studies indicate critical and partially non-redundant functions for the PCSK enzymes in the regulation of the immune system and cellular homeostasis.

6.1 PCSKs mediate both redundant and unique immunomodulatory functions

The current knowledge on the function of PCSKs is mainly based on *in vitro* cleavage assays using recombinant proteins. However, information on their redundant and unique functions in cellular homeostasis and pathologies is still elusive (Seidah & Prat, 2012). In the present study, we used both developmental stage and tissue-specific zebra fish samples (II), and human atherosclerotic patient data (III) to determine the expression patterns of the entire PCSK family in these physiological contexts.

We could characterize all but two PCSKs, PCSK4 and PCSK6, in the zebra fish genome, and FURIN and PCSK5 were represented as two isoforms. We found

similarities in the PCSK tissue expression profiles between zebra fish and higher vertebrates, including a neural tissue specificity for PCSK1-2. Further, zebra fish PCSK7 showed highly conserved characteristics in our homology alignment comparing the amino acids of the human PCSK7 to other organisms. Homology models of human and zebra fish PCSK7 revealed small differences in electrostatic properties, which might propose different substrate preferences between organisms. To our knowledge, a PCSK7 mutant zebra fish has not been published, and novel genome editing technologies such as CRISPR-Cas9 were not yet available at the time of the study (Hwang et al., 2013). Thus, we silenced PCSK7 with the antisense morpholino technology, which offers an effective, albeit a slightly questionable method for studying specific gene functions in developing zebra fish. MO silencing has been reported to result in p53-dependent off-target neural toxicity (Bedell et al., 2011; Eisen & Smith, 2008), which we controlled by silencing p53 together with PCSK7. In addition, an mRNA rescue experiment restored the function of PCSK7 partially, which could argue that a specific spatiotemporal expression of the endogenous zebra fish PCSK7 might be needed to fully rescue the silenced PCSK7 phenotype. Given the fact that PCSK7 was found to be indispensable for embryogenesis also in *Xenopus* (Senturker et al., 2012), we suggest that PCSK7 might have a non-redundant role in development in lower vertebrates. In mouse, PCSK7 deficiency is linked to memory and learning difficulties, but these mice are otherwise viable (Wetsel et al., 2013). The difference between the need for PCSK7 expression might be explained by spatiotemporal expression differences in lower and higher vertebrates, or by redundant functions of the other PCSKs in developing mammalian embryos, especially as zebra fish lack the PCSK4 and PCSK6 enzymes.

Further, a genome-wide mRNA analysis of the PCSK7 silenced embryos revealed a link between immunological pathways and PCSK7 activity. We found changes in PCSK7-dependent genes of both the innate and adaptive immunity, including *stat4, tgfβ1a*, and *csfra,* and PCSK7 demonstrated proteolytic activity on pro-TGFβ1 *in vitro*. All of these factors have well established functions in mammalian immunity, and there is evidence that these proteins would mediate parallel roles also in zebra fish (Lam et al., 2002; Lin et al., 2014; Mitra et al., 2010; Rombout et al., 2014), but the detailed mechanisms of their function in lower vertebrate immunity remains to be determined. The immunomodulatory function of PCSK7 is further supported by the fact that it is highly expressed in mammalian T cells (Elhage et al., 2015; Pesu et al., 2006) and myeloid cells (Lansac et al., 2006), and it might shape the function of Tregs (de Zoeten et al., 2009; Elhage et al., 2015). In addition, human PCSK7 is essential for the rescue of unstable antigen presenting MHC-I molecules

(Leonhardt et al., 2010) and it can activate TLR-7 (Hipp et al., 2013). Although the phenotype of PCSK7 deficient mice indicated that the enzyme is critical to memory and learning processes (Wetsel et al., 2013), it would be interesting to study these mice also during an immunological challenge other than LPS administration, where the survival of the knockout mice did not differ from the wild type ones (Refaie et al., 2012). Based on our novel results and previous studies, PCSK7 deficient mice might suffer from defective antigen presentation or lack of immune tolerance. Whether higher vertebrates have evolved more redundant PCSK functions to ensure the availability of critical biomolecules, or whether PCSK7 has, during evolution, acquired a critical regulatory role in development in lower vertebrates, remains to be investigated.

PCSKs proteolytically target many proproteins, which have been demonstrated to critically affect the progression of common diseases such as atherosclerosis. Earlier, scattered findings in the literature have suggested an accelerative role for FURIN, PCSK5, and PCSK6 in atherosclerosis (Perisic et al., 2013; Stawowy & Fleck, 2005; Stawowy et al., 2005a; Stawowy et al., 2005b), whereas PCSK8-9 have been shown to target systemic lipid metabolism (Maxwell & Breslow, 2004; Seidah & Prat, 2007). In contrast to the aforementioned studies, our analysis of PCSKs expression in atherosclerotic plaques revealed that only FURIN was significantly overexpressed in all vascular plaques, whereas PCSK5 was modestly downregulated (III). This contrary finding might arise from differences in the experimental set-up, as earlier studies mainly utilized immunohistochemistry and *in vitro* cell cultures to demonstrate the dominant overexpression of PCSK5 in rodent vascular arteries and sporadic human samples (Stawowy et al., 2005a; Stawowy et al., 2005b). Another recent PCSK expression profiling study in human atherosclerotic samples by Perisic and colleagues showed PCSK6 to be the most overexpressed convertase, whereas FURIN was downregulated in their sample set (Perisic et al., 2013). In contrast to our study, they used different vascular regions and compared them to iliac arteries, which could affect the outcome. In addition, technical aspects in gene expression analysis may lead to different results. Perisic and colleagues also demonstrated PCSK6 to co-localize with vascular smooth muscle cells in the fibrous cap, whereas we found FURIN mainly in the immune cells of plaques. In the future, it would be interesting to further explore the cell type specific functions for FURIN, PCSK5, and PCSK6 in atherosclerosis. In addition, our data also further support the hypothesis that PCSK8 and PCSK9 regulate central lipid metabolism rather than the local environment in atherosclerotic plaques, since the expression of these PCSKs was not found to be affected in our analysis. A more detailed understanding of the

redundant and unique PCSK-mediated functions in inflamed arteries could lead to therapeutic and diagnostic interventions, which are very much in demand, and ultimately to the reduction of coronary heart disease, which is the primary cause of mortality in developed countries (Libby et al., 2011).

6.2 Expression of FURIN is critical for immune cell function

The ubiquitously expressed FURIN has been demonstrated to have an essential role in cellular homeostasis and the pathogenesis of several diseases (Seidah & Prat, 2012; Thomas, 2002), including an immunoregulatory function in T cells and in peripheral tolerance (Pesu et al., 2006; Pesu et al., 2008). In the present study, we demonstrated that FURIN is critical for the differentiation of CD4+ T cells (I), and found that FURIN is induced also in macrophages in response to immune activation (III).

An immune challenge with the Th1 inducing pathogen *T. gondii* indicated that IL-12 is essential for FURIN induction also *in vivo,* confirming earlier results based on expression profiling and *in vitro* activation experiments (Lund et al., 2004; Pesu et al., 2006). All mice with FURIN deficient T cells succumbed to the *T. gondii* challenge during the chronic phase of the infection characterized by higher brain cyst counts as well as impaired antigen-specific Th1 and Th1-IL-10 responses, which play a critical role in the host response against *T. gondii* (Jankovic et al., 2007). Although these findings together with the antigen specific *ex vivo* stimulation of splenic CD4+ T cells point to a defect in Th1 cell function, we cannot truly rule out the impact of exhausted CD8+ T cells (Luetjen et al., 2006). These cells mediate a critical function in the immune response against *T. gondii* both in the acute and chronic phase of the infection by cytokine secretion and direct cytolytic abilities (Bhadra et al., 2011). Interestingly, in our conditional FURIN deficient mouse model also CD8+ T cells are targeted by the Cre-recombinase during the CD4+CD8+ double positive phase in the thymus (Lee et al., 2001; Pesu et al., 2006). Further studies are clearly needed to understand how FURIN regulates the cytotoxic function of CD8+ T cells. In addition, the unbalanced Th1/Th2 polarization was also demonstrated *ex vivo*, and the FURIN deficiency ultimately lead to Th2 hyperpolarization and increased IL-4Rα expression on the cell surface. Collectively, these novel findings prompted us to study the T cell specific FURIN interactome, which is discussed in section 6.3.

In addition to CD4+ T cells, we also demonstrated that FURIN was highly expressed in human macrophages, and that B cells migrated into the atherosclerotic plaques (III). Interestingly, a previous study showed that PCSK1 has a critical role

in macrophage function in mice (Refaie et al., 2012), and also FURIN has been linked to macrophage chemotaxis in response to an adipocytokine stimulus (Kappert et al., 2013). Importantly, a role for PCSKs in B cell biology has not, to our knowledge, been described. Given the fact that both of these cell types actively secrete several cytokines and process antigens, PCSKs could participate in the regulation of pro- and anti-inflammatory cytokine bioavailability or antigen presentation to further shape the course of an immune response. Notably, a few studies have demonstrated that both FURIN and PCSK7 regulate MHC-I loading complexes during antigen presentation via different mechanisms (Leonhardt et al., 2010; Medina et al., 2009). Whether this is a cell type or PCSK specific phenomenon remains to be determined. Further analyses in human peripheral blood immune cells showed that in response to a LPS stimulation monocytes upregulate FURIN expression, whereas a TLR-7/8 agonist stimulation in B cells failed to induce FURIN. Whether a different experimental setting or stimulus is needed for FURIN expression in B cells remains to be determined. Next, it would be interesting to study the role of FURIN in these cell types comprehensively by crossing the mice with loxP-flanked *furin* with immune cell specific Cre-recombinase promoters including LysM for myeloid cells (Clausen et al., 1999), and CD19 for B cells (Rickert et al., 1997).

A better understanding of the biology of FURIN would help us understand why the excessive or defective function of FURIN has been linked to various diseases and infections. Based on previous studies covering rheumatoid arthritis (Lin et al., 2012), multiple sclerosis (Shiryaev et al., 2009), and several cancers (Bassi et al., 2003; Bassi et al., 2001; Senzer et al., 2012), FURIN seems to play an important regulatory role in the immune system. In addition, several pathogens are activated by FURIN making it an important target for treating infections (Artenstein & Opal, 2011) or a possible diagnostic tool (Kumar et al., 2014; Matsushita et al., 2008; Migita et al., 2007). Although previous data on FURIN deficient T cells suggested that FURIN inhibition could be used to boost the immune response (Pesu et al., 2008), our novel findings demonstrate that FURIN silencing in T cells might misbalance Th cell polarization and make the host susceptible to intracellular pathogens.

6.3 Interaction studies reveal new concepts of PCSK-mediated cellular regulation

Protein-protein interactions offer a vital field of study for the goal of better understanding the physiological function of a particular protein. PCSKs

proteolytically modify their interaction partners from intra- or extracellular sources, and thus they play a major regulatory role in protein bioavailability, in addition they can indirectly participate in the regulation of gene expression. In this study, we deepened our understanding of the well-known PCSKs' targets TGFβ1 (I, II) and BAFF/APRIL (III), and found novel interactions candidates for FURIN (I) and characterized the PCSK7-dependent expression of several genes (II). Collectively, these findings reveal the sophisticated regulation behind protein activation in various cell types and during different immune challenges.

Given the fact that various members of the TGFβ superfamily are cleaved by several PCSKs (Nelsen & Christian, 2009), and that *in vitro* studies suggest a secondary role for PCSK7 in TGFβ processing (Dubois et al., 2001), we propose that the proteolytic regulation of TGFβ1 maturation is more complex than was initially though. Our novel finding indicates that zebra fish TGFβ1 expression is regulated also by PCSK7, and that at least *in vitro* both the human and zebra fish PCSK7s are capable of processing this cytokine (II). In addition, our data reveal that the two zebra fish FURIN isoforms are not equally efficient in TGFβ1a processing, FurinA being the major protease. A recent zebra fish study also demonstrated that FurinA, in particular, regulates the immune response during a *Mycobacterium marinum* infection (Ojanen et al., 2015), raising the question of whether FurinB even has a role in zebra fish immunology. As was discussed earlier, the expression of FURIN and TGFβ1 are tightly coupled (Blanchette et al., 1997). Interestingly, associations found in human studies (Turpeinen et al., 2011), and now in zebra fish, suggest that also PCSK7 participates in the regulation of TGFβ1 expression. Whether this arises from a direct proteolytic event, or is mediated through unknown indirect mechanisms, remains to be demonstrated. Lower vertebrates such as zebra fish demonstrate partially different PCSK expression profiles compared to mammals, arguing for the complex spatiotemporal regulation of TGFβ1a activation.

In mouse, the loss of TGFβ1 expression in T cells results in a partially similar phenotype as does the loss of T cell expressed FURIN (Li et al., 2007; Pesu et al., 2008). However, minor differences between these mice argue that FURIN must have additional substrates in T cells. In this study we verified that FURIN expression is not fully necessary for the T cell response to TGFβ1 stimulation (I), although FURIN defective Tregs have been shown to be incapable of achieving a functional peripheral tolerance (Pesu et al., 2008). Whether other PCSKs, including PCSK7, can act partially redundantly in T cells remains to be investigated. Collectively, our novel data on TGFβ1 regulation indicates that this central cytokine is tightly regulated by FURIN and PCSK7 in a context-dependent fashion.

In addition to deepening our understanding of established PCSK interaction partners including TGFβ1, we explored novel interactions by various techniques. Firstly, we used a direct affinity purification method to capture T cell specific FURIN interactions and identified them by mass spectrometry (I). Various cell lysis protocols were tested to enhance the amount of FURIN amount in the elutes, and relatively strict conditions might have resulted in the loss of weaker or transient interaction partners. Finally, we were able to characterize a novel interaction complex FURIN-RAC-DOCK2, and re-constitute this interaction *in vitro*. Notably, we used both wild type and inactive human FURIN fusion proteins to distinguish interactions specific for the intact protein. Our novel interaction candidates, the small GTPase RAC and its activator DOCK2 have been shown to regulate T cell biology via various mechanisms. Mechanistically, RAC/DOCK2 activity regulates actin rearrangements and cytoskeleton dynamics (Nishikimi et al., 2013). In peripheral T cells, RACs regulate TCR signaling and apoptosis (Arrieumerlou et al., 2000; Ramaswamy et al., 2007), interestingly they also promote Th1 polarization through the induction of IFNɣ (Li et al., 2000). In our functional experiments, FURIN was shown to regulate the polymerization of F-actin in Jurkat T cells. This is in accordance with a previous finding on FURIN mediated F-actin regulation on vascular smooth muscle cells (Kappert et al., 2010). However, to truly demonstrate the biological meaning of this novel finding, more physiological experiments would be needed. Whether FURIN interacts with RAC/DOCK2 as a chaperon or activating protease, or through a larger protein complex, remains to be determined. Further, quantitative mass spectrometry could offer a better resolution for capturing interactions, and a comparison between resting and activated T cells could reveal new aspects on the dynamics of FURIN-mediated T cell regulation.

In addition to direct protein-protein interactions, we investigated links between gene expression levels associated either with PCSK7 deficiency (II) or atherosclerosis (III). Interestingly, a microarray analysis of the PCSK7 deficient zebra fish revealed a strong link between PCSK7 and immune system genes, including the earlier discussed *tgfβ1a, stat4*, and *csfra* (II). Those genes associate with the innate and adaptive immune systems indicating that the PCSK7 could regulate zebra fish immunity. In addition, we saw changes in many developmental genes, which was also obvious from the lethal phenotype of the PCSK7 deficient zebra fish. A genome wide expression analysis on human atherosclerotic plaques offered a way to examine the entire PCSK family and their known substrate molecules in a disease model (III). Interestingly, two substrate cytokines of FURIN, BAFF and APRIL, were significantly upregulated in our data set. FURIN has a key role in the

regulation of the bioavailability of BAFF and APRIL, and the soluble forms of these cytokines further mediate essential regulation of B cells (Bossen et al., 2011; Migita et al., 2007; Vincent et al., 2014). The neutralization of BAFF has been shown to be an effective method for treating systemic lupus erythematous (Vincent et al., 2014), and our atherosclerotic data suggest a potential therapeutic use for the silencing of either FURIN, BAFF or APRIL. As a technical aspect, we included a Q-RT-PCR verification for selected genes in both publications (II, III). Importantly, these kinds of gene expression analyses do not reveal causal relationships, and thus additional methods have to be used to demonstrate causes and consequences in the biology of the PCSKs. Collectively, this study demonstrated critical roles for FURIN and PCSK7 in the regulation of the immune system, which enables the targeting of PCSK activity more specifically in therapeutic applications.

7 CONCLUSIONS AND FUTURE PERSPECTIVES

In the present study, the PCSK-mediated regulation of the immune system was investigated using various *in vivo* and *in vitro* methods. For the first time, we demonstrated that FURIN expression in T cells is critical for the host response against an intracellular parasite, *T. gondii* in a mouse model. We further showed that FURIN regulates T cell responses by balancing the Th1/Th2 subsets, and identified a novel FURIN interacting protein complex in human Jurkat T cells. These findings indicate that FURIN could regulate cytoskeleton dynamics and receptor trafficking in T cells, but further studies are needed before the role of FURIN in T cell biology can be fully disclosed. Further, we used zebra fish to study the evolutionarily most ancient PCSK, PCSK7, and in contrast to the mammalian homolog, found that the zebra fish PCSK7 was critical for embryogenesis. This argues for a non-redundant function for the non-mammalian PCSK7. Further, we studied PCSK7-mediated gene regulation and revealed a link between PCSK7 and immune system specific gene expression. We also showed that both human and zebra fish PCSK7s are efficient in processing pro-TGFβ1, which reveals new aspects of the regulation of this important cytokine.

Finally, we studied the expression of the genes for the PCSK family and their substrate molecules in human atherosclerotic plaques. Interestingly, we found out that FURIN was the major PCSK to be upregulated in the vascular samples, and a novel association of two FURIN substrate cytokines, BAFF and APRIL with atherogenesis was also revealed. In addition, FURIN expression was shown to be induced in response to immune activation in immune cells including macrophages of the innate system. Collectively, these findings demonstrate an essential role for PCSKs, especially FURIN and PCSK7, in the regulation of immune system. The therapeutic targeting of PCSKs and their substrate molecules holds great promise for future clinical interventions. Although our results point to the potential of targeting PCSKs in the treatment of immunological pathologies, they also clearly indicate that a more detailed understanding of the unique and redundant function of the PCSKs is still critically needed.

To study the biology of PCSKs in more details, efficient methods are needed to manipulate these proteases, their regulatory elements and substrates in an

endogenous context. Recent genome editing technologies that utilize site-specific endonucleases could offer an easy and efficient way to generate transgenic cell lines and animal models. The most prominent method at the moment is the single RNA-guided endonuclease Cas9, adapted from the bacterial adaptive immune system known as CRISPR (Cong et al., 2013; Hsu et al., 2014). Compared to conventional gene targeting, the CRISPR-Cas9 system can increase the likelihood of precise genome modifications by several orders of magnitude. A CRISPR-Cas9-mediated double strand break can be repaired in the cell by non-homologues end-joining (NHEJ), which is suitable for the generation of the small deletions or insertions needed to destroy the reading-frame in coding regions. A double strand break can also be repaired by homologues recombination (HR), which offers an excellent tool for precisely manipulating the genome and introducing custom mutations (Pelletier et al., 2015). In addition, modified versions of the CRISPR-Cas9 system can be used for large genetic screens, DNA labeling, or expression studies (Cong et al., 2013; Hsu et al., 2014). Altogether, novel genome editing technologies can provide us better ways to understand how PCSKs are regulated and to what extend they share redundant functions *in vivo*. Finally, this will help us decipher the biology of PCSKs in human health and disease states.

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10 ORIGINAL COMMUNICATIONS

Proprotein Convertase FURIN Constrains Th2 Differentiation and Is Critical for Host Resistance against *Toxoplasma gondii*

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The proprotein convertase subtilisin/kexin enzymes proteolytically convert immature proproteins into bioactive molecules, and thereby they serve as key regulators of cellular homeostasis. The archetype proprotein convertase subtilisin/kexin, FURIN, is a direct target gene of the IL-12/STAT4 pathway and it is upregulated in Th1 cells. We have previously demonstrated that FURIN expression in T cells critically regulates the maintenance of peripheral immune tolerance and the functional maturation of pro–TGF-β1 in vivo, but FURIN's role in cell-mediated immunity and Th polarization has remained elusive. In this article, we show that T cell–expressed FURIN is essential for host resistance against a prototypic Th1 pathogen, *Toxoplasma gondii*, and for the generation of pathogen-specific Th1 lymphocytes, including Th1–IL-10 cells. FURIN-deficient Th cells instead show elevated expression of IL-4R subunit α on cell surface, sensitized IL-4/STAT6 signaling, and a propensity to polarize toward the Th2 phenotype. By exploring FURIN-interacting proteins in Jurkat T cells with Strep-Tag purification and mass spectrometry, we further identify an association with a cytoskeleton modifying Ras-related C3 botulinum toxin substrate/dedicator of cytokinesis 2 protein complex and unravel that FURIN promotes F-actin polymerization, which has previously been shown to downregulate IL-4R subunit α cell surface expression and promote Th1 responses. In conclusion, our results demonstrate that in addition to peripheral immune tolerance, T cell–expressed FURIN is also a central regulator of cell-mediated immunity and Th1/2 cell balance. ***The Journal of Immunology*****, 2014, 193: 5470–5479.**

Upon encountering the cognate Ag, naive $CD4^+$ Th cells activate and polarize into functionally distinct subsets that include Th1, Th2, Th17, and T regulatory cells (Tregs). The fate of a naive Th cell depends on the length and strength of the Ag stimulus, as well as on surrounding cytokine milieu, both of which activate cellular signaling pathways and expression of Th subtype-specific genes (1). The first characterized subtypes, Th1 and Th2, determine the balance between cell-mediated and humoral immune responses. Th1 polarization is characteristically driven by a strong Ag stimulus in the presence of the cytokine IL-12. These trigger the activation of transcription factor STAT4 and elevated T-box 21 (T-bet) expression, followed by secretion of the Th1 type effector cytokines. Th2 polarization, in contrast, is initiated by a weak Ag stimulus and IL-2/STAT5 signaling, resulting in upregulation of GATA binding protein 3 (GATA-3) and IL-4–induced STAT6 activation (2). Th1 cells secrete IFN-γ and TNF cytokines that promote, for example, the activation of macrophages in intracellular infections, whereas the Th2 cytokines IL-4, IL-5, and IL-13 induce IgE class switching in B cells, as well as the activation of eosinophils, both of which are required to restrain parasitic infections (3).

Dysregulation of Th cells is associated with immune-mediated diseases, including infections, immunodeficiencies, autoimmunity, and allergic responses (4). Insufficient Th1 polarization, for example, increases susceptibility to mycobacterial infections, whereas activated Th1 cells are often overrepresented in autoimmune diseases (2). Consequently, much research effort has been aimed at identifying the detailed molecular mechanisms that control Th polarization events over the past two decades. We and others have previously shown that

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Abbreviations used in this article: CBA, cytometric bead array; cKO, conditional KO; DC, dendritic cell; DOCK2, dedicator of cytokinesis 2; GATA-3, GATA binding protein 3; IL-4Rα, IL-4R subunit α; KO, knockout; MS, mass spectrometry; mut, mutant; PCSK, proprotein convertase subtilisin/kexin; Q-RT-PCR, quantitative real-time PCR; RAC, Ras-related C3 botulinum toxin substrate; SDF-1α, stromal cell–derived factor-1α; STAg, soluble *T. gondii* Ag; T-bet, T-box 21; Treg, T regulatory cell; wt, wild type.

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a proprotein convertase subtilisin/kexin (PCSK) family protease FURIN is a direct target gene for IL-12/STAT4 and TGF-β1, and that it is highly expressed in human Th1 cells (5–7). PCSK enzymes are a family of nine proteases (PCSK1-2, FURIN, PCSK4-7, MBTPS1, PCSK9) that cleave and convert their immature target proteins into biologically active forms by catalyzing endoproteolytic cleavage at a target site typically made up of basic amino acids arginine and lysine (8). Accordingly, PCSK enzymes play a key regulatory role in a multitude of biological events, including development and hormone function (9). Dysregulated proprotein convertase activity also contributes to pathogenic cell behavior; therefore, interfering with PCSK activity is currently being considered as a potential treatment for many diseases including atherosclerosis (10), rheumatoid arthritis (11), and multiple sclerosis (12).

