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THE ROLE OF PROPROTEIN CONVERTASE FURIN IN THE ACTIVATION OF N-GLYCOSYLTRANSFERASE GNT-V

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TIIVISTELMÄ

Tutkimuksen tausta ja tavoitteet: PCSK:t ovat proteiinien maturaatiolle tärkeä entsyymiperhe joita tarvitaan useissa biologisissa prosesseissa. Myös toinen yhtäläisen tärkeä entsyymiperhe, N-asetyyliglykosyylitransferaasiperhe Gnt, vaikuttaa moniin biologisiin toimintoihin. Terveyden ja normaalien biologisten toimintojen ylläpitämisen lisäksi molemmat entsyymiperheet vaikuttavat myös sairauksien kehittymisessä. Immuunijärjestelmän toiminnalle tärkeät PCSK-entsyymi FURIINI ja N-asetyyliglykosyylitransferaasi Gnt-V ovat välttämättömiä normaalille T-solujen toiminnalle. Niiden ekspressiotaso kasvaa T-soluaktivaation myötä, ja niiden puutteella on yhteys lisääntyneeseen T-solujen reaktiivisuuteen ja autoimmuniteettiin. Lisäksi molempien aktiivisuus ja sijainti paikantuu pääasiassa Golgin laitteeseen. Tästä yhteydestä huolimatta niiden keskinäistä vaikutusta ei ole tutkittu. Tässä tutkimuksessa selvitettiin FURIINIn vaikutusta Gnt-V:n prosessointiin ja aktiivisuuteen.
Tutkimusmenetelmät: FURIINIn vaikutusta Gnt-V:n aktiivisuuteen hiiren villityyppisissä ja ehdollisesti FURIINI-puutteisissa T-soluissa tutkittiin Gnt-V-modifioituihin glykaaneihin sitoutuvalla L-PHA-leimalla ja virtaussytometrialla. Sitoutumisen erot ja erojen merkittävyys laskettiin R-ohjelmalla. Gnt-V:n pilkkoutumista *Mgat5*-transfektoiduissa sekä *Mgat5*- ja *FURIINI*-transfektoiduissa hamsterin CHO-K1-soluissa sekä FURIINIa tuottamattomissa RPE.40-soluissa, sekä lisäksi FURIINIlla stabiilitransfektoiduissa Jurkat-soluissa, tutkittiin western blotilla. Lisäksi selvitettiin FURIINIn vaikutus *Mgat*-geeniperheen mRNAn tuottoon hiiren naiiveissa CD8+ T-soluissa RNA-sekvensointianalyysillä.
Tutkimustulokset: FURIINI vaikuttaa merkittävästi Gnt-V:n aktiivisuuteen hiiren naiiveissa ja muisti-T-soluissa. Gnt-V-modifioitujen glykaanien määrä oli suurempi naiiveissa kuin muistisoluissa, ja suurempi villityypin kuin ehdollisen *FURIINI*-deleetion soluissa. FURIINI ei prosessoi Gnt-V:tä sen potentiaalisesta pilkkomiskohdasta, mutta lisää proteiinin fraktioitumista transfektoiduissa hamsterin RPE.40- ja CHO-K1-soluissa. Jurkat-T-soluissa FURIINIlla ei havaittu selvää määrällistä entsymaattista tai ei-entsymaattista vaikutusta Gnt-V:n fraktioitumiseen. FURIINI ei myöskään vaikuta *Mgat*-geeniperheen mRNA:n ekspressiotasoihin hiiren CD8-positiivisissa naiiveissa T-soluissa.
Johtopäätökset: FURIINI ei ole välttämätön Gnt-V:n prosessoinnille, mutta lisää sen hajotusta ja vaikuttaa epäsuorasti sen aktiivisuuteen. Gnt-V:n aktiivisuuden muuttuminen T-soluaktivaation myötä on mahdollinen mekanismi T-muistisolujen antigeeni-indusoidun aktivoitumisen herkistymisen taustalla.