PCSK enzymes are also central regulators in host defense; inhibiting PCSK activity can prevent the proteolytic activation of bacterial toxins and viral entry (8), and recently a FURIN-like convertase was shown to regulate human TLR7 processing and subsequent antiviral immunity (13). In addition, deleting PCSK1 expression in mice is associated with accelerated proinflammatory responses (14), whereas PCSK7 participates in rescuing the unstable MHC class I molecules on dendritic cells (DCs) (15) and regulates the bioavailability of TGF-β1a cytokine in zebrafish (16). Biochemical analyses have also demonstrated that the PCSK controlled proteolytic cascades are important in the functional maturation of several proteins critical for host defense such as integrins, matrix metalloproteinases, and cytokines (9, 17).

We have previously shown that the T cell–expressed proprotein convertase FURIN is essential for maintaining Treg-mediated peripheral immune tolerance. CD4cre-fur$^{f/f}$ mice develop an age-related systemic autoimmune disease that is characterized by circulating autoantibodies, overly activated CD4$^+$ and CD8$^+$ T cells, and overproduction of both Th1 and Th2 cytokines, IFN-γ, IL-4, and IL-13 (18). The accelerated immune responses in CD4cre-fur$^{f/f}$ mice could be chiefly attributed to reduced bioavailability of a central anti-inflammatory cytokine TGF-β1 in T cells. Notably, the role of FURIN in controlling the Th cell subsets appears complex and is not limited to CD4$^+$FOXP3$^+$ Tregs; inhibiting FURIN by a recombinant inhibitor (α1-antitrypsin Portland) or small interfering RNA can also abrogate the production of the Th1 effector cytokine IFN-γ by human CD4$^+$ T cells (5). In this study, we show that FURIN expression in T cells is critical for ensuring an appropriate immune response against a prototypic Th1 parasite, *Toxoplasma gondii*. Specifically, our data demonstrate that FURIN expression is needed for adequate Ag-specific Th1 cell generation, and lack of FURIN expression in T cells results in the inherent upregulation of IL-4R subunit α (IL-4Rα) on the cell surface and dominance of Th2 polarization. Using Strep-tag purification and mass spectrometry (MS), we further show that FURIN interacts with a Th1-promoting and IL-4Rα–inhibiting Ras-related C3 botulinum toxin substrate (RAC)/dedicator of cytokinesis 2 (DOCK2) protein complex and regulates T cell cytoskeleton dynamics by increasing its activity.

Materials and Methods

Mice

T cell–specific FURIN conditional knockout (cKO; CD4cre-fur$^{f/f}$) mice on C57BL/6 background have been described earlier (18, 19); IL-12p40 KO animals were purchased from Taconic. For OVA-specific Th polarization experiment, CD4cre-fur$^{f/f}$ and fur$^{f/f}$ littermate controls were crossed with mice bearing OTII TCR to generate CD4cre-fur$^{f/f}$–OTII mice, which have OVA-specific CD4$^+$ T cells. All mice were housed under pathogen-free conditions in accordance with the National Institutes of Health Animal Care and Use Committee (National Institutes of Health, Bethesda, MD) or the National Animal Experiment Board (Finland).

Cell culture and transfections

Jurkat E6-1 T cells (ATCC TIB-152) were cultured in RPMI 1640 medium and HeLa cells (ATCC CCL2) in DMEM medium, both supplemented with 10% FBS, L-glutamine, and antibiotics. To generate the stable wild type (wt) and inactive FURIN (mutant [mut])-expressing cell lines, we electroporated Jurkat E6-1 T cells (1025 μF, 260 V) with pcDNA3.1-hFURINwt-StrepIII, pcDNA3.1-hFURIN-D153Amut-StrepIII, or pcDNA3.1-StrepIII as control (a kind gift from Prof. Jukka Westermarck, Turku Centre for Biotechnology). Geneticin antibiotic (600 μg/ml) was added to the cell cultures 3 d posttransfection. cDNA-expressing cell lines were then selected with clonal dilution, and constant expression of recombinant protein was evaluated by Western blot analysis (anti-FURIN MON-152; Enzo LifeSciences). Cell lines with equal wt and mut FURIN expression were selected for the interactome and F-actin polymerization studies. HeLa cells were transiently transfected with 0.5–1 μg pcDNA-RAC1-V5, pCI-DOCK2-FLAG (kind gifts from Prof. Yoshinori Fukui, Kyushu University, Fukuoka, Japan), and pcDNA3.1-hFURIN-MYC-His using FuGENE 6 Transfection Reagent (Promega).

Cell purification, cytokine measurements, and flow cytometry

To study OVA-induced Th1/Th2 polarization, we purified naive CD4$^+$ CD44^{low}CD62L$^+$ T cells using flow cytometry from CD4cre-fur$^{f/f}$ or littermate control mice on an OTII background. Cells were labeled with CFSE (Invitrogen) and stimulated with graded concentrations of OVA-peptide (5–1000 nM) and sorted splenic CD11c$^+$CD49b$^-$ DCs under neutral conditions. After 3.5 d, the cells were activated with PMA (10 ng/ml) and ionomycin (1 μM; EMD), and intracellular expression of IFN-γ, IL-4, and CFSE was analyzed with flow cytometry.

For polyclonal T cell activation studies, naive T cells were isolated with CD4$^+$ CD62L$^+$ T Cell Isolation Kit (Miltenyi) from spleen and lymph nodes. Cells were activated for 72 h with plate-bound anti-CD3 and -CD28 (10+10 μg/ml) Abs in serum-free X-VIVO 20 medium (Lonza). IL-12 (10 ng/ml), TGF-β1 (0.5 ng/ml), or anti–IL-4 (2 μg/ml) were added to the cell cultures during the activation. Cytokines from cell culture medium were measured with cytometric bead array Th1/2/17 kit (BD Biosciences) or IL-4 ELISA (Peprotech), and RNA was collected for quantitative real-time PCR (Q-RT-PCR) experiments.

For the detection of pERK, CD4$^+$ T Isolation Kit (L3T4; Miltenyi) was used to isolate CD4$^+$ T cells from spleen and lymph nodes. Cells were stimulated with soluble anti-CD3 (10 μg/ml) for 5–60 min, and phosphorylation of ERK was detected by Western blot using ERK and pERK primary Abs (Cell Signaling Technology) and anti-rabbit HRP-biotin conjugated secondary Ab (R&D Systems). Signal intensities were analyzed using National Institutes of Health ImageJ software. IL-4Rα cell-surface expression was analyzed from spleens and lymph nodes by staining the cells with anti–IL-4Rα (BD Biosciences), -CD4, and -CD62L (eBioscience) Abs, and analyzed by flow cytometry. To detect tyrosine phospho-STAT6, we purified naive CD4$^+$ T cells from spleen and lymph nodes with the CD4$^+$CD62L$^+$ T cell isolation kit (Miltenyi) and activated them with IL-4 (1 or 10 ng/ml) for 15 min. The cells were fixed with 4% paraformaldehyde and permeabilized with cold 90% methanol overnight. Staining was performed in 0.5% Triton X-100/0.1% BSA/PBS with anti-pSTAT6 Ab (BD Biosciences). All FACS analyses were performed using FACSCalibur or FACSCanto instrument (BD Biosciences), and data were analyzed with FlowJo software (Tree Star).

Quantitative real-time PCR

Total RNA was isolated with RNeasy kit (Qiagen) and reverse transcribed into cDNA with iScript kit (BioRad). Gene expressions were analyzed using SsoFast EvaGreen supermix and CFX96 instrument (BioRad). Primer sequences and size of amplicons: T-bet (110 bp): forward, 5′-TCAACCAGCACCAGACAGAG-3′, reverse, 5′-AAACATCCTGTAATGGCTTGTG-3′; Gata-3 (75 bp): forward, 5′-TTATCAAGCCCAAGCGAAG-3′, reverse, 5′-TGGTGGTGGTCTGACAGTTC-3′; Il4ra (102 bp): forward, 5′-GACTGGATCTGGGAGCATCA-3′, reverse, 5′-CAGTCCACAGCGCTATCCAG-3′; 18s (143 bp): forward, 5′-GTGATCCCTGAGAAGTTCCAG-3′, reverse, 5′-TCGATGTCTGCTTTCCTCAAC-3′. The expression of each gene was normalized to ribosomal 18s gene. For FURIN and B2M housekeeping gene expression analysis in splenocytes after *T. gondii* infection, predesigned primers and probes from Applied Biosystems were used.

Immunofluorescence

Three days posttransfection, HeLa cells were fixed on coverslips with paraformaldehyde and stained with rabbit anti-FURIN (a kind gift from Prof. John Creemers, Katholieke Universiteit Leuven, Leuven, Belgium) and mouse anti-V5 for RAC (Invitrogen). Nucleus was stained with DAPI, and specific protein expression was visualized with anti-rabbit Texas Red or anti-mouse Alexa 488 (both Life Technologies), using ApoTome microscope and AxioVision software (Zeiss).

Immunoprecipitation and Western blotting

Transfected HeLa cells were lysed (lysis buffer: 50 mM Tris pH 7.5, 10% glycerol, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 50 mM NaF, 1 mM TCEP, and Complete Mini protease inhibitor from Roche) and precleared with protein G-Sepharose 4 FastFlow beads (GE Healthcare). Anti-FLAG Ab was used to capture DOCK2 and anti-MYC for FURIN, in parallel with Ab isotype and resin controls (all from Sigma-Aldrich). Protein elutes were separated with SDS-PAGE gel and transferred to nitrocellulose membrane. Immunodetection was performed using anti-FLAG or anti-MYC primary Abs and anti-mouse HRP-conjugated secondary Ab (R&D Systems). Visualization was done using the ECL Western blotting detection kit (GE Healthcare) and AGFA CP1000 imaging system.

F-actin polymerization

FURIN wt and control Jurkat E6-1 T cell lines were starved in RPMI 1640 supplemented with 1% FBS and stimulated with 250 ng/ml stromal cell–derived factor-1α (SDF-1α; Peprotech) for 0–120 s. Cells were immediately fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100, stained for polymerized F-actin (Phalloidin-FITC; Sigma-Aldrich), and analyzed with flow cytometry.

In vivo challenge with T. gondii

CD4cre-fur$^{f/f}$, IL-12p40 KO, and littermate control mice (6–10 wk old) were challenged i.p. with 20 pepsin-treated cysts of the avirulent ME49 strain of *T. gondii*. Nine days postinfection, the FURIN expression was quantified in splenic CD4$^+$ T cells by Q-RT-PCR, and serum cytokines were measured with CBA (BD Biosciences). Survival of FURIN T cell KO and littermate control animals was monitored for 100 d and quantified using log-rank (Mantel–Cox) test. Some of the infected animals were sacrificed on 38 d postinfection to analyze the number of *T. gondii* cysts in brain, as well as Ag-specific Th1/Th1–IL-10 polarization and cytokine production. In brief, brains were isolated and homogenized by sequential passage through 19- and 21-gauge needles, and the number of cysts was determined microscopically. Ag-dependent cytokine production was induced ex vivo by stimulating splenocytes in complete RPMI 1640 + 10% FBS with soluble *T. gondii* Ag (STAg; 5 μg/ml) for 72 h. For flow cytometric analysis, cells were stained for CD4, IFN-γ, and IL-10 (BD Biosciences). Cytokines were measured from cell culture supernatants with CBA (BD Biosciences).

Sample preparation for MS

Cell membrane fractions from Jurkat T cell lines were isolated (Mem-PER Eukaryotic Membrane Protein Extraction Kit; Thermo Scientific) and FURIN, as well as associated proteins, were affinity purified from lysates with Strep-tag columns (IBA). The presence and purity of recombinant FURIN in elutes was verified with both anti-FURIN (MON-152; Enzo Life Sciences) and anti-Strep (IBA) Abs (data not shown). Eluted proteins were separated by one-dimensional SDS-PAGE gel (Miniprotean precast gel; Bio-Rad) and visualized by silver staining.

Mass spectrometry

Target bands were cut from silver-stained gels, and after enzymatic protein digestion and extraction, peptides were identified by MS (Proteomics Facility, Turku Centre for Biotechnology). Analysis was performed by liquid chromatography/electrospray ionization–tandem MS on a nanoflow HPLC system coupled online to an Orbitrap Velos MS instrument. Database searches were performed by Mascot (version 2.2.6) against SwissProt (UniProt) protein sequence database (version 2010_09). Scaffold 3 software (Proteome Software) was used to further analyze identified proteins. Data were filtered through validation parameters (i.e., m.w. match, minimum 2 unique peptides, minimum ∼10% coverage).

Statistical analysis

Data represent mean ± SEM. Statistical significance was determined by nonparametric Mann–Whitney *U* test for mouse experiments and by two-tailed Student *t* test for cell line experiments, if not indicated otherwise. Survival after *T. gondii* infection was analyzed with log-rank (Mantel-Cox) test. The p values < 0.05 were considered statistically significant.

Results

T cell–expressed FURIN is essential for host resistance against T. gondii

We initially identified FURIN as a novel IL-12 target gene using microarray expression analysis on human peripheral blood T lymphocytes (5). Subsequent experiments showed that the FURIN mRNA and protein are particularly highly expressed in Th1 cells and that FURIN is directly regulated by STAT4 chromatin binding, rather than in a T-bet–dependent manner (20). *T. gondii* is an obligate intracellular parasite that triggers a profound Th1-mediated cellular immune response characterized by elevated production of IFN-γ and TNF cytokines. We first assessed whether *T. gondii* infection induces FURIN expression in splenic CD4$^+$ T cells by infecting both wt and IL-12–deficient mice with avirulent *T. gondii* ME-49 strain parasites. In line with previous in vitro data showing an IL-12–dependent FURIN regulation (5), parasite-induced FURIN expression was significantly impaired in IL-12–deficient animals at 9 d postinfection (Fig. 1A).

To study the potential role of FURIN in this prototypic Th1-inducing infection, we next infected mice in which FURIN was deleted specifically in T cells (CD4cre-fur$^{f/f}$) with *T. gondii*. Presence or absence of FURIN in T cells did not significantly influence survival or serum cytokine levels in the IL-12–dependent (21, 22), acute phase of parasite infection (Fig. 1B and Supplemental Fig. 1). However, *T. gondii*–infected CD4cre-fur$^{f/f}$ mice had modestly reduced numbers of IL-10–producing Th1 cells at 9 d postinfection (data not shown). Later in the course of infection, all infected CD4cre-fur$^{f/f}$ animals became moribund and ultimately succumbed to the infection (Fig. 1B). The lethality associated with the absence of FURIN in T cells could be due to either a host defense defect or an exaggerated inflammatory response (18). Analysis of parasite burden in moribund animals showed a significantly higher number of intracranial cysts in T cell FURIN KO mice, suggesting inadequate protective host response (Fig. 1C) (23).

To better define the role of FURIN in mounting appropriate *T. gondii*–specific CD4$^+$ T cell responses, we stimulated splenocytes from infected CD4cre-fur$^{f/f}$ or littermate mice with STAg for 72 h (Fig. 1D). Total splenocyte cell number was reduced in the declining CD4cre-fur$^{f/f}$ mice, but CD4$^+$/splenocyte ratios in the spleen were similar to those of the littermate control animals. The resistance to *T. gondii* infection is critically dependent on an adequate Th1 response and IFN-γ/TNF cytokine production (24–26), but also on the generation of Th1–IL-10$^+$ cells, which restrict the magnitude of the infection-induced inflammation (24, 27). STAg stimulation resulted in an increase of the numbers of both Th1 and Th1–IL-10 cells in control mice, whereas the generation of Ag-specific Th1 and Th1–IL-10 cells was significantly reduced in splenocytes isolated from CD4cre-fur$^{f/f}$ mice, indicating that FURIN promotes cell-mediated immune responses (Fig. 1D, 1E). Profiling the Ag-stimulated splenic cytokine production further showed a lower trend of IFN-γ levels accompanied with a significantly reduced production of another Th1 cytokine, TNF, but unchanged secretion of the innate immunity cytokines IL-6, IL-12, and MCP-1 (Supplemental Fig. 2). Together, our data demonstrate a critical role for proprotein convertase FURIN in T cell–mediated host resistance against *T. gondii* and in the generation of pathogen-specific Th1 and Th1–IL-10 responses.

FURIN regulates the Th1–Th2 balance

The failure of FURIN-deficient T cells to generate appropriate protective Th1 response prompted further investigation. To this end, we crossed CD4cre-fur$^{f/f}$ mice with TCR transgenic (OTII) mice and stimulated the Ag-specific naive CD4$^+$ T cells (OTII$^+$ CD4$^+$CD62L$^+$CD44$^-$) with varying concentrations of cognate antigenic peptide (OVA), and analyzed the production of Th subtype cytokines and cell proliferation. As reported previously (28), in neutral culture conditions, low-dose Ag (5 nM) drove a predominant Th2 polarization of wt CD4$^+$ cells, as attested by

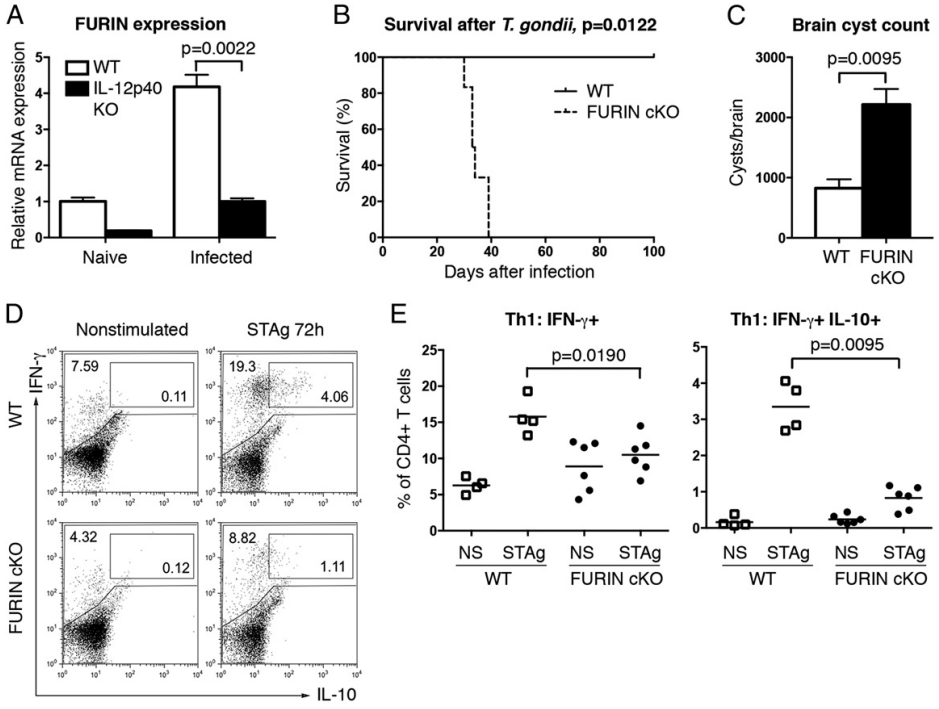


FIGURE 1. T cell–expressed FURIN is critical for the host defense to *T. gondii*. (**A**) wt and IL-12p40 KO ($n = 6$) mice were left uninfected (naive, $n = 1$) or infected ($n = 5$) i.p. with 20 *T. gondii* cysts (ME-49). Nine days postinfection, FURIN and B2M housekeeping gene expressions in splenic $CD4^+$ T cells were determined with Q-RT-PCR. FURIN expression was normalized to B2M, and relative expression in uninfected wt $CD4^+$ cells was arbitrarily set as 1. (**B**) Ten-week-old CD4cre-fur$^{f/f}$ and littermate control mice were inoculated i.p. with 20 *T. gondii* ME-49 cysts. Survival was analyzed with log-rank (Mantel–Cox) test between FURIN cKO mice ($n = 6$) and littermate controls ($n = 3$), experiment was repeated twice with identical results. (**C**) Brain cysts were calculated from moribund FURIN cKO mice ($n = 6$) and littermate controls ($n = 4$) on day 38 postinfection. (**D** and **E**) Splenocytes from *T. gondii*–infected FURIN cKO ($n = 6$) and littermate ($n = 4$) mice were left nonstimulated (ns) or stimulated ex vivo with STAg (5 μg/ml) for 72 h. Production of IFN-γ and IL-10 was measured with flow cytometry in $CD4^+$ T cells; representative cytokine stainings (D) and scatter plots (E) are shown. (A–E) Mean ± SEM. Statistics were calculated with Mann–Whitney *U* test except for survival analysis.

high IL-4/IFN-γ ratio (Fig. 2). Conversely, increasing Ag (10–100 nM) favored generation of Th1 (IFN-γ^+) cells. At a low OVA concentration, FURIN cKO $CD4^+$ cells showed modestly enhanced cell proliferation and increased the IL-4^+ Th2 cell proportions. Strikingly, stimulation of FURIN-deficient T cells to increasing Ag (10–100 nM) failed to induce normal proportions of IFN-γ^+IL-4^- Th1 cells. In contrast, a marked overabundance of IL-4 production sustained upon increasing the Ag dose up to 100 nM. These data show that, in neutral culture conditions, FURIN-deficient T cells have an intrinsic impairment in Th1 and dominance of Th2 differentiation, which could be a result of altered sensitivity to the effects of Ag dose or dysregulated cytokine signaling.

Th2 polarization in FURIN-deficient $CD4^+$ T cells is independent of TGF-β1 and is resistant to IL-12 inhibition

One aspect of the function of FURIN is its role in the maturation of pro–TGF-β1 through a site-specific proteolytic cleavage (29). Because TGF-β1 can have diverse effects on lymphocyte activation, we next addressed the possibility that the effect of FURIN was related to TGF-β1 or other serum cytokines (30). Naive wt and FURIN cKO T cells were activated with a strong polyclonal TCR stimulus (anti-CD3/28 10+10 μg/ml) in serum-free cell culture conditions, and IL-4 production was measured at different time points (Fig. 3A). Lack of FURIN expression was not associated with enhanced apoptosis, because cell numbers and Bcl-2 mRNA expression were unaffected (data not shown). Naive FURIN cKO $CD4^+$ cells produced significantly greater amounts of IL-4 as early as 24 h after TCR activation, indicating that FURIN deficiency results in accelerated Th2 responses also upon a strong polyclonal TCR activation and independently of serum cytokines.

We next investigated whether FURIN-deficient T cells respond correctly to Th1- and Treg/Th17-inducing cytokines IL-12 and TGF-β1, respectively (Fig. 3B, 3C). In keeping with the increased levels of IL-4, activated FURIN cKO $CD4^+CD62L^+$ T cells also had increased the GATA-3/T-bet ratio in neutral, serum-free cell culture conditions. However, addition of IL-12 to the cell cultures promoted IFN-γ production in both wt and FURIN cKO cells (Fig. 3C). Further, the lack of FURIN did not abolish the IL-12–triggered induction of STAT4 tyrosine phosphorylation in $CD4^+$ T cells or granzyme B expression in $CD8^+$ T cells (data not shown), which collectively indicates that FURIN is not critical for responsiveness to IL-12. In contrast, the production of Th2 type cytokines IL-4 and IL-13 or elevated GATA-3 expression could not be markedly inhibited by adding IL-12 into the FURIN-deficient $CD4^+$ T cell cultures. Previously, we have observed that FURIN-deficient T cells respond normally to TGF-β1 by upregulating the Foxp3 expression (18). FURIN-deficient cells

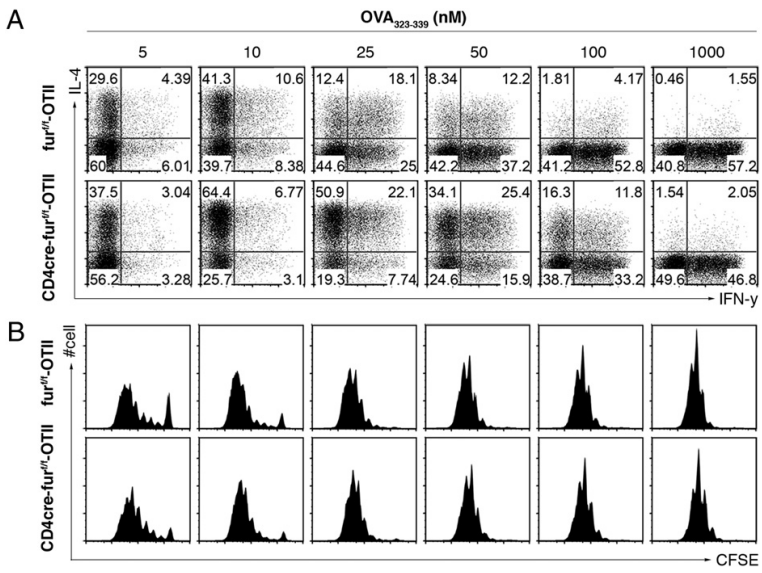


FIGURE 2. FURIN expression in CD4$^+$ T cells inhibits Th2 polarization. Naive CD4$^+$ T cells (CD4$^+$CD44^{low}CD62L$^+$) from CD4cre-fur$^{f/f}$–OTII or littermate control (fur$^{f/f}$-OTII) mice were labeled with CFSE and stimulated with graded concentrations of OVA-peptide/splenic DCs under neutral cell culture conditions. After 3.5 d, the primed T cells were stained for intracellular IFN-γ and IL-4. Cytokine production (**A**) and proliferation rate (**B**) were measured with flow cytometry. Experiment was performed twice with identical results.

remained Th2 biased (elevated GATA-3 expression and IL-13 levels) also in the presence of exogenous TGF-β1, but responded by showing downregulation of IL-4. Altogether, we conclude that the FURIN-deficient T cells respond correctly to IL-12 and TGF-β1 cytokines, and the inherent Th2 bias is independent of serum and resistant to Th1 and Th17/Treg polarization-inducing cytokines. These findings suggest that the FURIN-deficient Th cells could hyperpolarize to Th2 phenotype as a result of sensitized responses to Th2-promoting signals, such as autocrine IL-4 production.