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ABSTRACT

Background and aims: PCSKs are an important enzyme family in protein maturation and needed for several biological processes. Another, equally important enzyme family, N-glycosyltransferase Gnt, also affects many aspects of biological functions. In addition to being involved in maintenance of health and normal functions, they also affect development of diseases. PCSK enzyme FURIN and N-glycosyltransferase Gnt-V are involved in T cell biology and are vital for normal T cell functions and immune system in general. Their expression is increased upon T cell activation, and their deficiency is associated with increased T cell reactivity and autoimmunity. In addition, both are active and localized mainly in the Golgi apparatus. Despite their shared functionality and intracellular localization, their interrelatedness has not been studied. In this study we investigated the role of FURIN in Gnt-V proteolytic processing and activity.
Methods: The effect of FURIN on Gnt-V activity was studied by comparing L-PHA staining of mouse wild type T cells and T cells with a conditional deletion of FURIN. The amount of Gnt-V product-bound stain on the surface of T cells was detected using FACS and differences and significance was analysed with R software. FURIN´s cleavage activity on Gnt-V was studied with western blot using hamster CHO-K1 cells and *FURIN*-deficient RPE.40 cells transfected with human *Mgat5* vector with and without human *FURIN* vector. Also *FURIN* stable transfected Jurkat cell lines were used. In addition, FURIN´s effect on the mRNA levels of the *Mgat* family genes was studied using RNA sequencing.
Results: FURIN significantly affects Gnt-V activity in naive and memory T cells of mice. The amount of Gnt-V-modified glycans was greater in naïve than memory cells, and higher in wild type cells than in T cells with conditional *FURIN* knockout. FURIN does not directly process Gnt-V at its predicted cleavage site, but increases unspecific fractioning of the protein in transfected hamster CHO-K1 and RPE.40 cells. In Jurkat T cells, no clear quantitative enzymatic or non-enzymatic effect of FURIN on Gnt-V fractioning was detected. FURIN also does not affect *Mgat* family mRNA expression levels in CD8 positive naïve mouse T cells.
Conclusions: FURIN is not required for Gnt-V processing, but increases its degradation, and indirectly affects its activity. Change in the Gnt-V activity is a possible mechanism behind the sensitization of memory T cells to antigen-induced activation.

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Johanna Laakkonen

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ABBREVIATIONS

ACK	ammonium-chloride-potassium
AP-1	activator protein 1
APC	antigen-presenting cell
BCR	B cell receptor
BSA	bovine serum albumin
CD	cluster of differentiation
CDG	congenital disorder of glycosylation
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
Dol-PP	dolichol diphosphate
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EMT	epithelial-mesenchymal transition
ER	endoplasmic reticulum
Ets	E26 transformation-specific
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FGF-2	fibroblast growth factor 2
Fur KO	FURIN knockout
GEM	glycosphingolipid-enriched microdomain
GlcNAc	N-acetylglucosamine
Gnt-V	N-acetylglucosaminyltransferase-V
Gnt-1IP	Gnt-I inhibitory protein
IFNγ	interferon-gamma
Ig	immunoglobulin
IL	interleukin
iTreg cell	induced regulatory T cell
Lck	lymphocyte-specific protein tyrosine kinase
LDL	low-density lipoprotein
MACS	magnetic-activated cell sorting
L-PHA	phytohemagglutinin-L
MHC	major histocompatibility complex
MS	multiple sclerosis
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NFAT	nuclear factor of activated T-cells
OST	oligosaccharyl transferase
PACE4	Paired basic amino acids cleaving enzyme
PCSK	Proprotein convertase subtilisin/kexin
PC	Proprotein convertase
pMHC	peptide-major histocompatibility complex
PBS	phosphate-buffered saline
RPKM	Reads Per Kilobase of transcript per Million mapped reads
RT	Room temperature
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SG	secretory granule
SKI-1/S1P	Subtilisin kexin isozyme 1/Site 1 binding protein
SPPL3	signal peptide peptidase-like 3
STAT4	signal transducer and activator of transcription 4
TACE	tumor necrosis factor-alpha converting enzyme

TBS	Tris-base saline
TCR	T cell receptor
Tc cell	cytotoxic T cell
TGFβ	Transforming growth factor beta
TGN	trans-Golgi network
Th cell	helper T cell
TNFα	tumor necrosis factor alpha
Treg cell	regulatory T cell
UDP-GlcNAc	uridine diphosphate N-acetylglucosamine
WT	wild type

1 INTRODUCTION

Post-translational protein modifications are vital for biological functions in protein, cellular, and systemic level. These modifications are vital to maintain health and normal functions, but sometimes they also have a role in disease. One of these modification processes is called glycosylation, where additions of carbohydrate units to proteins result in specific glycan structures. These structures can help to guide the correct folding of newly produced proteins and protect them from cleavage (Zhang et al. 2014; Wahrenbrock and Varki 2006), while some of them can also guide the misfolded proteins towards degradation in proteasome (Moremen et al. 2012). Glycosylations also have a role in cell-cell interactions such as cellular signaling and recognition, and regulate functions such as activation of immune cells (Demetriou et al. 2001). Imbalance between separate glycosylation enzymes in the pathway can lead to disturbed glycosylation patterning (Mkhikian et al. 2011), that also seems to have a role in pathological conditions like autoimmunity (Demetriou et al. 2001; Grigorian et al. 2007; Grigorian et al. 2012b) and cancer (Pan et al. 2014; Munkley et al. 2016). Environmental and genetic factors, and their interactions, can be behind this imbalance of expression (Mkhikian et al. 2011). Another factor might be the processing of immature glycosylation enzymes: as in case of many other enzymes, activation or inactivation might be dependent of enzymatic cleavage during the functional maturation of a protein.