FURIN inhibits the cell-surface expression of IL-4Rα in naive CD4$^+$ cells

Previous work has also shown that the strength of TCR signaling can affect Th1 versus Th2 commitment by influencing the degree of ERK activation; early Th2 polarization events are inhibited by an

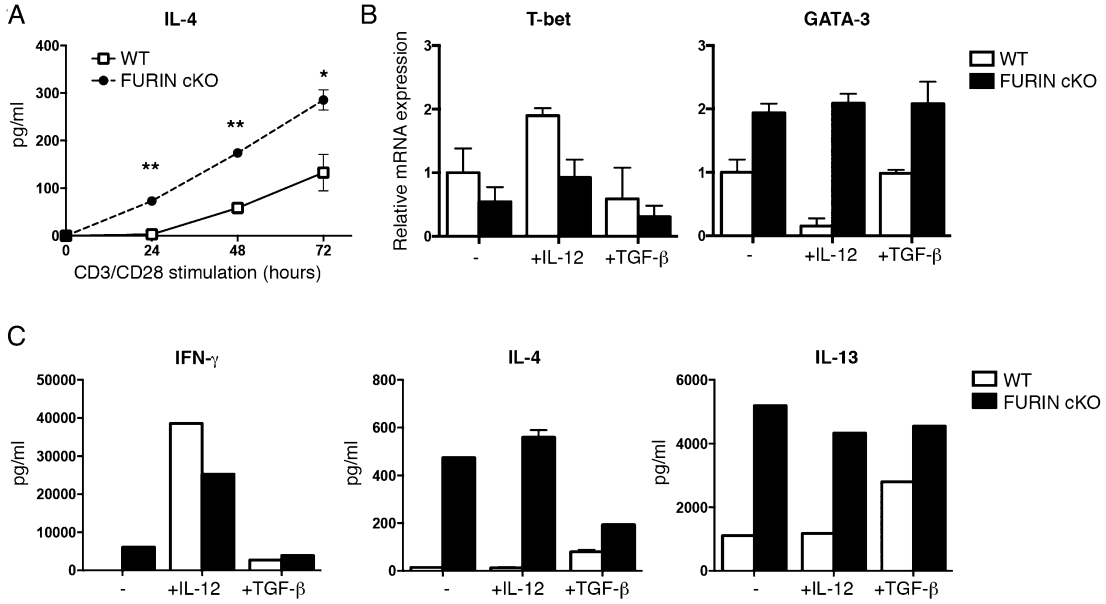


FIGURE 3. Th2 polarization in FURIN-deficient CD4$^+$ T cells is independent of serum cytokines and resistant to IL-12 and TGF-β1. Naive CD4$^+$ CD62L$^+$ T cells from CD4cre-fur$^{f/f}$ (cKO) or littermate control (wt) mice were stimulated with plate-bound anti-CD3/28 (10+10 μg/ml) and cultured in serum-free medium in the presence or absence of IL-12 (10 ng/ml) or TGF-β1 (0.5 ng/ml) as indicated for 72 h. (**A**) IL-4 production was measured with ELISA at different time points (n = 2–3 for both groups). The p values were determined with Student t test, *$p < 0.05$, **$p < 0.01$. (**B**) T-bet and GATA-3 expressions were measured with Q-RT-PCR. (**C**) Cytokines from cell culture supernatants were measured with CBA Th1/2/17 kit (BD Biosciences). Shown are representative experiments of two identical performed (B and C). Error bars indicate SEM.

elevated ERK activation upon a strong Ag stimulus (28). To investigate mechanisms by which FURIN normally inhibits Th2 differentiation, we measured TCR-dependent ERK activation and found that ERK phosphorylation was enhanced, not impaired, in FURIN-deficient $CD4^+$ cells (Fig. 4A). Moreover, FURIN-dependent Th2 bias was evident also in the presence of Th1 favoring strong TCR induction (Fig. 3). Thus, it seems unlikely that impairment of TCR signaling underlies derangement of Th1/Th2 differentiation. IL-2–mediated STAT5 activation and upregulation of GATA-3 expression is essential for the early Th2 commitment, independently of the IL-4/STAT6 signaling cascade (2, 31). To investigate whether FURIN regulates fate-determining transcription factors during T cell development, we measured thymic expression of GATA-3 and T-bet, and found that FURIN deficiency did not significantly affect the expression levels of either factor in single-positive $CD4^+CD8^-$ cells (Supplemental Fig. 3). Collectively, these data suggest that the FURIN deficiency causes Th2 hyperpolarization by regulating the Th2 phenotype amplification phase through the IL-4/STAT6 route rather than by disrupting the normal thymic development or downmodulating the initial TCR signal transduction.

To test directly whether the skewed Th2 phenotype of FURIN cKO cells was due to cytokine signaling, we inhibited the IL-4/STAT6 pathway by adding IL-4 neutralizing Ab (2 μg/ml) into serum-free $CD4^+CD62L^+$ T cell cultures and measured the mRNA expression of GATA-3. Blocking IL-4 function prevented the GATA-3 overexpression in in vitro activated FURIN cKO T cells (Fig. 4B). Interestingly, when the expression of IL-4Rα was analyzed, we observed that FURIN did not regulate *Il4ra* mRNA levels (Fig. 4C) but significantly inhibited the IL-4Rα protein expression on the naive $CD4^+$ T cell surface presumably through a posttranslational mechanism (Fig. 4D). To verify the biological relevance of the increased IL-4Rα surface expression, we measured the IL-4–induced STAT6 activation by flow cytometry. As expected, naive $CD4^+CD62L^+$ T cells from FURIN cKO mice repeatedly showed more pSTAT6 after a short IL-4 stimulus when compared with littermate control mice (Fig. 4E). In conclusion, we show that the lack of FURIN upregulates the expression of IL-4Rα on naive Th cells, which then contributes to sensitized IL-4/STAT6 signal transduction and a shift in Th1/Th2 balance.

FURIN interacts with RAC/DOCK2 and regulates T cell cytoskeleton dynamics

To find a potential mechanistic explanation for the FURIN-dependent dysregulation of IL-4Rα and consequent Th2 bias, we sought to identify novel FURIN-interacting proteins in T cells.

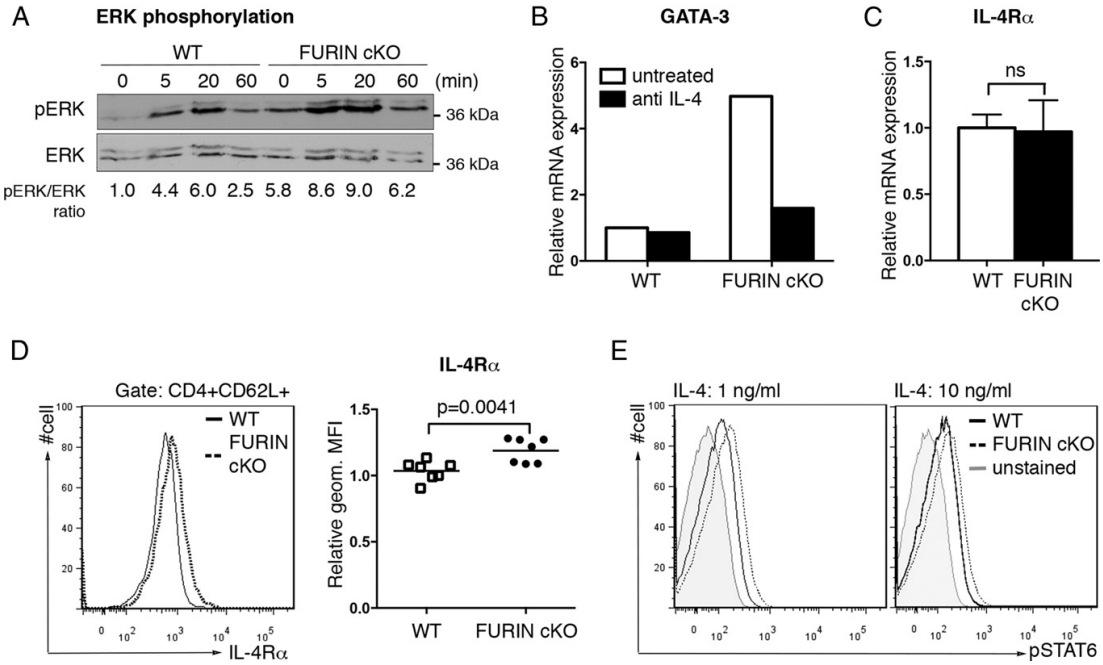
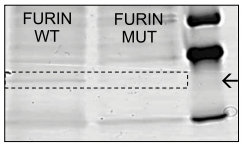


FIGURE 4. FURIN inhibits IL-4Rα cell-surface expression and IL-4–induced STAT6 phosphorylation. (**A**) $CD4^+$ T cells were isolated from CD4cre-fur$^{f/f}$ (cKO) and littermate control (wt) mice, and stimulated with soluble anti-CD3 (10 μg/ml) for indicated times. Total ERK and pERK were detected with Western blot, and pERK/ERK ratio was determined by measuring the intensity of the bands. Ratio from unstimulated wt sample was arbitrarily set as 1. Experiment was repeated twice with similar results. (**B**) Naive $CD4^+CD62L^+$ T cells from FURIN cKO and wt mice were cultured in serum-free media for 72 h in the presence of plate-bound anti-CD3/28 (10+10 μg/ml) activation and neutralizing anti–IL-4 Ab (2 μg/ml) as indicated. GATA-3 and housekeeping gene 18s expressions were determined by Q-RT-PCR, and relative expression in untreated wt sample was arbitrarily set as 1. Experiment was repeated twice with identical results. Error bars indicate SEM. (**C**) mRNA expression of Il4ra and 18s housekeeping gene was measured from steady-state naive $CD4^+CD62L^+$ T cells from FURIN cKO ($n = 4$) and littermate control ($n = 3$) mice. wt cell population was arbitrarily set as 1, and no statistical differences (ns) were observed between the groups (Mann–Whitney U test). Error bars indicate SEM. (**D**) Steady-state expression of IL-4Rα on naive $CD4^+CD62L^+$ T cells was determined with flow cytometry ($n = 7$ for both groups). Relative IL-4Rα expression (geometric median fluorescence [MFI]) in wt live cell population was arbitrary set as 1, and statistics were calculated with Mann–Whitney U test. (**E**) Phosphorylation of STAT6 was detected in $CD4^+CD62L^+$ T cells from FURIN cKO and littermate control mice. Cells were activated with IL-4 (1 or 10 ng/ml) for 15 min, and pSTAT6 was stained for FACS analysis. Geometric MFI values for pSTAT6: unstained 22, 26; wt 68, 85; and FURIN cKO 111, 121 (IL-4: 1, 10 ng/ml, respectively). Shown is a representative experiment of two performed.

A



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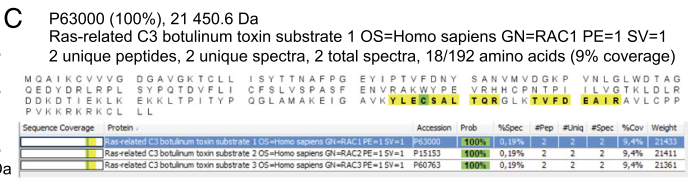
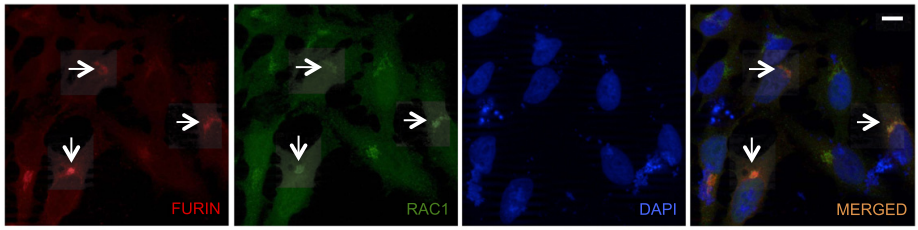


B

#	Identified proteins (12)	Gene name	Uniprot accession code	Molecular weight (kDa)	Number of peptides identified	Sequence coverage (%)	FURIN WT	FURIN MUT
1	Arginase-1	ARG1	P05089	35	4	10		x
2	ATP synthase subunit O, mitochondrial	ATP5O	P48047	23	3	15		x
3	Cystatin-A	CSTA	P01040	11	2	31		x
4	Desmocollin-1	DSC1	Q08554	100	3	4		x
5	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	P04406	36	1	4		x
6	Junction plakoglobin	JUP	P14923	82	15	26		x
7	Neutrophil defensin 1	DEFA1	P59665	10	1	10		x
8	Peroxiredoxin-1	PRDX1	Q06830	22	4	19	x	x
9	Proteasome subunit beta type-6	PSMB6	P28072	25	2	9		x
10	Ras-related C3 botulinum toxin substrate 1	RAC1	P63000	21	2	9	x	
11	Ras-related protein Rap-1b	RAP1B	P61224	21	3	16	x	x
12	Transgelin-2	TAGLN2	P37802	22	4	21	x	x

D



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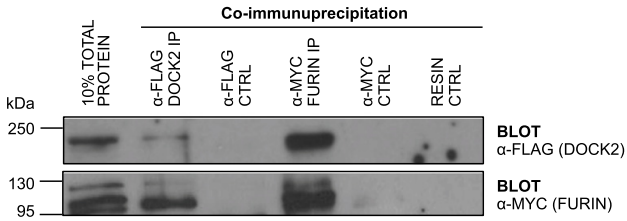

F

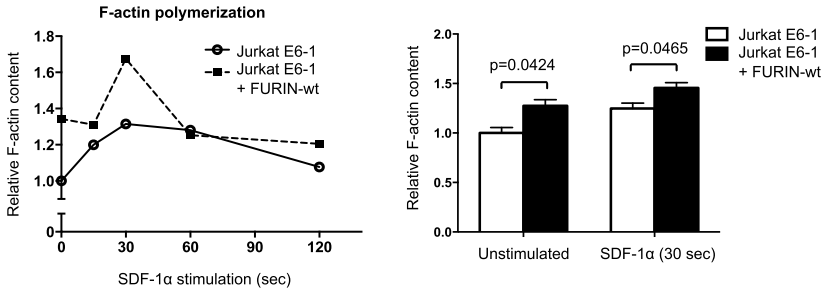

FIGURE 5. FURIN interacts with RAC/DOCK2 complex and regulates F-actin polymerization. Jurkat E6-1 T cell lines stably expressing either Strep-tagged human wt FURIN (pcDNA3.1-hFURINwt-StrepIII) or enzymatically inactive (D153A) mut (pcDNA3.1-hFURINmut-StrepIII) were generated as described in *Materials and Methods*. Cell membrane fractions were isolated, and FURIN as well as associated proteins were affinity purified from lysates with Strep-tag columns. (**A**) Eluted proteins were separated by one-dimensional SDS-PAGE gel and visualized by silver staining. (**B**) A 20-kDa band, the intensity of which was higher in the FURINwt-containing cell elutes, was cut from the gel, and after enzymatic protein digestion and extraction from the gel, peptides were identified by MS (Proteomics Facility, Turku Centre for Biotechnology); shown is a list of 12 potential FURIN interaction partners. (**C**) Peptide sequence coverages for FURINwt-specific interaction candidate RAC isoforms 1–3 are shown. HeLa cells were transiently transfected with pcDNA3.1-hFURINwt-MYC, pcDNA-RAC1-V5, pCI-DOCK2-FLAG encoding constructs. (**D**) Colocalization of RAC1 and FURIN was detected with immunofluorescence using anti-MYC and anti-V5 Abs and DAPI staining. Representative image is shown; arrows identify (*Figure legend continues*)

To this end, we first generated human Jurkat E6-1 T cell lines that stably expressed either wt or enzymatically inactive mut (32) human FURIN-Strep-Tag fusion proteins. FURIN interactomes were purified from cell membrane fractions using Strep-tag affinity purification and SDS-PAGE gels followed by peptide identification with MS (33). Analysis of a 20-kDa band, the intensity of which was higher in the wt FURIN containing elutes, resulted in the identification of 12 potential interaction partners for FURIN (Fig. 5A–C). Of these, only small GTPase RAC1–3 fulfilled the identification criteria (i.e., correct m.w., sufficient sequence coverage, two or more identified peptides) and was specifically present in lysates containing the enzymatically active FURIN. Of note, RAC has been reported to directly promote Th1 polarization by upregulating IFN-γ (34), and together with its activator DOCK2, RAC is also directly implicated in the regulation of IL-4Rα recycling and degradation in $CD4^+$ T cells (35, 36).

First, to validate our MS finding, we transfected HeLa cells with FURIN and RAC1 or DOCK2 encoding cDNAs. RAC1 and FURIN were found to be colocalized (Fig. 5D), and the association of FURIN and DOCK2 proteins was detected by coimmunoprecipitation (Fig. 5E), which collectively indicates the existence of FURIN/RAC1/DOCK2 complexes in the cells. Because the RAC/DOCK2 complex regulates the T cell cytoskeleton dynamics (36), we then investigated whether FURIN can also promote F-actin polymerization. The stable overexpression of wt FURIN significantly enhanced the level of F-actin polymerization in Jurkat T cells at both steady-state and in response to chemotactic signal SDF-1α (Fig. 5F). Taken together, our data show that FURIN interacts with RAC/DOCK2 complex and positively regulates the F-actin polymerization in T cells, which has previously been linked with downregulation of IL-4Rα on Th cell surface and inhibition of Th2 type immunity in vivo (34, 36, 37).

Discussion

Although the proprotein convertase FURIN has been shown to critically regulate diverse functions in homeostasis and pathology (9), its role in the T cell–mediated immune regulation is incompletely understood. Our initial studies revealed that FURIN is highly expressed in human Th1 type cells, where it promotes IFN-γ production (5). However, mice lacking T cell–expressed FURIN surprisingly presented with overly activated Th1 and Th2 cells, and inappropriate Treg function (18), which implied that FURIN has a multifaceted role in the $CD4^+$ T cell biology. In this study, when mice were infected with a prototypic Th1 pathogen *T. gondii*, FURIN was found to be upregulated in $CD4^+$ T cells in an IL-12–dependent manner. T cell–expressed FURIN was shown to be essential for host defense against the parasitic infection and the generation of Ag-specific Th1 and Th1–IL-10 cell responses. Activated FURIN cKO $CD4^+$ cells showed propensity to polarize toward Th2 phenotype in vitro, which was accompanied with elevated cell-surface IL-4Rα expression on naive Th cells. Finally, exploring the FURIN interactome using Strep-tag purification and proteomics resulted in identification of cytoskeleton modifying RAC/DOCK2 complex as a novel FURIN interaction partner. FURIN promoted actin polymerization in T cells, which has previously been shown to mediate IL-4Rα internalization. Altogether, our results unravel an IL-12/TGF-β1–induced protease FURIN as a central regulator of Th cell polarization and demonstrate its criticality in cell-mediated immune responses.

Transcriptional profiling studies on IL-12 target genes have offered mechanistic insights into Th1 differentiation. Cytokine receptors like IL-2Rα, IL-12Rβ2, and IL-18Rα, TPL2 kinase, and transcription factors such as IFN regulatory factor 1 are all induced by IL-12, and are required for optimal IFN-γ production and cell-mediated immunity (5, 24). In this study, we showed that IL-12 is important for FURIN upregulation upon *T. gondii* infection in vivo, which suggested a regulatory role for this protease in host defense against intracellular pathogens. FURIN is thought to be widely expressed, and it autoregulates its own enzymatic activity (8). Thus, the expression levels of FURIN serve as a critical determinant for its substrate processing activity also during an immune response. Recent findings show that not only IL-12 and TCR signals in T cells, but also LPS stimulation in macrophages can upregulate FURIN (10, 38). FURIN is also secreted from cells upon immune activation, and increased serum levels were recently found in chronic typhoid carriers (39). Assessing serum FURIN levels has thus potential to become a future biomarker in detecting the activation of immune systems in host.

FURIN has an essential function in proteolytic processing of the inactive pro–TGF-β1 precursor into its bioactive form (40, 41). Consequently, CD4cre-$fur^{f/f}$ mice largely phenocopy the immunological abnormalities (e.g., age-related systemic autoimmunity) seen in CD4cre-$tgfb^{f/n}$ mice (18, 42). However, FURIN-deficient Tregs were partly functional, and FURIN cKO effector $CD4^+$ cells appeared more resistant to the suppressive activity of wt Tregs (18) than what was reported in TGF-β1 deficiency, which implies the existence of additional targets for FURIN in T cells. PCSK substrate molecules and interaction candidates have been investigated by a variety of methods, including gene expression correlations, peptide sequencing, and microarrays (43–45). In this study, we used Strep-tag affinity purification and MS to identify T cell–specific FURIN interactions (33). By using both wt and inactive mut forms of FURIN, we could distinguish proteins that specifically interact with the active FURIN enzyme in human Jurkat T cells. Although we carefully optimized the cell lysis protocols to catch FURIN protein efficiently, it is possible that the relatively high stringency that was required for effective lysis may have resulted in loss of some transient interaction partners. In our experiments, the FURIN interacting proteins were first separated in one-dimensional SDS-PAGE gel, and identifications were focused on the silver-stained bands with different intensities, which may obviously limit the number of identifiable interaction partners.

The identified novel FURIN-associated proteins, small GTPase RAC and its activator DOCK2, have diverse roles in T cell biology. In peripheral T cells, RACs are essential for TCR signaling (46) and reactivation-induced apoptosis (47), but RAC can also promote Th1 polarization by upregulating signaling via p38 and NF-κB pathways and IFN-γ production (34). At the molecular level, the divergent functions of the RAC/DOCK2 complex are mediated through the modulation of actin rearrangements and associated with T cell cytoskeleton dynamics (48). Indeed, it has been previously described that FURIN regulates the actin cytoskeleton dynamics by processing integrins in vascular smooth muscle cells

FURIN and RAC1 colocalization. Scale bar, 10 μm. (**E**) Interaction between DOCK2 and FURIN was evaluated with coimmunoprecipitation and Western blotting using anti-FLAG and anti-MYC Abs. Experiment was repeated twice with identical results. (**F**) F-actin polymerization was analyzed in wt FURIN overexpressing and control (pcDNA3.1) Jurkat E6-1 T cells that were left untreated or stimulated with SDF-1α (250 ng/ml) for 15–120 s. Polymerized actin levels were quantified with flow cytometry ($n = 4$ for both groups). Error bars represent SEM. Student t test was used to calculate statistical significance.

(49). Our data show that FURIN promotes actin polymerization in Jurkat T cells, but decoding the detailed molecular mechanisms and associated target molecules remains uncharacterized.

The cytoskeleton-modifying activity of RAC/DOCK2 complex regulates the IL-4Rα trafficking and its surface expression on activated T cells, which then contributes to the strength of IL-4/STAT6 signaling and ensuing Th2 polarization (35, 36). Our flow-cytometric analyses showed that FURIN deficiency significantly upregulates IL-4Rα protein levels on naive $CD4^+$ T cells, which inherently express only low levels of this receptor chain (31). Accordingly, FURIN-deficient $CD4^+$ T cells mimicked the phenotype of DOCK2 KOs by showing elevated Th2 responses, such as high serum IgE level, but in contrast with DOCK2-deficient animals (36), the Ag-specific IFN-γ response to chronic intracellular parasite infection was reduced in FURIN cKO mice. The *Il4ra* mRNA expression in $CD4^+$ cells is initially primed by IL-2–induced STAT5 activation (31). FURIN-deficient Th cells showed unchanged *Il4ra* and *Il2ra* mRNA expressions, and the IL-2–induced STAT5 phosphorylation in $CD4^+$ cells was intact (Fig. 4C and data not shown). In contrast, neutralizing IL-4 effectively blocked the excessive GATA-3 expression in FURIN cKO T cells, and the IL-4–induced STAT6 activation was sensitized. Collectively, our results thus suggest that FURIN regulates Th1/Th2 balance by coordinating cytoskeleton dynamics and the IL-4Rα expression on the cell surface, which is essential for cytokine-dependent amplification of Th2 type cells. However, it is possible that by promoting RAC activity, FURIN enhances also directly IFN-γ and associated Th1 polarization (34). Further studies using endogenously expressed proteins and primary T cells are clearly required to decode the physiological molecular mechanisms by which the FURIN-mediated RAC activation regulates Th cell balance.

Cell-mediated immune responses are essential for host control over intracellular pathogens, such as *Mycobacteria*, *Toxoplasma*, *Leishmania*, and certain viruses. CD4cre-$fur^{f/f}$ mice succumbed to *T. gondii* infection, arguing for the criticality of T cell–expressed FURIN in the generation of protective immunity. In the acute phase of *T. gondii* infection, FURIN was dispensable for survival, generation of Th1 cells, and normal serum cytokine responses. The early Th1 cell generation has been shown to depend on high levels of APC-produced IL-12 (21), which functions FURIN independently. However, increased *T. gondii* burdens in the brains of CD4cre-$fur^{f/f}$ mice demonstrated that FURIN has a T cell–intrinsic role in controlling parasite growth. The analysis of Ag-specific Th responses showed that FURIN regulated the generation of sustainable protective (Th1) and tolerogenic (Th1–$IL\text{-}10^+$) cell populations. An analogous dual role has previously been reported for IL-27, which was initially characterized as a driver of Th1 type responses (50) and later shown to support suppressive IL-10–producing Tregs in *T. gondii* infection (51). In contrast, the lack of FURIN in T cells did not appear to promote the generation of single-positive $CD4^+IL\text{-}10^+$ cells in *T. gondii*–infected mice. These data could indicate that in the presence of elevated parasite burdens, FURIN deficiency is not able to contribute to the complementary induction of Th2 polarization. Importantly, a direct measurement of IL-4 and other Th2 cytokines postinfection would be required to clarify this aspect. Also, cytotoxic $CD8^+$ T cells critically restrict *T. gondii* in the chronic phase, and the exhaustion of $CD8^+$ T cells leads to uncontrollable parasite growth and encephalitis (52). Notably, in recall assays, we used STAg to induce T cell responses, which activates $CD4^+$ cells through MHC class II. However, the role of FURIN in the regulation of $CD8^+$ T cell biology is currently unknown, and we can thus not rule out the fact that *T. gondii*–infected CD4cre-$fur^{f/f}$ mice could die because of impaired $CD8^+$ T cell function. Importantly, our previous data demonstrate that FURIN deficiency accelerates T cell responses (18), which, in theory, can promote the exhaustion of $CD8^+$ T cell responses in the brain.

Inhibiting the PCSK function has a potential to become a future means to treat immune-mediated diseases such as cancers, atherosclerosis, and infections (10–12). In addition, administering recombinant FURIN can alleviate the overactive immune cells in autoimmune diseases (11). Understanding how FURIN regulates cell-mediated immunity and Th balance is thus critical when such treatments are considered for clinical use. Our earlier results demonstrate that deleting FURIN activates T cells, which could be beneficial in treatment of cancers, for example. However, our results show that T cell–expressed FURIN is critical for host resistance to *T. gondii* and inhibits Th2 polarization. Therefore, interfering with the PCSK activity in patients may compromise protection against intracellular pathogens and result in allergic responses.

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Disclosures

The authors have no financial conflicts of interest.

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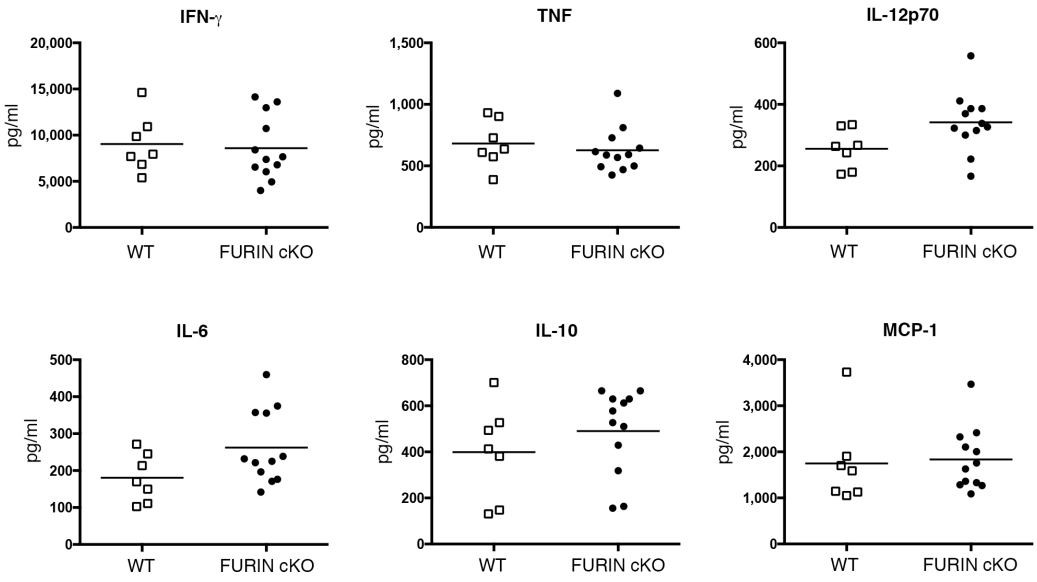

Figure S1. Serum cytokine levels in *T. gondii* infected CD4cre-fur$^{f/f}$ and littermate animals. CD4cre-fur$^{f/f}$ (n=12) and littermate control (n=7) mice were inoculated i.p. with 20 pepsin-treated ME-49 cysts. Serum cytokines were measured 9 days post infection with CBA inflammation kit (BD). Statistically significant differences between the groups could not be detected with Mann-Whitney test. Data is pooled from two identical experiments.

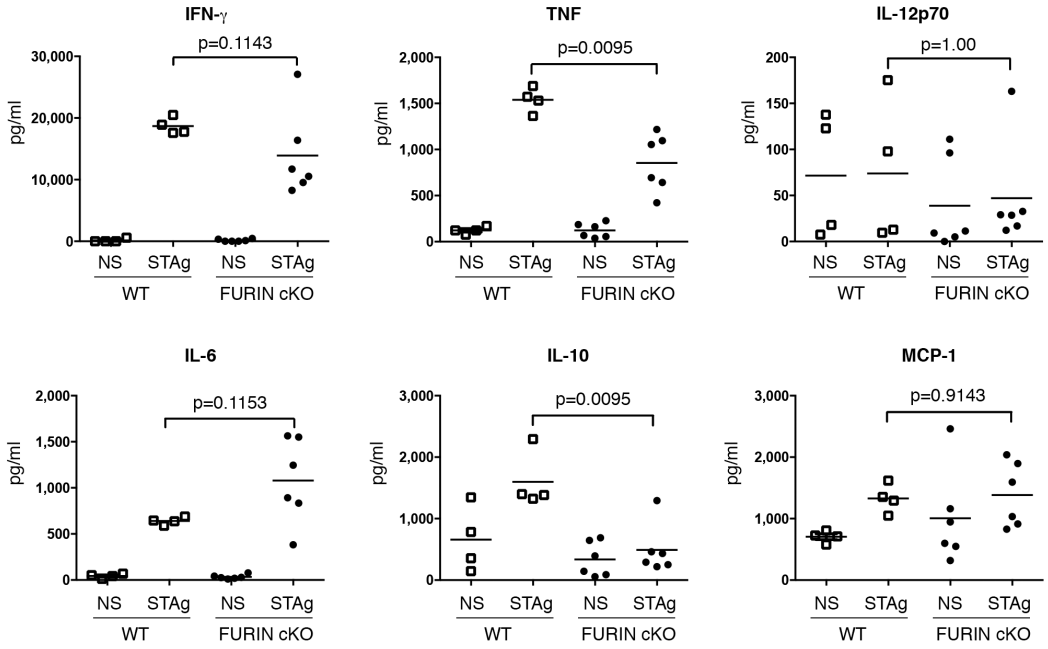

Figure S2. **T-cell-expressed FURIN regulates the production of Th1 type cytokines in response to *T. gondii* antigen stimulation**. Splenocytes from *T. gondii* infected CD4cre-fur$^{f/f}$ (n=6) and littermate (n=4) mice (38 dpi) were left unstimulated (ns) or stimulated ex vivo with soluble *T. gondii* antigen (STAg, 5 µg/ml) for 72 hrs. Cytokine levels were determined from supernatants with CBA kit (BD). Statistics were calculated with Mann-Whitney test.

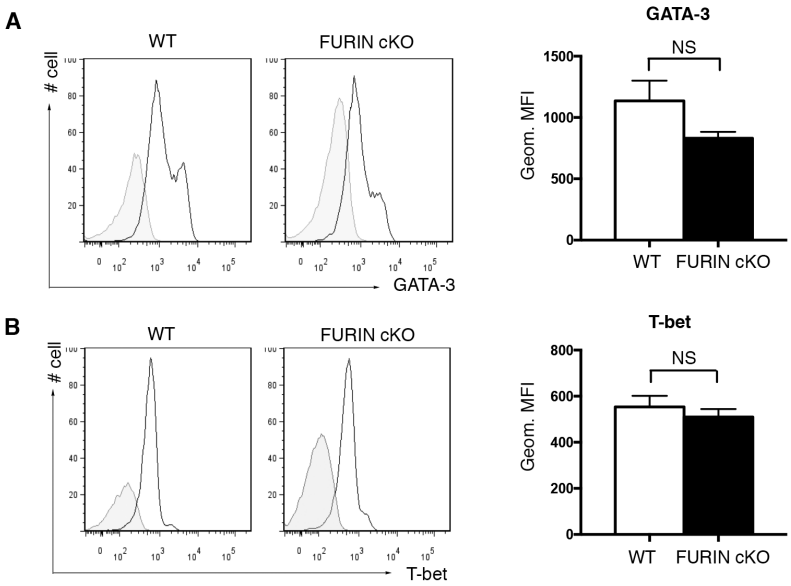

Figure S3. FURIN does not regulate the expression of GATA-3 or T-bet in thymic CD4+CD8- cells. Thymocytes from 12 weeks old CD4cre-fur$^{f/f}$ (n=4) and littermate (n=3) mice were isolated and stained with CD4, CD8, T-bet and GATA-3 antibodies (eBioscience). Representative histograms (black stained cells, grey unstained cells) and quantification of geometric MFIs are shown for GATA-3 (A) and T-bet (B). No statistical differences (ns) were found using Mann-Whitney test.

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Proprotein Convertase Subtilisin/Kexin Type 7 (PCSK7) Is Essential for the Zebrafish Development and Bioavailability of Transforming Growth Factor β1a (TGFβ1a)*[S]

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Background: The *in vivo* importance of PCSK7 in the vertebrates is currently poorly understood.
Results: Inhibiting PCSK7 in zebrafish results in various developmental defects and dysregulation of gene expressions.
Conclusion: PCSK7 is essential for zebrafish development and regulates the expression and proteolytic cleavage of TGFβ1a.
Significance: PCSK inhibitors are considered future therapeutics for human diseases; understanding the biological role of PCSK7 is therefore critical.

Proprotein convertase subtilisin/kexin (PCSK) enzymes convert proproteins into bioactive end products. Although other PCSK enzymes are known to be essential for biological processes ranging from cholesterol metabolism to host defense, the *in vivo* importance of the evolutionarily ancient PCSK7 has remained enigmatic. Here, we quantified the expressions of all *pcsk* genes during the 1st week of fish development and in several tissues. *pcsk7* expression was ubiquitous and evident already during the early development. To compare mammalian and zebrafish PCSK7, we prepared homology models, which demonstrated remarkable structural conservation. When the PCSK7 function in developing larvae was inhibited, we found that PCSK7-deficient fish have defects in various organs, including the brain, eye, and otic vesicle, and these result in mortality within 7 days postfertilization. A genome-wide analysis of PCSK7-dependent gene expression showed that, in addition to developmental processes, several immune system-related pathways are also regulated by PCSK7. Specifically, the PCSK7 contributed to the mRNA expression and proteolytic cleavage of the cytokine TGFβ1a. Consequently, *tgfβ1a* morphant fish displayed phenotypical similarities with *pcsk7* morphants, underscoring the importance of this cytokine in the zebrafish development. Targeting PCSK activity has emerged as a strategy for treating human diseases. Our results suggest that inhibiting PCSK7 might interfere with normal vertebrate development.

Seven proprotein convertase subtilisin/kexin (PCSK1,[3] PCSK2, FURIN, and PCSK4–PCSK7) enzymes modulate the biological activity of immature proproteins by catalyzing limited proteolysis at sites containing a stretch of basic amino acid residues. Consequently, PCSK enzymes are important regulators of a multitude of biological events, including development, host defense, and hormone function (1). Although the archetype PCSKs possess closely related, even redundant, biochemical properties *in vitro* and often share substrate molecules, studies with genetically targeted animals and humans carrying inactive PCSK alleles argue for substrate specificity. FURIN (2), PCSK5 (3), and PCSK6 (4) are essential for normal mammalian development, whereas the inactivation of PCSK1 (5), PCSK2 (6), and PCSK4 (7) result in tissue-restricted phenotypes that range from infertility to defects in the neuroendocrine system. Genetic inactivation has also demonstrated a specific function for the more recently identified and biochemically unique PCSK family members MBTPS1 (8) and PCSK9 (9) in cholesterol and lipid metabolism. In contrast, the biological function of the evolutionarily most ancient enzyme, PCSK7, has remained enigmatic. Scattered references in the literature postulate that PCSK7-deficient mice have little, if any, phenotypic abnormalities, but a comprehensive analysis of these animals has not yet been published (10–12).

It has been demonstrated that similarly to *FURIN*, *PCSK5*, and *PCSK6* the expression of *PCSK7* is ubiquitous and that it

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[3] The abbreviations used are: PCSK, proprotein convertase subtilisin/kexin; MO, morpholino; RC, random control; dpf, days postfertilization; hpf, hours postfertilization; GO, gene ontology; hu, human; zf, zebrafish; QRT-PCR, quantitative RT-PCR; e, exon; BMP, bone morphogenetic protein.

exerts its function mainly in the trans-Golgi network and on the cell surface (13, 14). Importantly, previous biochemical studies indicate that PCSK7 operates often redundantly with other PCSKs, especially FURIN. For example, the activity of PCSK7 can be replaced in the site-specific cleavage of pro-BMP4 (15), pro-PDGF (16), pro-NGF (17, 18), and pro-VEGF-C (19). However, PCSK7 seems to be solely responsible for rescuing an unstable MHC I in post-endoplasmic reticulum compartments (20) and for the proteolysis of pro-EGF (21). In addition, a genome-wide association study recently revealed that an SNP (rs236918) in the *PCSK7* gene region is associated with an increase in the serum level of soluble transferrin receptor, thus implying that PCSK7 could have a role in iron metabolism in humans (22). This observation was later confirmed by showing that PCSK7 is the only convertase that sheds transferrin receptor into the medium in several cell lines (23).

Most mammalian proteins and genes have orthologs in the zebrafish, which makes them a feasible model for studying the role of PCSK enzymes in vertebrate biology *in vivo*. Previously, the functions of FURIN (24), PCSK5 (25), MBTPS1 (26), and PCSK9 (27) have been assessed in developing zebrafish. Inactivation of FURIN and MBTPS1 in zebrafish results in defective skeletal and cartilage formation, respectively, whereas PCSK5 and PCSK9 are important for neurological development. To decode the poorly defined PCSK7 function in vertebrate biology *in vivo*, we inhibited PCSK7 in zebrafish using morpholinos (MOs). The phenotypes of the *pcsk7* morphants clearly demonstrate that PCSK7 has a critical and non-redundant role in early zebrafish development.

EXPERIMENTAL PROCEDURES

Genes Studied—Blast searches were used to look for zebrafish orthologies for human *PCSK* genes. The following zebrafish *pcsk* genes were found: *pcsk1*, ENSDARG00000002600; *pcsk2*, ENSDARG00000019451; *furinA*, ENSDARG00000062909; *furinB*, ENSDARG00000070971; *pcsk5a*, ENSDARG00000067537; *pcsk5b*, ENSDARG00000060518; *pcsk7*, ENSDARG00000069968; *mbtps1*, ENSDARG00000014634; and *pcsk9*, ENSDARG00000074185.

Zebrafish Lines—Embryos used in all experiments were obtained through natural crosses of wild-type AB strain individuals. Adult fish used in QRT-PCR expression analyses as well as in MO experiments were also of the wild-type AB strain. All fish used in the experiments were maintained under standard conditions at 28.5 °C. The care and analyses of the animals were in accordance with the Finnish Laboratory Animal Welfare Act 62/2006, the Laboratory Animal Welfare Ordinance 36/2006, and Authorization LSLH-2007-7254/Ym-23 by the national Animal Experiment Board.

QRT-PCR Expression Analyses—Expression of *pcsk* genes was measured both in various adult zebrafish tissues (female/male gonads, liver, kidney, intestine, eye, gill, brain, skin, and tail) as well as in whole developing embryos of different ages (1–7 days postfertilization (dpf)). Conventional quantitative real time PCR with a reverse transcribed cDNA template from total RNA was used. *ef1a* gene (ENSDARG00000020850) was utilized as a housekeeping gene (28). Primers used in the QRT-PCR analyses are presented in Table 1.

TABLE 1
Primers used in QRT-PCR analyses
F, forward; R, reverse.

Gene	Sequence 5′–3′
pcsk1	F CGGGAAAAGGAGTGGTCAT
	R GGTGGAGTCGTATCTGGG
pcsk2	F CGGATCTGTATGGAAACTGC
	R GCCGGACTGTATTTTATGAATG
furinA	F AGCATGTTCAAGCGCAG
	R CCAGTCATTGAAGCCCTCA
furinB	F CCAAGGCATCTACATCAACAC
	R ACACCTCTGTGCTGGAAA
pcsk5a	F AAGCCATGGTACCTGGAAGA
	R GGTCAGAGCTGGATTTGCTT
pcsk5b	F TGTTCCTCGACCCTTACCAC
	R ATCTCGCCATGTCAGGAAAG
pcsk7	F AGAGTGTTGGACGGGC
	R TGCCTAATGGATGCGGT
mbtps1	F GATGTTATAGGTGTTGGAGGG
	R TCACGATGTCAGGCTTCA
pcsk9	F AGGGCAAGGGTACTGTG
	R TGTTTAGGGTGCGACTGA
ef1a	F CTGGAGGCCAGCTCAAACAT
	R ATCAAGAAGAGTAGTACCGCTAGCATTAC
tgfβ1A	F CAGGATGAGGATGAGGACTA
	R CAGCCGGTAGTCTGGAATA

Morpholinos—Prior to morpholino design 8–10 individual fish of the AB strain were sequenced for each gene to verify the sequence identity with the published sequence and to find genomic regions homomorphic enough for MO design. The *furinA* and *furinB* MOs were designed to hit ATG sites, whereas the *pcsk7* MOs target exon-intron boundaries in exon 3 (*pcsk7* e3 MO) and exon 8 (*pcsk7* e8 MO). These exons contain codons for amino acids of the catalytic triad of the PCSK7 enzyme. The morpholino for *p53* was commercially predesigned, and the random control (RC) morpholino is a random base mixture at every position intended for use as a negative control.

The sequences for the gene-specific MOs used were as follows: *furinA*, TCAATGAGGCAAGCCTGAGATCCAT; *furinB*, ACAGCAGGATCAAGCGGCCCTCCAT; *pcsk7* e3, AGGACTCTGGAAAACACACAGGTTT; *pcsk7* e8, CTTTATGGTTTGTGGATGTACCTGT; *p53*, GCGCCATTGCTTTGCAAGAATTG; and *tgfβ1a*, TCAGCACCAAGCAAACCAACCTCAT. Morpholinos were designed and synthesized by GeneTools (Philomath, OR) and stored dissolved in distilled water (−20 °C) at a 1 mM concentration.

Morpholino and RNA Injections—For injections, 0.25–1.0 pmol of each MO was used. A rhodamine dextran tracer was used to control the injections, and unsuccessfully injected embryos were removed at 1 dpf. All injections (1–2 nl) were administered into the yolk sac of a one- to four-cell stage zebrafish embryo. 0.2 M KCl was used as buffer in the injection solutions. The RNA used in the RNA rescue experiments was transcribed from *pcsk7* cDNA with the SP6 mMESSAGE mMACHINE kit (Ambion, Austin, TX) according to the manufacturer's instructions (imaGenes, Berlin, Germany). All MO injection experiments were controlled with embryos injected with RC MO (GeneTools) as well as with non-injected embryos. Injections were carried out using a PV830 Pneumatic PicoPump (World Precision Instruments) and a Nikon SM7645 microscope. For visual analysis and live fish pictures, a SteREO Lumar V12 microscope with the AxioVision Rel. 4.8 program (Carl Zeiss) was used.

pcsk7 Histological Staining—The larvae were fixed in 4% paraformaldehyde, PBS solution and embedded in 2% agarose. Next, the samples were dehydrated in an alcohol series (70%, 96%, absolute ethanol, and xylene; 1 h in each). The dehydrated samples were embedded in paraffin, and 5-μm transverse sections were cut. These were then fixed on glass slides at 58 °C, deparaffinized in xylene (2 × 4 min) and in absolute, 96, and 70% ethanol (each 1–2 × 3 min), and rinsed with distilled H_2O. The hematoxylin-eosin staining was performed as follows: Mayer's hematoxylin, 2 min; running tap water, 2 min; water, 1.5 min; 70% ethanol, 15 s; eosin Y, 15 s; 96% ethanol, 30 s twice; absolute ethanol, 1 min twice; and xylene, 1–4 min. Thereafter, the slides were mounted, and pictures were taken using an Olympus SZX16 microscope and a Color View Soft Imaging System camera.

pcsk7 in Situ Hybridization—Whole mount *in situ* hybridization was performed for *pcsk7* as described in detail in Thisse and Thisse (29) to analyze the detailed expression pattern of the gene. 2-dpf embryos were used. An antisense probe was used for specific detection of the *pcsk7* mRNA, and a sense probe was used to control for unspecific background staining.

RNA Microarray—100 ng of total RNA (triplicate samples pooled from 18–35 embryos/group; two time points of 6 and 24 hpf) isolated from fish injected with RC (0.5 pmol) or *pcsk7* e3 (0.5 pmol) + *p53* (0.75 pmol) MO was amplified and Cy3-labeled. Altogether, 1.65 μg of sample was then hybridized on the arrays (Agilent 4x44K Zebrafish GE v3; 65 °C, overnight). Chips were scanned using an Agilent Technologies G2565CA Scanner, and numeric data were obtained from Agilent Feature Extraction software v10.7.1 (The Finnish Microarray and Sequencing Centre, Turku, Finland). Only features having corresponding Entrez ID available were retained. The expression value for each gene is calculated in three phases. 1) If the same probe appears in the raw data file more than once, the row from the data file that has the highest average expression across all samples is used to represent that probe. 2) If the same gene appears in the raw data file more than once, the probe that has the highest average expression across all samples is used to represent that gene. 3) The resulting gene expression values are quantile-normalized across samples. Genes with a median expression >10 on a linear scale in either the *pcsk7* morphant or control samples were included in further analyses. A two-sample *t* test was used to test the differential expression of each gene between the morphant and control samples using the $\log_2$ gene expression values. To control for the false discovery rate, the resulting *p* values from the *t* test were used to calculate *q* values for each gene as described (30). Genes with a *q* value <0.05 were retained. A $\log_2$ ratio between the median of *pcsk7* morphant samples and controls was calculated for each gene, and genes with an absolute $\log_2$ ratio ≥2 were considered differentially expressed. A gene ontology enrichment analysis was conducted using hypergeometric distribution testing. Here, we tested whether the number of differentially expressed genes annotated to a gene ontology (GO) term was larger than could be expected by chance. The resulting *p* values serve as an indication of the possible enrichment of each GO term.

To summarize the GO categories, each enriched term was annotated to a high level GO term. A high level GO term refers to terms that are directly linked to the root term biological process, molecular function, or cellular component. Finally, the number of terms under each high level GO term was calculated. Here, we included the 15 most enriched terms into the analysis. The original raw data of the microarray are available in the Gene Expression Omnibus database.

Cloning, cDNA Constructs, and Western Blotting—FURIN-deficient RPE.40 cells (a kind gift from Prof. J. Creemers, KU Leuven, Belgium) were grown in Ham's F-12 medium supplemented with 10% fetal bovine serum and antibiotics. The cDNA sequence encoding the zebrafish *tgfβ1a* gene was amplified from the wild-type AB zebrafish cDNA (7 dpf) by PCR (forward oligo, 5′-GGAGAATTCGCCATGAGGTTGGTTTGCTTGGTGCTG; and reverse oligo, 5′-CATGGTGGTGAGGAACTGCAAGTGCAGTGGTACCGGA). The insert was subcloned into pcDNA3.1-myc-His plasmid and validated by sequencing. Human *TGFβ1* (ATCC) subcloned into pcDNA3.1-myc-His, pSVL-hu*FURIN* (ATCC), pcDNA3-hu*PCSK7*-FLAG (a gift from Prof. J. Creemers), pME18S-FL3-zf*furinA*, pME18S-FL3-zf*furinB*, or pCMV-SPORT6.1-zf*pcsk7* (imaGenes) was transfected in RPE.40 cells using FuGENE® 6 transfection reagent (Promega). 48 h after transfections, supernatants were collected, and cells were lysed in Triton X-100 lysis buffer (20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 20% glycerol, 0.1% Triton X-100, 1 mM EDTA, 50 mM NaF, 1 mM tris(2-carboxyethyl)-phosphine hydrochloride) supplemented with protease inhibitors (Complete Mini, Roche Applied Science). Equal amounts of proteins were separated by SDS-PAGE, and immunodetection was performed using anti-myc (M5546 Sigma) primary antibody and anti-mouse HRP secondary antibody (HAF007, R&D Systems). Visualization was done using the ECL™ Western blotting detection kit (GE Healthcare) and AGFA CP1000 imaging system. Signal intensities were analyzed using NIH ImageJ software.

RESULTS

Quantification of pcsk Gene Expression in Zebrafish—The zebrafish genome contains orthologs for most mammalian genes. We blasted the human PCSK protein sequences against zebrafish genome databases and found unambiguous orthologs for all but two members (PCSK4 and PCSK6) of the mammalian PCSK family. Two isoforms for both human *FURIN* (*furinA* and *furinB*) and *PCSK5* (*pcsk5a* and *pcsk5b*),which probably arose from a genome duplication that occurred early in the evolution of teleost fish, were present in the zebrafish genome (31).

We first analyzed the expression of *pcsk* genes in the whole developing zebrafish using a QRT-PCR approach (1–7 dpf). In these experiments, different *pcsk* genes showed variable expression patterns. For example, the *pcsk* family members with reported implications in neuronal development (*pcsk1*, *pcsk2*, *pcsk5a*, and *pcsk9*) were significantly up-regulated during the early development (Fig. 1*A*). In contrast, the expression of other *pcsk* genes remained relatively constant during the first 7 days of life. However, a subtle but significant up-regulation of the *furinB*, *pcsk5b*, and *pcsk7* mRNAs could be observed at 2.5 dpf. In accordance with previous studies in mammals, our experiments assessing tissue-specific *pcsk* expression in adult fish

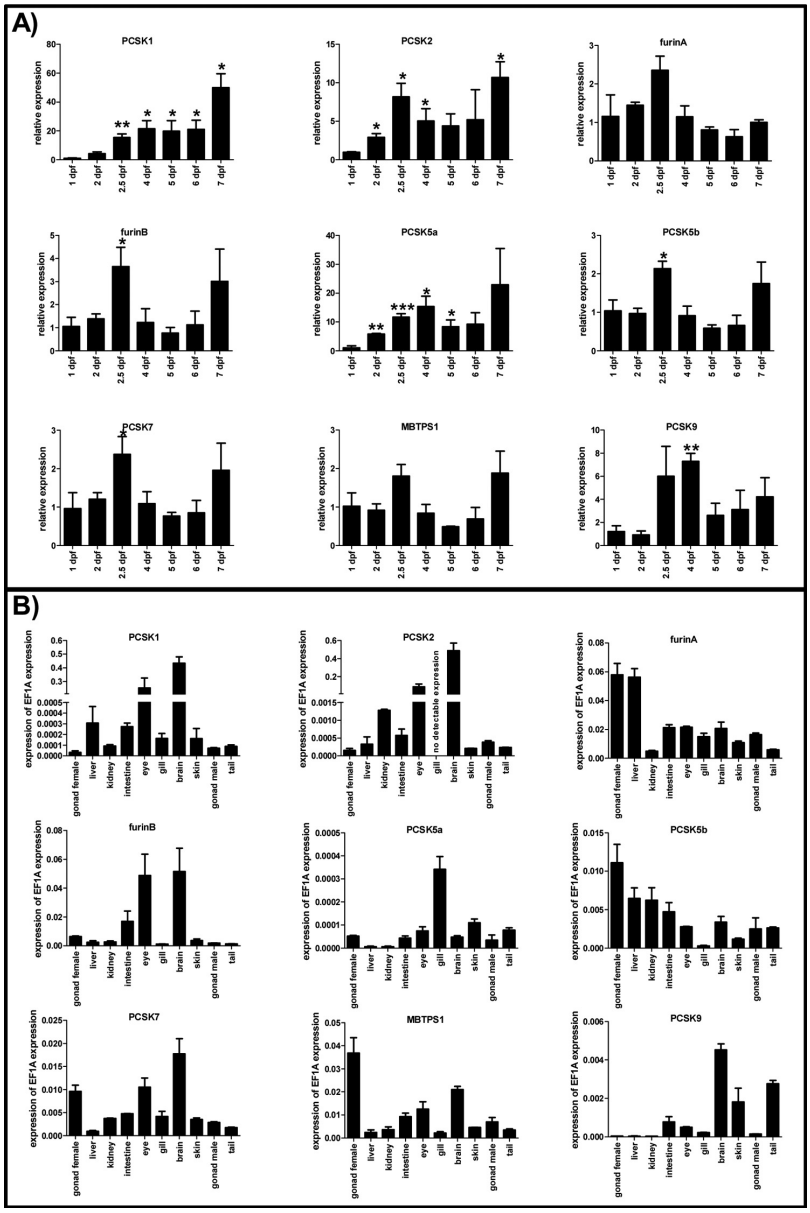


FIGURE 1. **Expression of *pcsk* genes in zebrafish.** QRT-PCR analysis was used to analyze the relative expression of the *pcsk* genes in developing zebrafish larvae (1–7 dpf) (*A*) and various adult zebrafish tissues (*B*). *A*, *pcsk* gene expression levels were normalized to the housekeeping gene *ef1a*, and the normalized gene expression on 1 dpf was given a value of 1. Other time points are shown as relative to this. *Asterisks* denote statistical significance for differences in comparisons between 1 dpf and other time points: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ in Welch-corrected two-tailed Student's *t* tests. Experiments in *A* were performed with three biological replicates (each sample consisted of 15–30 individual larvae depending on the age of larvae). *B*, in adult tissue analyses, gene expression levels related to that of *ef1a* are shown. Experiments in *B* were performed twice in technical replicates with essentially similar results. *Error bars* represent S.D. Note the diverse scales on the *y axis*.

showed particularly high expression of *pcsk1* and *pcsk2* in eye and brain (32, 33) (Fig. 1*B*). Apart from the lack of *pcsk2* in gill tissue, variable degrees of expression of all proprotein convertase enzymes could be detected with QRT-PCR in the tested tissues. Interestingly, two orthologs of *FURIN* and *PCSK5* showed (to some extent) complementary tissue expression profiles. The *pcsk7* expression was highest in female gonads, brain, and eye in adult zebrafish. The magnitude of *pcsk5a/b* and *pcsk7* expression levels was generally lower than that of the *furin* genes. These data corroborate that *pcsk7* is present in

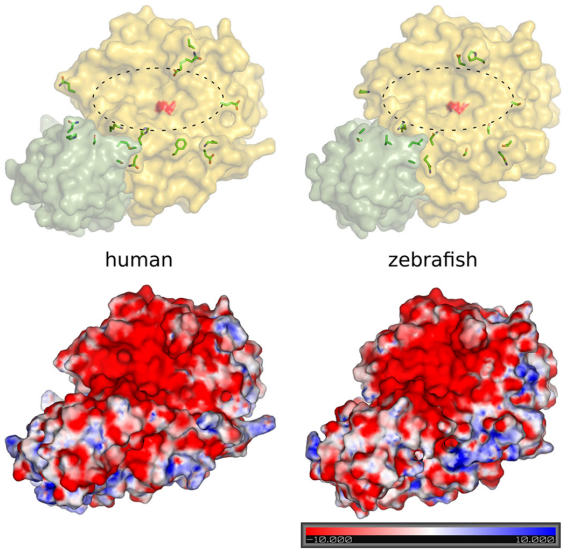


FIGURE 2. **Homology models of human and zebrafish PCSK7.** *Upper part*, *yellow*, catalytic domain; *green*, P domain. The catalytic site is marked with an *oval*, and the surface of the catalytic serine is highlighted in *red*. Amino acid differences close to the conserved substrate binding site are shown in *stick* presentation. *Lower part*, electrostatic potentials (*kT/e*) for the models were calculated using APBS 1.3 (57) and visualized in PyMOL 2.7. *Red-white-blue* color indicates the ±10 *kT/e* electrostatic potential plotted on the protein surface.

both developing larvae and multiple adult zebrafish tissues together with other convertase enzymes. This allows the reliable assessment of the specific role of the *pcsk7* in vertebrate biology.

Homology Modeling of PCSK7—To assess putative structural and electrostatic differences between mammalian and zebrafish PCSK7, we prepared a homology model of the protein with Modeler 9v10 (34) using published crystallographic structures of mouse FURIN and yeast Kexin as templates. Our modeling efforts showed evident structural conservation in catalytic and P domains of PCSK7 between the human and zebrafish (Fig. 2). We then analyzed the binding of a substrate to PCSK7 by superimposing the crystallographic structure of subtilisin (Protein Data Bank code 1CSE (35)) with the PCSK7 models. These data demonstrated that most of the potential substrate-binding residues are also conserved between human and zebrafish. The alignment of the PCSK7 catalytic and P domains of several species further demonstrated that the active sites are close to identical except for a non-conserved loop preceding the catalytic histidine (Fig. 3). The human sequence VENG in this loop is replaced by GPSD in zebrafish, which potentially affects substrate specificity at the P2 or P1′ site (Figs. 2 and 3). Notably also, a little further away from the active site, differences in the charge distribution can be observed (Figs. 2, *lower part*, and 3). In conclusion, PCSK7s in less developed organisms display remarkable structural similarities but subtle electrostatic differences with mammalian counterparts. This indicates that studying the zebrafish PCSK7 function can also give insights into other vertebrate homologs.

Inactivation of PCSK7 in Zebrafish Larvae Results in Developmental Lethality—Previous *in vitro* experimental data show that FURIN can often replace PCSK7 (19, 36, 37). To compare and contrast how these enzymes regulate zebrafish development, we first interfered with *furinA* and *furinB* translation using morpholino technology. In accordance with a previous report (24), the simultaneous blocking of *furinA* and *furinB* with MOs resulted in a reduction in ventral jaw structures and consequently an open mouth phenotype (data not shown). Disruption of *furinA*/*B* translation also reduced the survival of fish larvae; only 30.6% (22 of 72 fish) of MO-injected fish were alive on 7 dpf. We next designed two distinct morpholinos that block the pre-messenger RNA splicing at exon-intron boundaries around the catalytic site containing exons 3 and 8 in the zebrafish *pcsk7* gene (e3 and e8 MOs). Injecting the *pcsk7*-targeting MOs alone or in combination resulted in numerous defects that reduced the survival of fish larvae dramatically and resulted in 100% mortality by 7 dpf (Fig. 4). The survival rate and gross phenotypes observed were MO dose-dependent, and the development of several organs, including the brain, eye, heart, otolith, and tail, was severely affected.

Because *p53*-dependent off-target neural toxicity has been estimated to affect 15–20% of all morpholino injections (38, 39), we co-injected a *p53* MO together with the *pcsk7*-silencing MOs. The phenotypes and survival of morphant fish remained similar compared with fish not injected with the *p53* MO (Fig. 4). This indicates that the observed lethality of the *PCSK7* morphant was not due to *p53*-mediated toxicity. To further validate the specificity of the *pcsk7* MO phenotypes, we set up an RNA rescue experiment. When *in vitro* transcribed *pcsk7* RNA was co-injected together with e3 MO into the developing larvae, the severity of the MO phenotypes was reduced, and the survival improved ($p < 0.0001$, log rank $\chi^2 = 38.94$, $df = 1$) (Fig. 5, *A* and *B*). To verify that the *pcsk7* MOs specifically disrupt the splicing of the *pcsk7* pre-mRNA molecules, we amplified and sequenced the affected exon regions from the morphant fish (Fig. 6, *A* and *B*, and data not shown). Our results demonstrated that injecting the e3 MO results in three mRNA products with different lengths, whereas the e8 MO deletes a 58-bp fragment at the end of exon 8. These data show that *pcsk7* morpholinos target *pcsk7* pre-mRNA and that *pcsk7* has a critical and non-redundant role in zebrafish development.

PCSK7 Regulates Brain, Otolith, and Eye Development—Our expression analyses implicated *pcsk7* as prominently present in adult zebrafish eye and brain tissues. To investigate the *pcsk7* expression pattern in larvae, we used RNA *in situ* hybridization on wild-type fish at 2 days postfertilization. These experiments demonstrated that *pcsk7* is most abundant in the entire cranial area and the eye already in developing wild-type zebrafish (Fig. 7, *A* and *B*).

The observed gross anatomical differences and location of early *pcsk7* expression prompted us to analyze in more detail the head region phenotypes seen in the developing zebrafish. A dramatic underdevelopment of the entire brain and eyes was observed in histological sections (Fig. 7, *C* and *D*). The cranium of *pcsk7* morphants contained mostly a non-cellular, meshlike substance, whereas the brain remained underdeveloped and was located abnormally downward between the eyes. In addi-

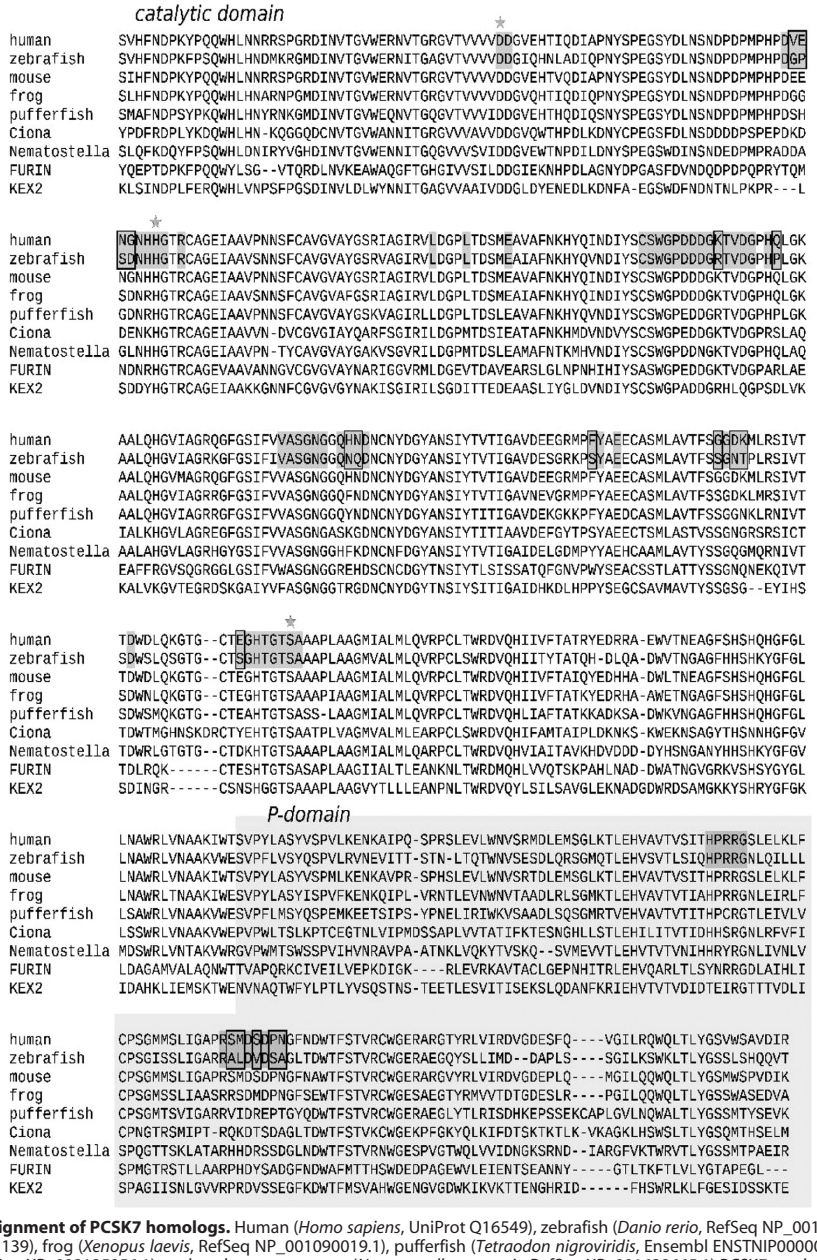


FIGURE 3. **ClustalW alignment of PCSK7 homologs.** Human (*Homo sapiens*, UniProt Q16549), zebrafish (*Danio rerio*, RefSeq NP_001076494.1), mouse (*Mus musculus*, UniProt Q61139), frog (*Xenopus laevis*, RefSeq NP_001090019.1), pufferfish (*Tetraodon nigroviridis*, Ensembl ENSTNIP00000017367), vase tunicate (*Ciona intestinalis*, RefSeq XP_002125956.1), and starlet sea anemone (*Nematostella vectensis*, RefSeq XP_001638665.1) PCSK7 catalytic and P domains were aligned with those of mouse FURIN (Protein Data Bank code 1P8J (58)) and yeast Kexin (*Saccharomyces cerevisiae*, Protein Data Bank code 2ID4 (59)). The catalytic triad is marked with *stars*, and potential substrate-binding residues are in *dark gray*. Residues showing differences in Fig. 2 are highlighted with *rectangles*.

tion, the cellular structure in the eyes of MO-injected fish was unorganized, and photoreceptor, outer and inner plexiform, and inner nuclear and ganglion cell layers were undefined. However, the retinal pigment epithelium and lens structures were also evident in morphant fish. The zebrafish with a nonfunctional PCSK7 also had an abnormal number of otoliths. Injection of the e3 MO resulted in a reduction in otolith number (one otolith per ear in >92% of fish, $p = 7.9e{-}63$), whereas the fish injected with the e8 MO in contrast had an increased amount of otoliths per ear (three otoliths per ear in >26% of fish, $p = 2.4e{-}11$). All evaluated 149 control fish had the normal two otoliths per ear (Fig. 8, *A–E*). These data show that in

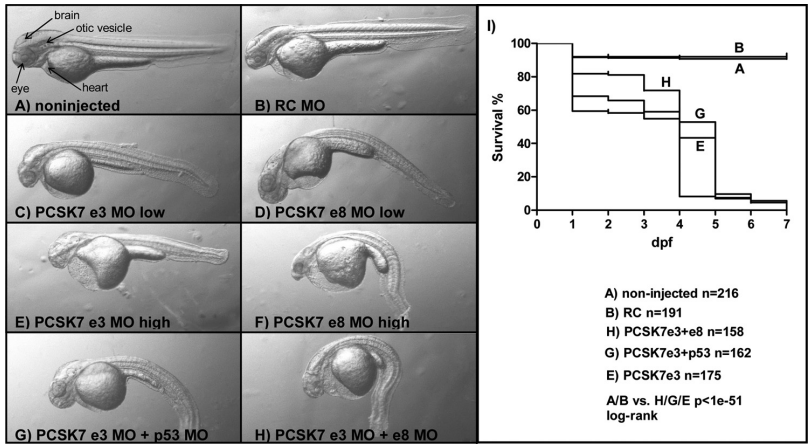


FIGURE 4. **Phenotypes and survival of *pcsk7* morphant fish.** Zebrafish larvae (2 dpf) were non-injected (*A*) or injected with RC MO (0.5 pmol) (*B*), *pcsk7* e3 MO (0.25 pmol) (*C*), *pcsk7* e8 MO (0.25 pmol) (*D*), *pcsk7* e3 MO (0.5 pmol) (*E*), *pcsk7* e8 MO (0.5 pmol) (*F*), *pcsk7* e3 + *p53* MO (0.5 + 0.75 pmol) (*G*), or *pcsk7* e3 + e8 MO (0.25 + 0.25 pmol) (*H*). *A–H*, 35× *magnification*. *I*, survival of fish injected with *pcsk7* e3 MO (0.5 pmol), *pcsk7* e3 + e8 MOs (0.25 + 0.25 pmol), and *pcsk7* e3 + *p53* MOs (0.5 + 0.75 pmol) was significantly lower than that of RC morphant (0.5 pmol) or uninjected fish ($p < 1e-51$ for all comparisons by log rank test). *pcsk7* e8 MO reduced the survival of larvae similarly to e3 MO: all larvae died by 6 dpf ($n = 98$ larvae; e8 MO, 0.5 pmol; data not shown).

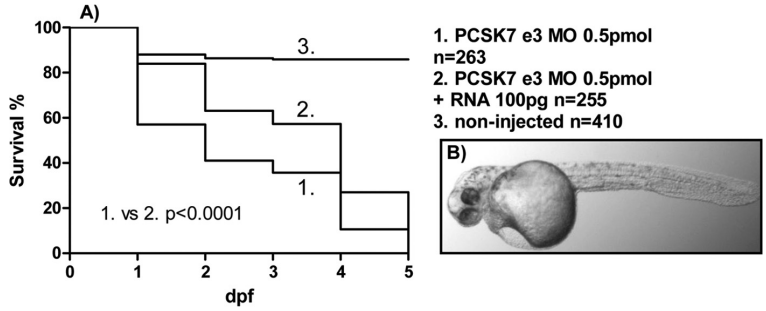


FIGURE 5. ***pcsk7* mRNA improves the survival and reduces the severity of the *pcsk7* morphant phenotype.** *A*, survival of zebrafish uninjected or injected with *pcsk7* e3 MO alone or together with *in vitro* transcribed *pcsk7* mRNA was monitored daily. Data are pooled from two independent experiments. *B*, 2-dpf zebrafish larvae co-injected with *pcsk7* e3 MO (0.5 pmol) and *pcsk7* mRNA (100 pg).

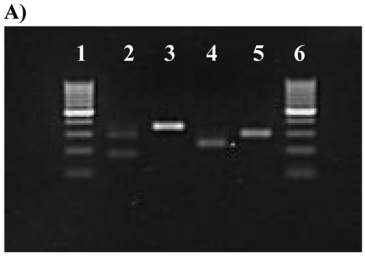
accordance with its spatiotemporal expression PCSK7 is important for several developmental processes of the vertebrate cranial organs.

Analysis of PCSK7-dependent Gene Networks—Genome-wide expression analyses can be used to clarify the biological relevance of gene-specific studies. Therefore, to comprehend the gene networks that are affected in the *pcsk7* morphant fish, we performed a genome-wide mRNA expression study in whole fish larvae after PCSK7 inhibition. We chose to evaluate gene expression at two different time points, 6 and 24 hpf. At the gastrula stage (6 hpf), most of the zygotic genes have commenced transcript accumulation and often peak in their expression. In contrast, after 24 hpf, the genome-wide gene expression profile remains relatively steady as was demonstrated in a previous analysis with wild-type zebrafish (40).

Conventional PCSK enzymes are highly redundant in substrate processing. Therefore, it is important to evaluate whether disrupting PCSK7 function causes any significant changes in the expression of other proprotein convertases (41), which might contribute to or even compensate for the observed phenotype. At both 6 and 24 hpf, the other conventional *pcsk* genes were not found to be significantly up-regulated in *pcsk7* morphant fish. *pcsk7* expression, however, was significantly reduced in the morphant zebrafish (log ratio of −1.95 and $p = 5.77e-6$ at 24 hpf). The decrease in the *pcsk7* mRNA level is presumably due to the cellular deletion of the defectively spliced *pcsk7* pre-mRNA molecule.

To understand which major biological, molecular, and cellular processes are dependent on the intact PCSK7 enzyme, we first performed a GO enrichment analysis using hypergeometric distribution testing. At 6 hpf, as expected from the observed morphant phenotypes, terms related to development (for example otic placode formation) and transcription regulation were abundantly enriched. Consequently, at 24 hpf, genes related to metabolism were also commonly up-regulated. Strikingly, at both 6 and 24 hpf, genome-wide mRNA expression samples showed a clear enrichment in immune system-related terms with *immune system* being the most enriched ($p = 0.0004$) term at 24 hpf. Several cytokine signaling-affiliated terms could also be found among the most significantly enriched terms at both time points (supplemental Table S1 and Fig. 9).

A)



B)

Primer	Sequence
e3 F	ATGACATGAAGCGAGGGATG
e3 R	CGATGGCCTCCATACTGTCT
e8 F	AACCTATGCGGAGGAGTGTG
e8 R	TGATGGAAACCAGCTCCATT

FIGURE 6. ***pcsk7* MO injections result in erratic splicing of the pre-mRNA.** *A*, total RNA was isolated from the control (RC MO) and different *pcsk7* morphant fish and reverse transcribed into cDNA, which was amplified by PCR and run on an agarose gel. From the *left*, *lane 1*, 100-bp molecular weight marker; *lane 2*, *pcsk7* e3 MO; *lane 3*, control (=RC MO with *pcsk7* e3 primers); *lane 4*, *pcsk7* e8 MO; *lane 5*, control (=RC MO with *pcsk7* e8 primers); *lane 6*, 100-bp molecular weight marker. Sequencing showed that 345- and 288-bp DNA fragments represent intact, wild-type *pcsk7* mRNA that can be detected in RC MO samples with the e3 (*lane 3*) and e8 primers (*lane 5*), respectively. The *pcsk7* e3 MO injection resulted in three differentially sized fragments: (i) a fragment corresponding to the completely deleted exon 3 (the shortest band in *lane 2*), (ii) a fragment with a 58-bp deletion from the start of exon 3 followed by a 49-bp polymorphic region either from exon 3 or from exon 4 and further supplemented with the untouched end of exon 3 (the middle band in *lane 2*), and (iii) a faint band of wild-type exon 3 (the longest band in *lane 2*). The *pcsk7* e8 MO injection deleted a 58-bp fragment from the end of exon 8 (*lane 4*) resulting in a truncated *pcsk7* mRNA molecule. *B*, sequences for the primers used for the sequencing described in *A*. *F*, forward; *R*, reverse.

A more detailed analysis of the PCSK7-dependent genes in the microarray revealed the dysregulation of several immune system-, neurological system-, and otolith/otic vesicle-related genes (supplemental Table S2 and Fig. 9). For example, the expression levels of important regulators of both the adaptive and innate immunity, *stat4*, *tgfβ1a*, and *csfra*, were greatly reduced at 6 hpf in the *pcsk7* morphants, whereas otolith/ear-related *foxi1* (down-regulated at 6 hpf) and *pax2a*, *msxc*, and *fsta* (up-regulated at 6 hpf) were also dysregulated. In addition, a multitude of differentially expressed neurological genes, including several protocadherin and fibroblast growth factor genes, were found to be dependent on a functional PCSK7.

We have demonstrated previously that another PCSK family member, FURIN, is a key regulator of both T helper 1 type immune responses and T regulatory cell-mediated peripheral immune tolerance (42, 43). These crucial events of adaptive immunity are dependent on transcription factor STAT4 function and cytokine TGFβ1 signaling, respectively. We noticed that in our microarray experiment both of the aforementioned genes were significantly repressed when *pcsk7* expression was blocked. To confirm this finding, we performed a QRT-PCR analysis that revealed a down-regulation of both *tgfβ1a* and *stat4* by 11.7- and 5.1-fold, respectively, at 6 h postfertilization in an RC *versus pcsk7* + *p53* comparison (three biological replicates, one-tailed $p = 0.037$ for both genes in Student's t test with Welch correction; data not shown). These findings imply that, in addition to FURIN, PCSK7 may also have an important regulatory role in developing adaptive immune responses.

PCSK7 Regulates the Expression of tgfβ1a in Zebrafish—In mammals, FURIN is the major proprotein convertase enzyme that regulates the bioavailability of the anti-inflammatory TGFβ1 cytokine (43, 44). From previous experiments, it is also noteworthy that TGFβ1 can directly up-regulate the expression of *furin* (45). However, PCSK7 can also proteolytically process the TGFβ superfamily cytokines. Mammalian PCSK7 is sug-

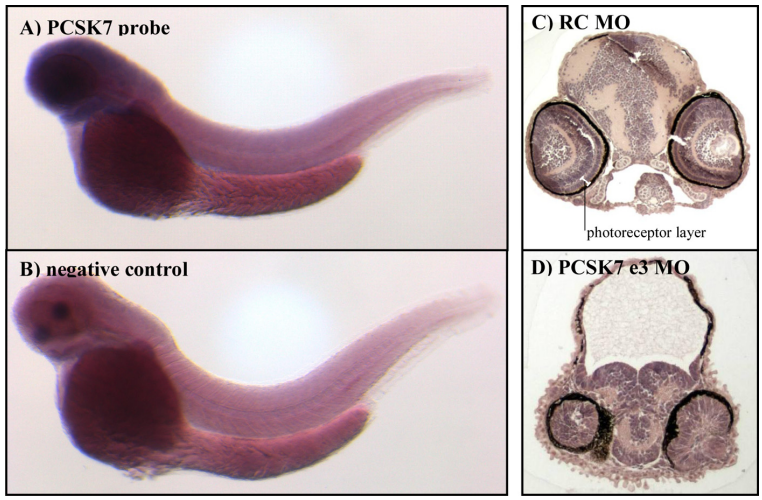
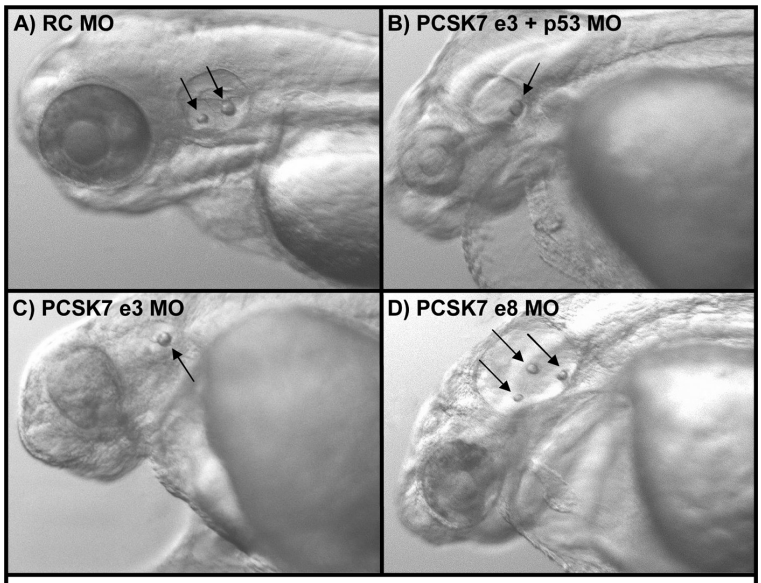


FIGURE 7. ***pcsk7* is expressed in the head region, and *pcsk7* morphant fish have cranial developmental defects.** *pcsk7* expression (*dark blue*) was analyzed with RNA *in situ* hybridization in wild-type zebrafish larvae at 2 dpf. *A*, *pcsk7* antisense probe. *B*, negative control (*pcsk7* sense probe). *Right-hand panels* show hematoxylin-eosin-stained transverse sections of zebrafish cranial region of RC (*C*) and *pcsk7* e3 morphant (*D*) (3 dpf).



E)

		at 2dpf			
MO	conc. pmol	1 otolith per ear (%)	2 otoliths per ear (%)	3 otoliths per ear (%)	p*
RC	0.5	0	149 (100%)	0	
PCSK7 e8	0.25	0	109 (97.3%)	3 (2.7%)	NS
PCSK7 e8	0.5	0	57 (73.1)	21 (26.9%)	2.4e-11
PCSK7 e3	0.25	88 (86.3%)	12 (11.7%)	2 (2.0%)	1.9e-55
PCSK7 e3	0.5	94 (92.2%)	6 (5.9%)	2 (2.0%)	7.9e-63
PCSK7 e3+p53	0.5+0.75	93 (86.9%)	14 (13.1%)	0	3.3e-55

* 2-sided 2x3 Fisher exact test PCSK7 MO vs RC

FIGURE 8. ***pcsk7* morphant fish have an abnormal number of otoliths.** Zebrafish were injected with RC MO (0.5 pmol) (*A*), *pcsk7* e3 + *p53* MO (0.5 + 0.75 pmol) (*B*), *pcsk7* e3 MO (0.5 pmol) (*C*), or *pcsk7* e8 MO (0.5 pmol) (*D*), and otoliths (*arrows*) were visualized on 3 dpf. Quantification of otoliths is presented in *E*. *NS*, not significant.

gested to cleave pro-BMP4 in a developmentally regulated fashion, and it possesses up to one-third of the capacity of FURIN in pro-TGFβ1 processing *in vitro* (15, 44). Furthermore, the mRNA expression correlation of human *PCSK7* with *TGFβ1* is very strong, which may indicate a physiological role for PCSK7 in pro-TGFβ1 processing (46). As described above, we observed that *tgfβ1a* (ENSDARG00000041502), the zebrafish counterpart for the mammalian *TGFβ1*, was markedly down-regulated in the microarray analysis at 6 hpf in *pcsk7* morphant fish (supplemental Table S2 and Fig. 9). This coordinated expression of *pcsk7* and *tgfβ1a* prompted us to address the importance of PCSK7 for *tgfβ1a* expression and activation and to assess whether the lack of TGFβ1a could contribute to the PCSK7-dependent phenotype.

We first wanted to investigate whether zfPCSK7 can directly proteolytically process zfpro-TGFβ1a. To this end, the cDNA encoding TGFβ1a was amplified from wild-type zebrafish and subcloned into a myc-His expression plasmid. After verification by sequencing (data not shown), we then co-expressed *tgfβ1a* together with zebrafish *furinA*, *furinB*, or *pcsk7* in FURIN-deficient RPE.40 cells. For comparison, we also co-expressed human *TGFβ1* together with human *FURIN* and *PCSK7* cDNAs. Our results clearly demonstrate that in addition to FURIN both zebrafish and human PCSK7s are able to process and promote the release of bioactive TGFβ1 (zebrafish, 16 kDa; human, 14 kDa) into cell culture supernatants (Fig. 10*A*). In line with previous reports, the PCSK7s possessed approximately one-third of the activity of FURIN in the TGFβ1 maturation (44).

We then assessed whether the *tgfβ1a* mRNA expression also remains down-regulated in *pcsk7* morphant fish in a later developmental stage and found that *tgfβ1a* gene expression is significantly repressed also at 48 hpf ($p = 0.0052$; Fig. 10*B*). To investigate how the lack of TGFβ1a contributes to the *pcsk7* morphant phenotype, we injected zebrafish with a TGFβ1a-blocking MO. In these experiments, we observed clear similarities between the *tgfβ1a* and *pcsk7* morphants; both showed tail abnormalities, pericardial swelling, and an abnormal number of otoliths (24.4% of *tgfβ1a* morphants (21 of 86) had three otoliths per ear ($p = 2.16e{-}9$ in Fisher 2 × 2 test; Fig. 10, *C* and

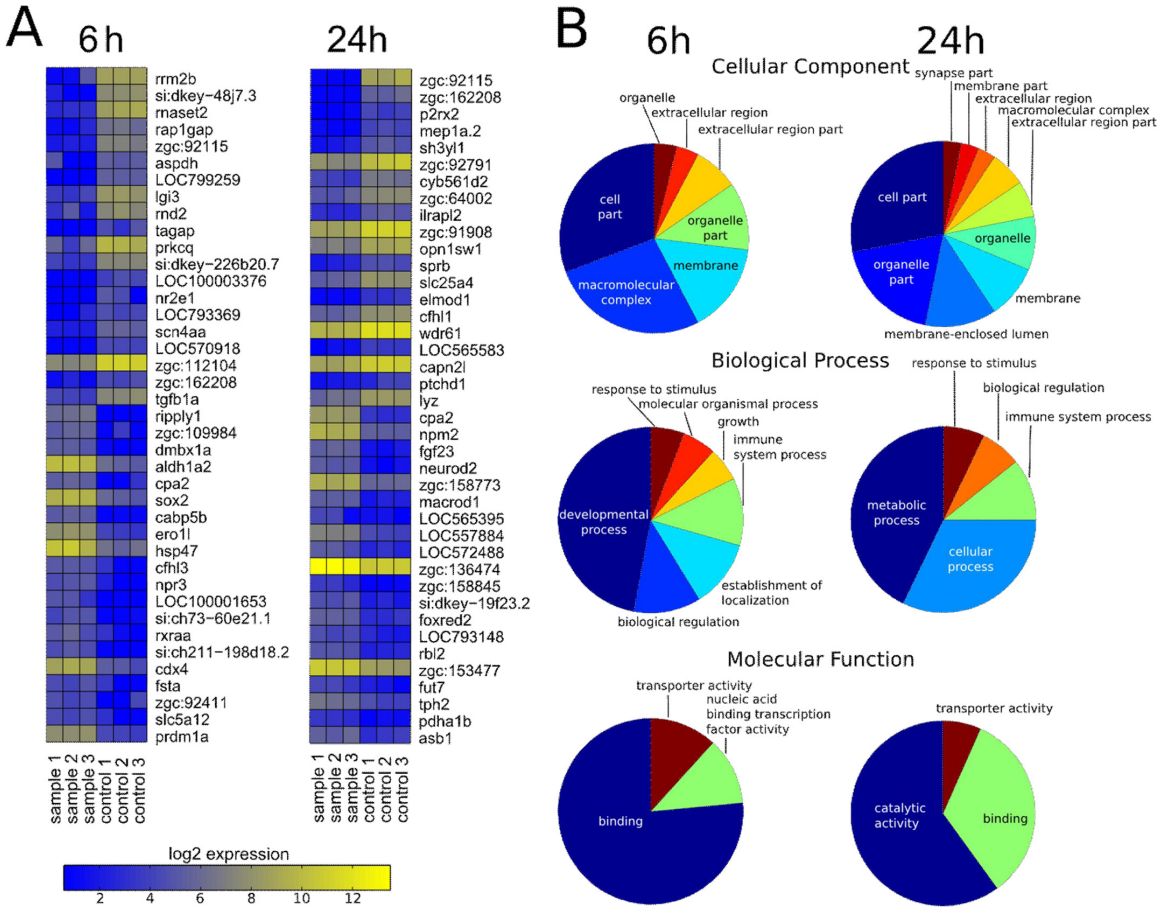


FIGURE 9. **Heat map of the most differentially expressed genes and high level summary of gene ontology enrichments between control and *pcsk7* morphant fish.** Samples were prepared, and data were analyzed as described under "Experimental Procedures." *A*, zebrafish genes that have an identifiable human homolog are shown in the heat maps. *B*, summaries from the enriched gene ontology terms. Pie charts show enriched high level categories for cellular component, biological process, and molecular function at 6 and 24 hpf. In each chart, the *size* of the *wedge* corresponds to the number of terms enriched under the given high level category.

D)). Taking these observations together, our data suggest that in zebrafish the intact PCSK7 enzyme is important for the expression and bioavailability of mature TGFβ1a and that a defect in this contributes to the observed phenotype of the *pcsk7* morphant fish.

DISCUSSION

Regardless of extensive studies on proprotein convertase enzymes in vertebrate biology, the function and significance of the evolutionarily ancient PCSK7 has remained largely unclear. In an effort to fill this gap, we studied the function of PCSK7 in zebrafish and observed that it is critical for the development of zebrafish larvae. The *pcsk7* morphant fish display severe developmental defects that lead to 100% mortality within the first 7 days of life. The lack of functional PCSK7 enzyme interferes with the organogenesis of several key elements, including the brain, eyes, and otic vesicles. In addition, our genome-wide gene expression and biochemical analyses demonstrate that PCSK7 regulates genes important for organogenesis and immunology and that it is specifically capable of contributing to the function of cytokine TGFβ1a in developing fish larvae.

To first validate the feasibility of zebrafish as a model for the analysis of the PCSK7 function in vertebrate biology, we surveyed the expression of all identifiable proprotein convertases. In accordance with previous reports in mammals, the zebrafish PCSK enzymes also show variation in their developmental and tissue-specific expression. Zebrafish homologs for the neuroendocrine system-specific PCSK1 and PCSK2 were particularly highly expressed in the fish neural tissues, and the homologs of previously reported ubiquitous enzymes, such as FURIN, PCSK5, and PCSK7, were found to be relatively widely expressed similarly to earlier reports using mammals (17, 18, 47). PCSK7 shares several common substrate molecules with PCSK5 and FURIN at least in *in vitro* analyses (48). We found that *pcsk7* was co-expressed in different tissues and developing fish together with other PCSK enzymes that have been reported

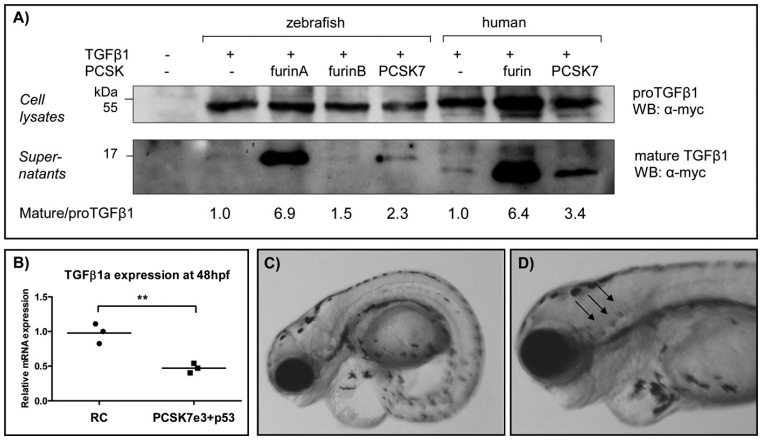


FIGURE 10. **PCSK7 regulates TGFβ1a, which affects the zebrafish larva development and otolith formation.** *A*, FURIN-deficient RPE.40 cells were transiently transfected with zebrafish *tgfβ1a*-myc or human *TGFβ1*-myc together with z*furinA*, z*furinB*, z*pcsk7*, hu*FURIN*, or hu*PCSK7*. Pro-TGFβ1 (45 kDa) and mature TGFβ1 (16 kDa in zebrafish and 14 kDa in human) expressions were detected with Western blotting (*WB*). Mature/pro-TGFβ1 ratios were quantified using NIH ImageJ software, and ratios in cells transfected only with *tgfβ1* cDNAs were given an arbitrary value of 1. Equal loading of cell lysates and supernatants was verified by Ponceau S staining (data not shown). The experiment was repeated twice with similar results. *B*, *tgfβ1a* mRNA expression was measured by QRT-PCR from *pcsk7* e3 + *p53* and RC morphant embryos at 48 hpf (**, $p = 0.0052$, two-tailed Student's *t* test). *C* and *D* depict the phenotypes (4 dpf) of zebrafish injected with *tgfβ1a* + *p53* MOs (*tgfβ1a* MO, 1.0 pmol; *p53* MO, 1.5 pmol). *tgfβ1a* morphants had incorrect otolith numbers: *tgfβ1a* MO, 65 fish with two otoliths per ear, 21 fish with three otoliths per ear; RC MO, 120 fish with two otoliths per ear, 0 fish with three otoliths per ear ($p = 2.16e{-}9$ in Fisher 2×2 test).

to compensate its biological function. Taken together, our quantitative *pcsk* expression data demonstrated obvious similarities between the mammalian and fish *pcsk* expression. Consequently, deleting the PCSK7 function in developing fish can also give important insights into the specific biological function of this poorly defined proprotein convertase in other vertebrates.

We and others have previously reported notable evolutional conservation of the catalytic and P domains in the PCSK enzymes (46, 49). To specifically address structural and electrostatic properties in the PCSK7 enzymes, we generated homology models of mammalian and fish PCSK7s and investigated the PCSK7 sequences in several species. In conclusion, our modeling data support the idea that the catalytic and P domains in zebrafish PCSK7 share most structural features with the human counterpart. Therefore again, investigating the fish PCSK7 function is also likely to generate novel insights into the PCSK7 function in other vertebrates.

The crucial role of many of the PCSK enzymes in vertebrate development is indisputable. For example, FURIN-deficient mouse embryos show defective ventral closure and axial rotation and die during the 2nd week of embryonic development (2). In addition, a mutation in *furinA* causes significant embryonic lethality in zebrafish despite the duplication of the *furin* gene (24). These fundamental phenotypes can be explained by a lack of processing of PCSK substrate molecules; in early mouse development, the significance of the proper activation of the TGFβ family cytokines, such as BMPs and NODAL (50), is particularly emphasized. In contrast, a thorough functional analysis of a mammalian model for PCSK7 is not available in the literature. A few scattered references to *Pcsk7* knock-out mice, however, suggest either complete redundancy or at least non-critical functions in mammalian development (10–12). Our analysis of the *pcsk7* morphant fish and a recent publication by Senturker *et al.* (51) using a *Xenopus* model system demonstrate that PCSK7 is indispensable at least in lower vertebrates. Both studies come to the conclusion that a lack of PCSK7 function leads to severe defects especially in the neural system and eye. In zebrafish, these defects lead to 100% mortality within the 1st week postfertilization.

A complete knock-out or an inactive mutation of the gene would give the definitive answer for the biological relevance of PCSK7 in zebrafish. However, to our knowledge, PCSK7-null zebrafish have not been produced. To overcome this limitation, we analyzed how the lack of an active PCSK7 protein affects the larva development. Using morpholinos to assess the biological function of a protein has provoked some controversy. In some cases, morpholinos can cause unspecific phenotypes due to p53-dependent off-target neural toxicity. However, in our experiments, silencing the p53 pathway did not alter the conclusive role of PCSK7. In addition, sequencing the MO-truncated *pcsk7* mRNA showed that the e8 morpholino efficiently removes the end of exon 8 (58 bp) and shifts the reading frame in the catalytic domain. This replaces roughly 50 C-terminal residues of the catalytic domain by 23 residues of non-native sequence followed by a stop codon. In contrast, the e3 morpholino results in the complete deletion of exon 3 of PCSK7. This removes a codon encoding one of the amino acids of the catalytic triad. In addition, the correct reading frame is lost for the remaining protein. As a consequence, PCSK7 proteins translated in both morphants lack the entire P domain, which is needed for correct folding of the enzyme (52). A PCSK7 without the P domain is unlikely to traverse to the secretory pathway, and it might eventually be degraded in the cell.

Co-injecting *pcsk7* mRNA with morpholino significantly improved the survival of morphant larvae and partially restored the defected phenotypes, but a complete rescue could not be achieved. The partial improvement of phenotype by RNA res-

cue has also been reported by others (53). Therefore, it is probable that a more natural spatiotemporal RNA expression of *pcsk7* than what could be achieved using RNA injections into the yolk sac would be needed to cancel out all the effects of *pcsk7* MO.

Little information has been available on PCSK7-dependent biological processes at the genomic level. Our GO enrichment analysis revealed several biological, molecular, and cellular functions that were significantly altered if PCSK7 function was inhibited. These findings corroborated the observed gross phenotype of the *pcsk7* morphants by highlighting the enrichment of several development-related GO terms. Noteworthy, the enriched GO terms also included the otic placode formation, which is a novel finding and suggests, together with the abnormal amount of otoliths in the *pcsk7* morphants, a specific and non-redundant role for PCSK7 in ear development. In the future, it will be interesting to assess whether genetic alterations in human *PCSK7* (22) play a role in hearing- or balance-related phenotypes.

One of the few specific PCSK7 functions *in vitro* is the rescue of an unstable MHC I-peptide complex (20). Analyses of microarray data on our *pcsk7* morphant fish revealed that PCSK7 in addition to having a role in several developmental pathways is indeed linked strongly to immunological processes. Differentially expressed genes included several genes that are key regulators of both the adaptive and innate immunity, such as *tgfβ1a*, *stat4*, *csf1a*, *ccr7*, and various MHC genes. The host defense-linked GO terms containing these genes were among the most enriched categories already at 6 hpf and remained significantly overrepresented until 24 hpf. It has been shown earlier that TGFβ1 up-regulates the convertase FURIN, which is the major proteolytic activator of this central anti-inflammatory cytokine (44). Interestingly, our genome-wide expression analysis demonstrated that when functional PCSK7 is not available during early development *tgfβ1a* was among the most down-regulated genes at 6 hpf. The strength of down-regulation was weaker at later phases of development, but *tgfβ1a* expression remained significantly down-regulated up to 2 days postfertilization.

Importantly, *tgfβ1a* and *pcsk7* morphant fish shared several phenotypic similarities, further suggesting that PCSK7 has a role in the regulation of the TGFβ1a cytokine in developing zebrafish. The detailed mechanisms behind the PCSK7-dependent regulation of the TGFβ1a bioavailability are not fully understood. Mature TGFβ1 cytokine is known to promote its own function by up-regulating the expressions of its own mRNA and the converting enzyme FURIN. Accordingly, because we and others have shown that PCSK7 can also process and activate pro-TGFβ1 at one-third of FURIN proteolytic capacity, the reduced PCSK7 expression can interfere with this feed-forward loop and result in reduced *tgfβ1a* expression (Fig. 10) (44). However, it is equally probable that *in vivo* PCSK7 promotes the TGFβ1a function in an indirect manner that does not involve direct proteolysis (21).

In addition to its fundamental inhibitory role in immunity (54, 55), TGFβ1 directly controls cell differentiation and proliferation. It is noteworthy that TGFβ1 deficiency in mice causes significant intrauterine lethality (56). Our experiments concur with this multifunctionality of TGFβ1 and suggest that in zebrafish TGFβ1a plays a role in various developmental processes, including otolith formation.

Proprotein convertases have a fundamental role in both health and disease. Interfering with PCSK activity holds promise for future treatment of a plethora of diseases ranging from infections to atherosclerosis. These efforts are often compromised by the lack of specificity of inhibitors of PCSK family members. If the inhibitors are considered for clinical use, it is of utmost importance to also understand the biological significance of PCSK7. Our data presented here underscore the importance of PCSK7 in zebrafish neural development and more specifically genomic processes associated with immunity. These can also be important factors to take into account when extrapolating the unwanted effects of general PCSK inhibitors in disease settings.

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Proprotein convertases in human atherosclerotic plaques: The overexpression of FURIN and its substrate cytokines BAFF and APRIL

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ABSTRACT

Background: Proprotein convertase subtilisin/kexin (PCSK) enzymes cleave proproteins into mature end products. Previously, MBTPS1 and PCSK9 have been shown to regulate cholesterol metabolism and LDL receptor recycling, whereas FURIN and PCSK5 have been suggested to inactivate lipases and regulate inflammation in atherosclerosis. Here, we systematically analyzed the expression of PCSKs and their targets in advanced atherosclerotic plaques.
Methods and results: Microarray and quantitative real-time PCR experiments showed that FURIN (42.86 median fold, $p = 2.1e{-}8$), but no other PCSK, is universally overexpressed in the plaques of different vascular regions. The mRNA expression screen of PCSK target proteins in plaques identified many known factors, but it also identified the significant upregulation of the previously overlooked furin-processed B cell activating cytokines APRIL (TNFSF13, 2.52 median fold, $p = 3.0e{-}5$) and BAFF (TNFSF13B, 2.97 median fold, $p = 7.6e{-}6$). The dysregulation of FURIN did not associate with its htSNPs or the previously reported regulatory SNP (−229, rs4932178) in the promoter. Immunohistochemistry experiments showed the upregulation of FURIN in the plaque lymphocytes and macrophages where it was co-expressed with BAFF/TNFSF13B and APRIL/TNFSF13.
Conclusions: Our data unequivocally show that FURIN is the primary PCSK that is dysregulated in the immune cells of advanced human atherosclerotic plaques, which implies a role for this enzyme in plaque pathology. Therefore, drugs that inhibit FURIN in arteries may modulate the course of this disease.

1. Introduction

Coronary heart disease (CHD) is a leading cause of both morbidity and mortality in developed countries. A primary cause of CHD is atherosclerosis, a compound chronic disease process in which focal endothelial injury, inflammation and erratic lipid metabolism have been implicated. Sclerotic arteries contain deposits of both low-density lipoprotein (LDL) and infiltrating inflammatory cells. The inflammation is further accelerated by the oxidation of LDL, which results in the worsening of the endothelial injury, proliferation of the vascular smooth muscle cells and enhanced extracellular matrix fibrinogenesis [1]. The aforementioned processes are controlled by a complex network of factors including lipoproteins, vascular growth factors, chemokines, integrins and tissue remodeling agents.

Abbreviations: GWEA, genome-wide expression array; LDA, low-density array; TREG, T regulatory cell; Th1, T helper 1; PC, proprotein convertase; PCSK, proprotein convertase subtilisin/kexin; MBTPS1, membrane-bound transcription factor peptidase site 1; CHD, coronary heart disease; LDL, low-density lipoprotein; HDL, high-density lipoprotein; VLDL, very low-density lipoprotein; TVS, Tampere Vascular Study; LITA, left internal thoracic artery; AHA, American Heart Association; TNFSF, tumor necrosis factor superfamily; MMP, matrix metalloproteinase; LDLR, low-density lipoprotein receptor.

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Many proteins that contribute to atherogenesis, such as VEGF and matrix metalloproteinases, are synthesized in the cell as inactive proproteins [2,3]. Proprotein convertase subtilisin/kexin (PCSK) enzymes are a family of nine proteases (PCSK1, PCSK2, FURIN, PCSK4–PCSK7, MBTPS1 and PCSK9) that cleave and convert their immature target proteins into a biologically active form [4]. Consequently, by controlling the activity of pro-atherosclerotic factors, PCSK enzymes play a central role in the pathogenesis of atherogenesis. For example, the lack of MBTPS1 function in a mouse liver has been shown to result in reduced cholesterol and fatty acid synthesis [5]. FURIN, PCSK6 and/or PCSK5 cleave and inactivate both endothelial and lipoprotein lipases, which play a critical role in HDL, VLDL and chylomicron metabolism [6]. In addition, it has been suggested that FURIN and PCSK5 modulate the inflammatory response in atherosclerosis. Specifically, PCSK5 and FURIN have been shown to cleave matrix metalloproteinases and integrins, and PCSK expression levels have been shown to increase during macrophage differentiation [3]. It is noteworthy that because of the increased embryonic mortality of PCSK-deficient animals, most of the evidence regarding the functions of PCSK5 and FURIN relies on *in vitro* experimental models.

Recently, much attention has also been focused on the direct role of PCSK9 in atherogenesis. PCSK9 regulates the serum cholesterol level by binding to the LDL receptor and targeting it for degradation. Therefore, gain-of-function mutations in the PCSK9 gene result in enhanced LDLR degradation in the liver and elevated serum cholesterol, whereas the downregulation of PCSK9 has the opposite effect [7].

To discover putative therapeutic targets and understand the development of human atherosclerosis, we have launched the Tampere Vascular Study (TVS), which is a collection of unique sample materials from advanced plaques of the major vascular territories and control vessels [8,9]. For the first time, we have systematically investigated the expression levels of the whole PCSK gene family and their reported targets in advanced human atherosclerotic plaques. Our clinical data unequivocally show that FURIN is the major plaque-expressed convertase. In contrast to previous reports [10], PCSK5 is statistically significantly downregulated in sclerotic arteries in our clinical samples. We also show that the FURIN-processed pro-inflammatory cytokines APRIL/TNFSF13 and BAFF/TNFSF13B are novel factors in atherosclerosis.

2. Materials and methods

2.1. Vascular samples

The vascular plaque samples from the femoral and carotid arteries and the abdominal aortas used in the study were obtained during open vascular procedures on the patients as a part of the ongoing Tampere Vascular Study [8]. The left internal thoracic artery (LITA) samples that were used as controls were obtained during coronary artery bypass grafting. All the samples were obtained from patients subjected to open vascular surgical procedures in the Division of Vascular Surgery and Heart Center, Tampere University Hospital, Finland. The samples were classified according to the recommendation by the American Heart Association (AHA). The study has been approved by the Ethics Committee of Tampere University Hospital, and the clinical investigation followed the principles of the Helsinki declaration. The demographic data of the samples used in the study are presented in Table 1.

Table 1
Demographics of the study samples.

	LDA samples	GWEA samples
n	36	26
Carotid plaque (*n*)	9	9
Aortic plaque (*n*)	9	7
Femoral plaque (*n*)	9	4
Control arteries (*n*)	9	6
Age, median, year (SD)	73.0 (10.2)	69.0 (11.1)
Males (%)	75.0	73.1
Body mass index, median kg/m^2 (SD)	25.3 (4.1)	25.2 (4.1)
Dyslipidemia (%)	30.6	38.5
Hypertension (%)	66.7	76.9
History of smoking (%)	69.5	80.8
Type 2 diabetes (%)	16.7	19.2
AHA Class V–VI (%), of the atherosclerotic arteries	77.8	90.0

2.2. RNA isolation and genome-wide expression analysis (GWEA)

Endarterectomy samples ($n = 26$) consisting of the intima and inner media from the carotid and femoral arteries and the aortic regions were obtained. The harvested samples (5–10 mm × 5–10 mm) were immediately immersed in RNALater (Ambion, Austin, TX). Total RNA was isolated with Trizol (Invitrogen, Carlsbad, CA) and the RNAEasy Kit with the RNase-Free DNase Set (Qiagen, Valencia, CA) according to the manufacturers' instructions. The expression levels of >23,000 genes were analyzed using Sentrix Human-8 Expression BeadChips (Illumina, San Diego, CA). The accuracy of the method used to quantify the gene expression was previously verified [11]. This procedure has been described in more detail in our previous work [8].

2.3. Quantitative real-time PCR

The expression results from the GWEA analyses were ascertained with TaqMan low-density arrays (LDAs; Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Nine samples from each artery (aorta, carotid artery, femoral artery and LITA, 36 total samples) were analyzed. The gene expression assays that were used were as follows: PCSK1 Hs01026107_m1, PCSK2 Hs01037347_m1, FURIN Hs00159829_m1, PCSK4 Hs00399493_m1, PCSK5 Hs00196400_m1, PCSK6 Hs00159844_m1, PCSK7 Hs00237114_m1, MBTPS1 Hs00186886_m1, PCSK9 Hs03037355_m1, TNFSF13 Hs00182565_m1 and TNFSF13B Hs00198106_m1. Five hundred nanograms of total RNA was transcribed to cDNA using the High Capacity cDNA Kit (Applied Biosystems). For the PCR, the LDAs were loaded with 7 μl of cDNA, 43 μl of H2O, and 50 μl of PCR Universal Master Mix (Applied Biosystems). Duplicate samples were used, and both the cDNA synthesis and PCR reactions were validated for inhibition. Ribosomal protein S9 (Hs02339426_g1) was used as a housekeeping gene, and the qPCR results were analyzed with SDS 2.2 software using the $2^{-\Delta\Delta C_T}$ method (Applied Biosystems).

2.4. Immunohistochemistry

Immunohistochemistry (IHC) was performed using the *N*-Histofine® Simple Stain MAX PO staining method (Nichirei Biosciences Inc., Tokyo, Japan) and paraffin-embedded vascular samples without any counterstaining; one exception was that TNFSF13B-immunoreactivity (IR) was detected with the ABC-method (Vestastain Elite kit, Vector Laboratories, Burlingame, CA). FURIN-IR was visualized with either a mouse monoclonal antibody (clone Mon-152, dil. 1:20, Axxora Europa, Lausen, Switzerland) or rabbit polyclonal antibody (dil. 1:100, a kind gift from Dr. Iris Lindberg, University of Maryland, Baltimore, MD), and the TNFSF13-IR cells were recognized with a rabbit polyclonal antibody (dil. 1:100, Abcam, Cambridge, UK). TNFSF13B-IR was visualized

Table 2
Genome-wide gene expression analyses of PCSK and target genes in arterial plaques. Values represent mean fold changes relative to the expression in non-atherosclerotic left internal thoracic artery control samples from the Tampere Vascular Study. Only statistically significant *p* values are presented.

Gene	Ensembl	Fold change			*p* value		
		Carotid artery	Aorta	Femoral artery	Carotid artery	Aorta	Femoral artery
PCSK1	ENSG00000175426	−1.01	−1.05	−1.08			
PCSK2	ENSG00000125851	−1.14	−1.07	−1.12	0.01		
FURIN	ENSG00000140564	1.46	1.71	1.45	0.00002	<10e−6	0.01
PCSK4	ENSG00000115257	1.09	1.01	1.07			
PCSK5	ENSG00000099139	−2.28	−1.55	−1.66	0.00008	0.01	
PCSK6	ENSG00000140479	1.35	1.18	1.16	<10e−6		0.05
PCSK7	ENSG00000160613	−1.03	1.08	−1.05			
MBTPS1	ENSG00000140943	−1.39	−1.31	−1.33	<10e−6	0.01	0.04
PCSK9	ENSG00000169174	1.02	1.01	1.04			
MMP1	ENSG00000196611	3.32	5.16	3.75	<10e−6	0.02	0.03
MMP11	ENSG00000099953	2.91	3.79	4.28	<10e−6	0.01	0.01
DTR	ENSG00000113070	1.07	4.78	3.54		0.00004	<10e−6
ADAMTS4	ENSG00000158859	1.05	5.35	4.64		<10e−6	0.01
ADAM8	ENSG00000151651	2.51	2.75	2.44	<10e−6	0.01	0.03
CXCL10	ENSG00000169245	2.46	2.74	1.65	0.00006	<10e−6	0.0001
TNFSF13B	ENSG00000102524	2	1.64	1.62	0.00009	0.04	0.04
PENK	ENSG00000181195	1.32	1.58	2.04			
MMP14	ENSG00000157227	1.99	2.39	2.41	<10e−6	0.01	0.03
CXCL9	ENSG00000138755	1.88	1.86	1.55	<10e−6	<10e−6	0.05
TNFSF13	ENSG00000161955	1.72	1.49	1.5	<10e−6	0.05	
ITGA5	ENSG00000161638	1.07	1.32	1.71			
MMP25. variable 1	ENSG00000008516	−1.64	1.79	−1.17	0.05		
VEGFC	ENSG00000150630	1.36	1.46	1.58	<10e−6	<10e−6	<10e−6
ADAM10	ENSG00000137845	1.56	1.43	1.44	0.000003	<10e−6	<10e−6
FBN1	ENSG00000166147	1.06	1.3	1.82			0.01
IGF1	ENSG00000017427	−1.19	1.8	1.49			
IGF2	ENSG00000167244	−1.51	1.84	1.31	0.00008	0.01	
ADAMTS2. variable 1	ENSG00000087116	1.1	1.1	1.43			0.03
BMP1. variable BMP1-3	ENSG00000168487	1.06	1.02	1.43			
BMP1. variable BMP1-6	ENSG00000168487	1.14	1.25	1.47		<10e−6	
PDGFB	ENSG00000100311	1.34	1.12	1.51	0.05		0.05
MMP25. variable 2	ENSG00000008516	−1.39	1.31	−1.21	0.03		
ADAM17	ENSG00000151694	1.22	1.22	1.33	0.02	0.01	0.02
ITGA4	ENSG00000115232	1.31	1.28	1.17	<10e−6		
MMP2	ENSG00000087245	−1.06	−1.07	1.39			
NOTCH1	ENSG00000148400	1.08	1.28	1.24			0.05
POMC	ENSG00000115138	1.14	1.24	1.12			
LPL	ENSG00000175445	2.19	2.01	1.64	<10e−6		
LIPG	ENSG00000101670	1.07	1.04	1.16			
SEMA3A	ENSG00000075213	1.05	1.21	1.24			0.05

with a rat monoclonal antibody (dil. 1:20, Abcam). The vascular cell types were identified with mouse anti-human muscle actin (dil. 1:30, clone HHF35; DakoCytomation, Glostrup, Denmark) and mouse anti-human endothelial cells (dil. 1:70, CD31, clone JC70A; DakoCytomation). Mouse anti-human CD68 (dil. 1:70, clone PG-M1, DakoCytomation) was used as marker of monocytes and macrophages. T-lymphocytes were recognized with the mouse anti-CD3 antibody (dil. 1:150, eBioscience Inc., San Diego, CA), and B-lymphocytes were labeled with mouse anti-CD20 (dil. 1:1000, DakoCytomation). The samples were subjected to the microwave antigen retrieval treatment. Endogenous peroxidase activity was extinguished by treating the samples with 0.3% H_2O_2 for 30 min. Subsequently, the samples were incubated overnight with the primary antibodies and then treated with the appropriate *N*-Histofine staining reagent for 30 min. ImmPACT™ (Vector Laboratories, Burlingame, CA) diaminobenzidine solution with nickel-intensification was used as the chromogen. All of the antibodies were diluted in PBS containing 1% BSA and 0.3% Triton X-100. The controls included the omission of the primary antibody or its replacement with non-immune sera. No staining was observed in the controls. The co-localization of FURIN, TNFSF13B and TNFSF13-IRs with different markers was studied in adjacent 5 μm sections (mirror image sections). The samples were stained as described above. Alternatively, double-color IHC stainings were performed with the MultiVision Polymer Detection System from Thermo Fisher according to the manufacturer's instructions. For TNFSF13B-IR, the ABC method was used for HRP staining. In some experiments, the sections were photographed after the first staining reaction (blue) was performed. The second staining reaction (brown) was induced afterwards to verify the specificity of the double staining method. The double-labeled cells appear dark blue/black. The photographs were obtained using a Nikon FXA-100 microscope and a PCO Sensicam digital camera (Kelheim, Germany).

2.5. The FURIN gene SNP effect on FURIN expression in artery wall tissues

The genotyping of FURIN haplotype-tagging single-nucleotide polymorphisms (htSNPs, FURIN gene region ±10 kb, $r^2 \geq 0.8$, rs4702, rs3759929, rs4932370, rs12903530, rs4526996, rs4932374 and rs1894401) was performed using commercial TaqMan assays and equipment. The genomic DNA used was extracted from peripheral blood leukocytes using the QIAamp® DNA Blood Minikit and Automated Biorobot M48 Extraction (Qiagen, Hilden, Germany). The effect of these htSNPs on FURIN expression was analyzed in

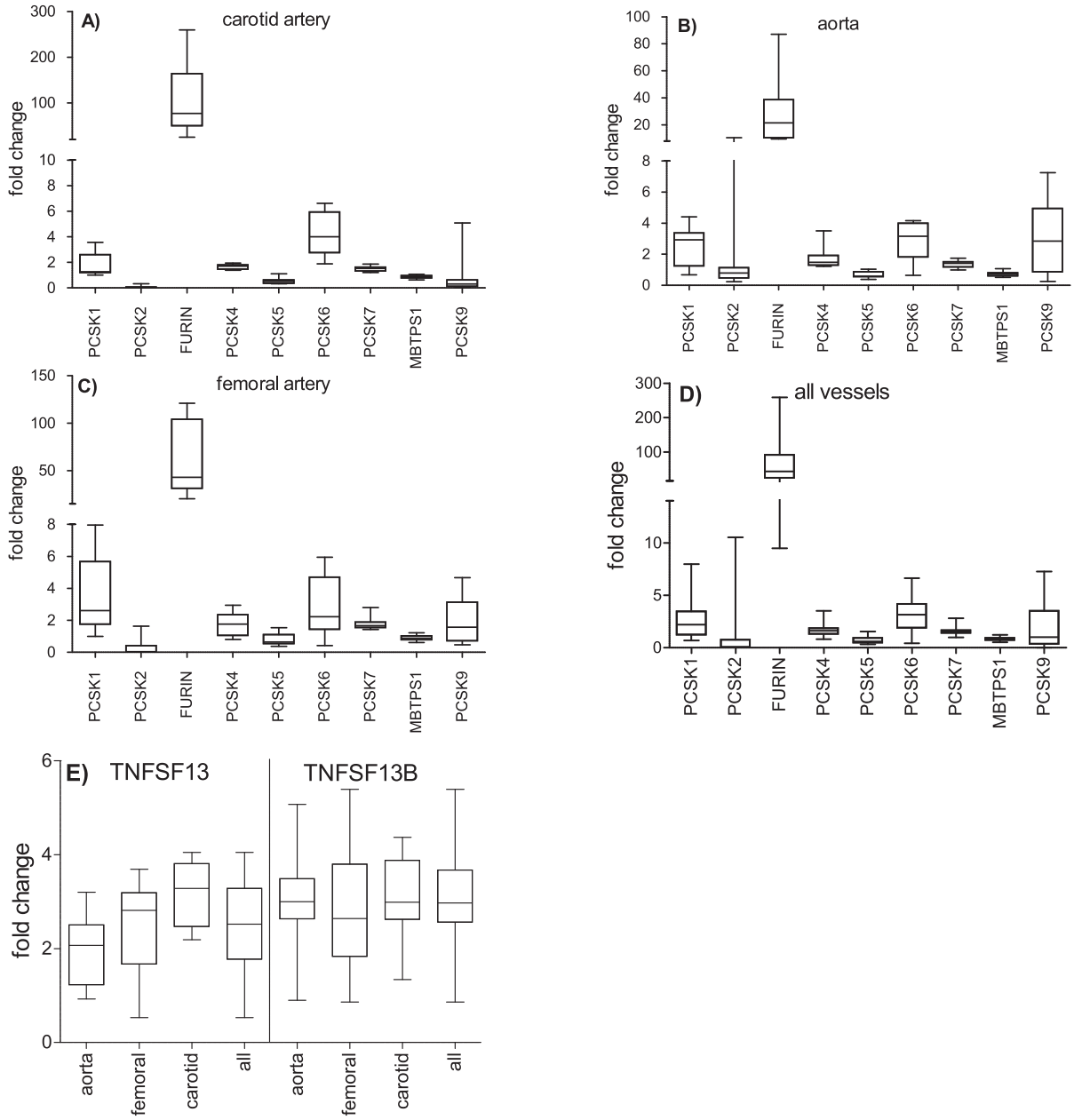

Fig. 1. (A–E) Relative expression levels of PCSKs and TNSF cytokines in atherosclerotic arteries. The median gene expression values of diseased arteries were compared to the expression of non-atherosclerotic control samples by using QRT-PCR. (A–D) PCSK expression levels in the carotid artery, aorta, femoral artery and all arteries combined, respectively. (E) The TNSF13 and TNSF13B expression levels in different arterial beds. The median, 1st and 3rd quartiles and minimum and maximum values are shown.

21 atherosclerotic artery samples containing histologically verified atherosclerosis. Kruskall–Wallis analyses were performed to compare the expression levels between genotypes.

2.6. Quantitative analysis of FURIN expression in leukocytes

Human CD14+ monocytes and CD4+ T helper cells were isolated from the peripheral blood with magnetic beads according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes and T cells were cultured in complete RPMI containing 10% FBS and were activated *in vitro* with LPS (1 μg/ml, Sigma–Aldrich) or plate-bound anti-CD3 and anti-CD28 (5 μg/ml each, eBiocience, San Diego, CA), respectively, for 24 h. Total RNA was isolated with RNAeasy, and FURIN expression was quantified with QRT-PCR using primers and probes from Applied Biosystems (Hs00159829_ml) and a BioRad CFX96 apparatus.

2.7. Statistical analyses

In the LDA analyses, the mean expression values of genes in duplicate samples from different arteries were compared to the expression of the same gene in a LITA control sample. The best representation of the LITA sample medians was used. The expression values were further divided by the median of the LITA samples. A non-parametric Mann–Whitney *U* test was used to detect the expression differences between the atherosclerotic and control tissues. The analyses were performed using SPSS v17.0 software (SPSS, Chicago, IL).

3. Results

3.1. The expression of PCSK and target genes in advanced atherosclerotic plaques

The mRNA expression levels of all nine PCSK genes as well as those of 31 previously identified target genes [12] were analyzed in a GWEA as previously described [8,9,13]. The expression in all (n = 20) atherosclerotic samples and in different arterial beds (aorta n = 7, carotid artery n = 9 and femoral artery n = 4) was compared with the non-atherosclerotic LITA samples (n = 6). FURIN was the only PCSK gene that was constantly and statistically highly significantly overexpressed in all of the sclerotic arterial beds (>1.4-fold change compared to the control, $p < 0.01$) (Table 2). Most of the other PCSK genes were found to be either sporadically or insignificantly enhanced. Interestingly, the PCSK5 and MBTPS1 genes were markedly downregulated in the arteries (PCSK5: carotid artery – 2.28-fold change, p = 8.0e−5; aorta – 1.55-fold change, p = 0.01 and MBTPS1: carotid artery – 1.39-fold change, p < 10e−6; aorta – 1.31-fold change, p = 0.01; femoral artery – 1.33-fold change, p = 0.04).

Of the 31 PCSK target genes studied, 24 were overexpressed and three downregulated in one or more of the atherosclerotic arterial beds (Table 2). Consistent with previous findings where PCSKs were implicated in the plaque inflammatory processes, the most highly overexpressed genes included several types of metalloproteinases (MMP1, MMP11, MMP14, ADAM8, ADAM10 and ADAMTS4) and chemokines (CXCL9 and CXCL10) [8,10,14]. PCSKs also regulate the activity of several TNF family cytokines that control lymphocyte-mediated immunity. Recent findings revealed that TNF family cytokines, such as TWEAK (TNFSF12) and LIGHT (TNFS14), can modulate atherogenesis [15,16]. Intriguingly, our screen identified the highly elevated expression of two structurally and functionally related B lymphocyte stimulators BAFF/TNFSF13B (carotid artery (p < 10e−6), femoral artery (p = ns) and aorta (p = 0.05)) and APRIL/TNFSF13 (carotid artery (p = 0.00009), femoral artery (p = 0.04) and aorta (p = 0.04)) in the atherosclerotic plaques.

To confirm the GWEA results, quantitative RT-PCR analyses with TaqMan LDA were performed (Fig. 1). These included all nine PCSK genes and TNFSF13 and TNFSF13B, which were the only targets that were highly (>1.4 fold change) and significantly overexpressed in arteries and not previously connected to inflammatory processes in atherosclerosis. Similarly to the GWEA results, the expression of FURIN was strikingly enhanced (femoral artery median 42.86-fold, p = 4.1e−5; carotid artery median 77.12-fold, p = 4.1e−5; aorta median 21.39, p = 4.1e−5) and that of PCSK5 and MBTPS1 significantly repressed in atherosclerotic arteries (PCSK5: carotid artery 0.65-fold, p = 7.8e−4 and aorta 0.58-fold, p = 4.0e−3; MBTPS1: carotid artery 0.88-fold, p = 0.040 and aorta 0.71-fold, p = 1.2e−3, Fig. 1A–D). The PCSK target genes TNFSF13 and TNFSF13B both showed statistically significant overexpression in all atherosclerotic beds when compared to the LITA sample (TNFSF13: carotid artery 3.28-fold, p = 4.1e−5; femoral artery 2.81-fold, p = 4.0e−3 and aorta 2.07-fold, p = 4.0e−3; TNFSF13B: carotid artery 2.99-fold, p = 8.2e−5; femoral artery 2.64-fold, p = 1.9e−3 and aorta 3.0-fold, p = 7.8e−4, Fig. 1E). In general, the FURIN, TNFSF13 and TNFSF13B genes thus showed the most prominent and significant overexpression in carotid arteries, where atherosclerotic plaques are often more fibrotic, ulcerated and inflamed in nature [17].

To analyze whether the gene expression levels were also altered during the progression of atherosclerosis, we analyzed the levels of FURIN, TNFSF13 and TNFSF13B using a microarray analysis of 57 samples of variably AHA-classified atherosclerotic lesions (3 samples of the AHA III lesion, 4 samples of the AHA type IV lesion, 20 samples of the AHA type V lesion and 30 samples of the AHA type VI lesion [18]). This analysis showed no statistically significant association between disease progression and changes in the gene expression levels (Kruskall–Wallis and Mann–Whitney U tests, data not shown).

Previously, changes in BAFF/TNFSF13B and APRIL/TNFSF13 levels in the serum have been observed in inflammation-related diseases, such as systemic lupus erythematosus [19]. To assess whether the elevated expression levels of TNFSF13 and TNFSF13B in the plaques were also reflected in the patients' sera, an ELISA analysis was performed in patients with severe atherosclerosis and the controls. We did not observe any statistically significant differences between the patients and the controls; these data likely suggest a local function for these cytokines in the plaque pathology (Supplementary Fig. S1).

3.2. Immunohistochemistry of FURIN, APRIL/TNFSF13 and BAFF/TNFSF13B

We next wanted to identify the plaque cell types that express the FURIN, APRIL/TNFSF13 and BAFF/TNFSF13B proteins. To this end, we performed an immunohistochemical (IHC) analysis of the dysregulated genes together with marker genes for several different cell types. The macrophages (CD68+) in the intima of atherosclerotic arteries showed the co-expression of FURIN, BAFF/TNFSF13B and APRIL/TNFSF13 in both mirror image and double-color IHC staining experiments (Fig. 2A), which is in accordance with the previously published findings [20,21]. Moreover, the double-color IHC stainings of FURIN together with BAFF/TNFSF13B and APRIL/TNFSF13 directly identified the plaque cells where FURIN was co-expressed with its target proteins (Fig. 2B). We have previously shown that FURIN is expressed in peripheral blood CD3+ T lymphocytes; there, it regulates cell-mediated immunity and peripheral immune tolerance [22,23]. Consistent with these results, obvious FURIN expression was found in the CD3+ cells in the plaque (Fig. 2C), which may represent the highly expressing FURIN T helper type 1 cells that were previously shown to regulate plaque inflammation [24]. Interestingly, most CD20+ B cells that infiltrated into the adventitia of advanced plaques were also strongly positively stained with the FURIN antibody (Fig. 2C). We did not identify any co-localization between FURIN and the markers of endothelial or smooth muscle cells (data not shown).

3.3. Immunoactivation induces FURIN expression

To compare the FURIN expression levels between resting and activated human lymphocytes and myeloid cells, we performed a QRT-PCR analysis on purified peripheral blood leukocytes (Fig. 2D). As we have previously shown, the T cell activation via a T cell receptor induced FURIN in CD4+ T cells [22,23]. Interestingly, FURIN expression in myeloid cells was also found to be upregulated through TLR4 mediated activation (LPS) of human CD14+ myeloid cells, which suggests that a role for this proprotein convertase also exists in the regulation of innate immune responses. In contrast, an ssRNA virus mimicking the TLR7/8 agonist (R848) stimulation of peripheral blood B cells did not result in similar FURIN induction (data not shown).

3.4. The SNP cis effect on FURIN expression

A SNP (rs4932178, −229 C/T) on the FURIN gene promoter regulates the expression of FURIN and consequently contributes to the outcome of the hepatitis B virus infection in the Han Chinese population [25]. To test whether FURIN expression was genetically regulated by SNPs in FURIN or the nearby gene region in our clinical cohort, we selected seven htSNPs covering the whole FURIN locus. The SNP *cis* effect on FURIN expression was analyzed in all artery samples (data normalized by Robust Multiarray

Average/Robust Spline Normalization). None of the htSNPs, including the SNP rs4932370 that tags the *Fur* regulating rs4932178 (D' = 0.989, r^2 = 0.862 in the HapMap CEU + TSI samples), were found to be significantly associated with the elevated FURIN expression levels in the artery wall (carotid artery, femoral artery, abdominal arteries and all arteries combined) (Supplementary Table SI).

4. Discussion

Proprotein convertase enzymes play a multifaceted role in the pathogenesis of atherosclerosis and consequent CHD. Earlier studies have suggested that FURIN and PCSK5 regulate inflammation and are overexpressed in plaques, whereas MBTPS1 and PCSK9 are essential for liver lipid metabolism [3,5,7]. In the present study, we show that FURIN is the most dysregulated PCSK in human atherosclerotic arteries. The QRT-PCR analysis showed that the expression of FURIN in a diseased vessel was up to 260 times higher than FURIN expression in a healthy one, whereas no other PCSK was consistently induced in our clinical samples. The majority of FURIN protein expression is localized in the plaque lymphocytes and macrophages where FURIN is coordinately expressed with its target proteins, the pro-inflammatory cytokines BAFF/TNFSF13B and APRIL/TNFSF13.

In light of the results from this and previous studies, PCSKs are one of the most exciting candidate molecules for future drug development [26]. Therefore, understanding their biology in human atherogenesis is critical. Notably, our clinical data do not suggest a role for PCSK5 in human atherosclerotic plaques. PCSK5 has been previously shown to be dominantly expressed in several cell types of rodent sclerotic arteries and sporadic human samples [27], where it is proposed to regulate the cell–matrix interactions through the activation of tissue-remodeling MMPs [3,10]. In contrast, our GWEA and QRT-PCR analyses using clinical samples showed a statistically significant downregulation of PCSK5 expression in the carotid and aorta plaques. The reasons for this discrepancy between the studies are not fully clear. One possible

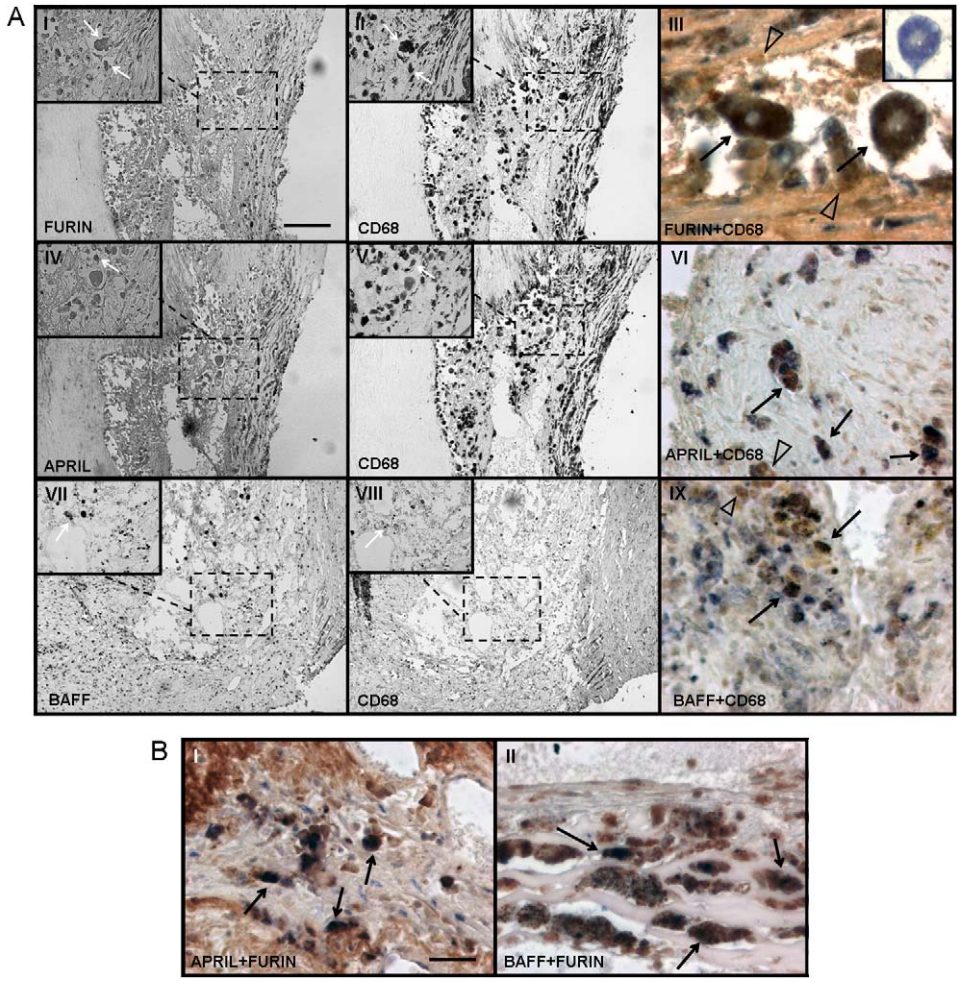

Fig. 2. (A–D) FURIN, APRIL/TNFSF13 and BAFF/TNFSF13B expression in plaque inflammatory cells. (A) FURIN, APRIL/TNFSF13 and BAFF/TNFSF13B expression levels in CD68+ macrophages. The IHC of the mirror image sections shows the co-localization of FURIN (anti-mouse monoclonal ab I), APRIL/TNFSF13 (IV) and BAFF/TNFSF13B (VII) with the CD68 macrophage marker (II, V and VIII, respectively). The double-color IHC shows the co-expression of the aforementioned molecules in the plaque intima-infiltrating macrophages (CD68 in blue; FURIN, rabbit polyclonal ab (III), APRIL/TNFSF13 (VI) and BAFF/TNFSF13B (IX) in brown, AHA type V–VI). The inserts in the mirror images show higher magnifications (panels I, II, IV, V, VII and VIII) and a double-stained cell after CD68 staining alone (panel III). The arrows indicate CD68+ cells with FURIN, APRIL/TNFSF13 or BAFF/TNFSF13B expression; the arrowheads identify single labeled cells (FURIN (III), APRIL/TNFSF13 (VI), BAFF/TNFSF13B (IX)). (B) FURIN (blue, anti-mouse monoclonal ab) is co-expressed in the same cells with APRIL/TNFSF13 (I, brown) and BAFF/TNFSF13B (II, brown). (C) FURIN is expressed in the plaque T and B cells. The mirror image sections show the co-localization of FURIN (anti-mouse monoclonal ab I and IV) with the T cell marker CD3 (II) and B cell marker CD20 (V). The double-color IHC-stainings show the co-expression of FURIN (III, VI brown, rabbit polyclonal ab) together with the CD3 (III) and CD20 (VI) markers in blue in the plaque infiltrating cells (intima, AHA type V–VI). The inserts in the mirror images show higher magnifications (panels I, II, IV and V) and double-stained cells after CD20 staining alone (panel VI). The arrows indicate the double-stained cells; the arrow-heads indicate the single labeled cells (FURIN (III and VI)). (D) CD4+ T cells and CD14+ myeloid cells were purified from human peripheral blood. The cells were activated *in vitro* as described in Section 2. FURIN expression was quantified by QRT-PCR and normalized to the housekeeping gene 18S. The experiment was performed twice with similar results.

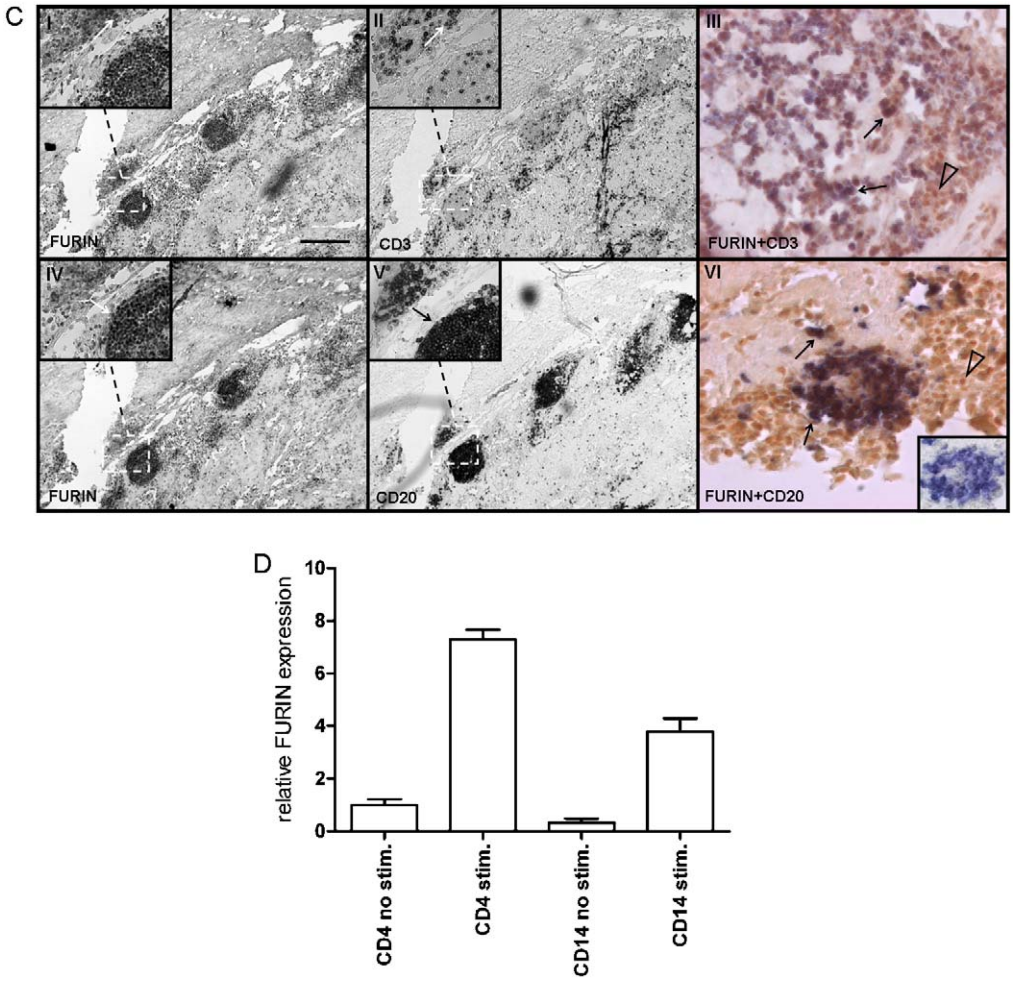

Fig. 2. (A–D) (*Continued*).

explanation is that there may be methodological or species-related differences in PCSK expression patterns. The previous findings that showed that PCSK5 was expressed in human atherosclerosis relied solely on immunohistochemistry and *in vitro* cell cultures and did not include the quantification of the gene expression in the patient plaques [10,27]. When interpreting the results of this study, it must be considered that due to obvious ethical reasons, we were not able to obtain the corresponding healthy control arteries from the carotid, aortic and femoral regions. Therefore, the histologically non-atherosclerotic internal thoracic artery samples are generally used as a control in the literature. However, we performed a comparative western blot analysis that compared FURIN expression in the atherosclerotic plaque and the macroscopically healthy adjacent part of the same femoral artery (Supplementary Fig. S2). These data are in accordance with our comparative mRNA results and further indicate that the overexpression of FURIN is concentrated in the plaque region, whereas even in atherosclerotic arteries, the healthy wall region expresses little FURIN.

Both MBTPS1 and PCSK9 play a well-characterized role in lipid metabolism. While MBTPS1 activates cholesterol synthesis and LDLR transcription by cleaving its substrates [26], PCSK9 directly regulates serum LDL levels through the degradation of the liver LDLR [28]. We show here that PCSK9 expression in the plaques was mostly neutral, while MBTPS1 was slightly downregulated in diseased samples. To our knowledge, this is the first published report on these PCSKs in atherosclerotic samples, and our findings further strengthen the current view that these enzymes are important for central liver lipid metabolism rather than peripheral plaque formation.

Many CHD-related molecules are synthesized as proproteins that need to be activated by PCSKs before they can perform their biological functions. We were able to confirm the upregulation of many previously published genes and identify the novel overexpression levels of BAFF/TNFSF13B and APRIL/TNFSF13, which are pro-inflammatory TNF superfamily cytokines that activate B lymphocytes. Although all arterial beds primarily showed similar expression levels of these genes, their overexpression was generally the highest in the carotid plaques, where inflammatory and fibrotic modifications are often prominent [17]. The accumulating evidence suggests that TNF family cytokines are important for CHD pathogenesis in experimental settings and that patients whose autoimmune disease is treated with a TNFα blocker display a reduced number of cardiovascular disease events [29]. However, the inhibition of TNFα function has associated risks; patients on TNFα blockers can develop an anaphylactic allergy, and they are more prone to severe infections, such as *M. tuberculosis*. BAFF/TNFSF13B and APRIL/TNFSF13 target B cells, which play an ambiguous role in atherogenesis. In different experimental settings, B cells have been shown to both promote and inhibit atherogenesis; recently, anti-BAFF antibodies and B cell-depleting anti-CD20 have been suggested to act as potential treatments for atherosclerosis [24,30]. It is noteworthy, however, that we did not identify any differences in the patient serum levels of BAFF/TNFSF13B in our ELISA analysis (Supplementary Fig. S1).

Further, it must also be considered that the expression analyses from clinical human samples cannot prove a causal relationship between increased gene expression and the progression of any disease. It is obviously possible that the overexpression of FURIN, BAFF/TNFSF13B or APRIL/TNFSF13 reflects arterial wall adaptation or even healing reactions rather than plaque evolution. Therefore, further functional studies are required before interfering with these proteins can truly be considered for future treatment.

We also found that FURIN expression was significantly increased by the activation of macrophages and T lymphocytes, whereas the standard stimulus of B cells failed to upregulate FURIN. The role of FURIN in immune cells has been incompletely studied; its role in B cell function has not been addressed at all. Further studies regarding the role of FURIN in immune cell function may help us to understand the biology of immune-mediated diseases, such as atherosclerosis. In our study, FURIN was not found to be genetically regulated. However, it is also noteworthy that the cytokines IL-12 and TGFβ-1 that regulate FURIN expression have both been implicated in arterial wall pathology, which may imply a *trans* rather than *cis* manner of FURIN regulation [24].

In conclusion, the observed overexpression of FURIN and its target molecules indicates a role for proprotein–PCSK pairs in the formation of peripheral atherosclerotic plaques. Studying these factors further will provide insights into the pathogenesis of this complex ailment and help us to understand how current and future treatments could be more specifically and efficiently used to treat this detrimental disease.

Disclosure of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2011.08.011.

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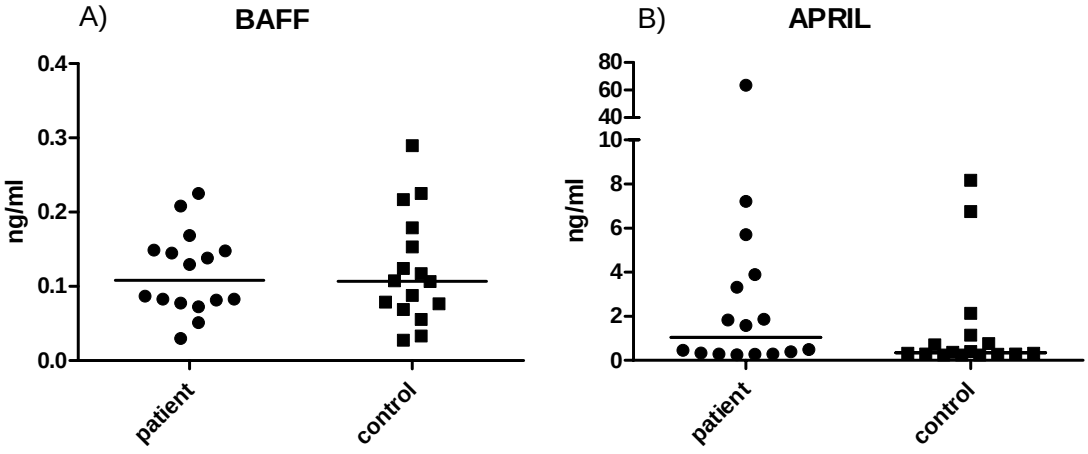
Supplementary Table I FURIN htSNP effect on gene expression. P-values for Kruskal-Wallis association analyses between seven htSNPs and FURIN expression in three different atherosclerotic arteries and their combined (all) group are presented. Statistically significant association between the SNPs studied and FURIN expression was not observed.

	artery			
	carotid	**femoral**	**abdominal**	**all**
rs4702	0.50	0.12	0.08	0.83
rs3759929	0.34	0.30	0.90	0.17
rs4932370	0.63	0.30	0.90	0.33
rs12903530	0.35	1.00	0.25	0.79
rs4526996	0.85	1.00	0.91	0.90
rs4932374	0.39	0.88	0.91	0.83
rs1894401	0.28	0.13	0.69	0.25

Supplementary Figure S1. BAFF (A) and APRIL (B) levels in sera of atherosclerotic patients and controls. Cytokine expression analysis in serum was performed with ELISA (R&D Systems, Quatikine Human BAFF/BLys (DBLYS0) and human APRIL/TNFSF13 (DY884)) on 16 patients with >50% stenosis in ≥2 arteries (of left main coronary artery, left anterior descending artery, left circumflex artery and right coronary artery) and 16 sex-, BMI- and age-matched healthy controls without stenosis in any of the coronary arteries. Statistically significant differences between patient and control groups were not observed.



Supplementary Figure S2. Western blot analysis of FURIN expression in an atherosclerotic femoral artery. Tissue samples from an atherosclerotic plaque and an adjacent macroscopically healthy part of the same artery were lysed as described earlier by Cromheeke *et al* (*Cardiovasc Res (1999) 43 (3): 744-754)*. Equal amounts of protein were run on SDS-PAGE, and western blot to detect FURIN (anti-furin, MON-152, Axxora) and actin (anti-actin, MAB150IR, Millipore) was done as described in earlier by Pesu et al *(Blood. 2000 Jan 15;95(2):494-502)*.