Another well-studied enzyme family responsible for the maturation processes of variety of a proteins is the proprotein convertase (PCSK) family. PCSKs are vital for functions such as development (Bessonnard et al. 2015; Essalmani et al. 2008; Roebroek et al. 1998) and reproduction (Li et al. 2000; Tadros et al. 2001), but also have a role in the infectivity of some viruses (Coutard et al. 2020; Izaguirre 2019; Lenz et al. 2001; Vincent et al. 2003) and the development of diseases (Duhamel et al. 2015; Jaaks and Bernasconi 2017). Of the nine members of this family, FURIN is one of the most studied as it is expressed in most mammalian tissues. In immunological context, FURIN has been found to be especially interesting due to its role in T cell functions. In addition to T cell activation, FURIN is also important in T cell regulation (Pesu et al. 2008), as it is needed for the production of several bioactive cytokines vital for immune responses (Dubois et al. 1995; Endres et al. 2003; Ortutay et al. 2015). The lack of FURIN in T cells disturbs their normal responses to stimulus, making them

hyperreactive (Pesu et al. 2008), and this increases the susceptibility to develop systemic autoimmune diseases.

The expression and activity of FURIN and N-acetylglucosaminyltransferase-V (Gnt-V) are both associated with the regulation of T cell activation and functions. Their upregulation is induced upon T cell activation and attenuates T cell reactivity and inflammatory phenotype, and deficiency of either of those causes T cells to be hyperactive and is associated with autoimmunity (Demetriou et al. 2001; Pesu et al. 2008). Despite this shared function and subcellular localization, their association hasn´t been studied. In this master´s thesis, we investigate the effect of FURIN on Gnt-V activity and expression.

2 REVIEW OF THE LITERATURE

2.1 Adaptive immune system

Animals constantly encounter numerous pathogens, some potentially harmful and some even lethal to the host. To have protection from diseases caused by viruses, bacteria, eukaryotic parasites, or tumor cells, animals have developed a variety of mechanisms to be able to recognize, respond, and eliminate these harmful invaders. These protective mechanisms include physical and chemical barriers, protective molecules, and cells, together called immunity. Immune system has developed to discriminate self and non-self-structures, the latter also including altered-self structures such as those in tumor cells. Immune system can be divided into evolutionarily ancient innate and more recently developed adaptive immune system, the former existing in some form in all animals and plants, and the latter only in vertebrates (Luckheeram et al. 2012). Some of the characteristic differences are summarized in *table 1*. The innate immune system, including protective molecules such as those of complement system, and innate immune cells, can recognize general foreign structures of a pathogen with pattern recognition receptors, following the engulfing and degradation of the pathogen. Of the innate immune cells, phagocytic dendritic cells and, with lesser amount, macrophages, are a vital link between innate and adaptive immunity. They can process engulfed foreign structures and act as professional antigen presenting cells (APC) for adaptive immune cells, enabling the activation of fine-tuned adaptive immune response. Other, nucleic non-immune cells, also act as APCs, presenting structures produced inside the cells. Compared to the highly antigen-specific adaptive immune system, innate immune responses are straightforward and fast, but can only react to limited number of different antigenic structures. To provide optimal protection to the host, the innate and adaptive immune systems act together in strictly coordinated manner (Luckheeram et al. 2012; Warrington et al. 2011).

Table 1: Examples of characteristic differences between innate and adaptive immune system. *TCR = T cell receptor; BCR = B cell receptor.*

	INNATE	**ADAPTIVE**
Effector cells	granulocytes, macrophages, dendritic cells	T cells, B cells
Effector molecules	complement proteins	antibodies
Antigen recognizing receptors	pattern recognition receptors	TCR, BCR
Specificity of receptors	general	highly specific
Memory	no	yes
Response time (primary infection)	hours	days
Response time (secondary infection)	hours	hours

A few days after infection the second line of protection, the adaptive immune system, activates. Adaptive immune system has humoral and cellular components involved, including B lymphocytes and the antibodies they produce, and T lymphocytes that can be divided into CD4+ helper T cells, CD8+ cytotoxic T cells, and immunosuppressive T regulatory cells. Precursors of both cell types are developed in bone marrow, but while *T* cells are matured in *t*hymus, maturation of *B* cells continues in *b*one marrow. Adaptive immune system works in an extremely pathogen-specific manner: the first encounter with a specific pathogen causes primary response, where the activation of antigen-specific T and B cells lead to clonal expansion of these activated cells, secretion of cytokines, and production of high-affinity antibodies by B cells (plasma cells). As a result of successful primary response, some of the activated B and T cells become memory cells. These cells enable immunological memory, allowing adaptive immune system to react faster and more efficiently to recurring attacks by microorganisms (Warrington et al. 2011). Being able to adapt from primary to secondary responses is the key feature of adaptive immune system (Ademokun and Dunn-Walters 2010; Chaplin 2010).

The central molecules in adaptive immune reactions are T cell receptors (TCRs) that interact with major histocompatibility complexes (MHC) I and II on the surfaces of APCs, immunoglobulins (Ig) secreted by B cells, and B cell receptors (BCRs), also called surface Ig receptors. They are vital in the recognition of non-self antigens, and allow the immune system to recognize and destroy harmful invaders while leaving normal structures of the host organism intact and unharmed (Chaplin 2010; Warrington et al. 2011). All nucleic cells express MHC I and present processed self-structures as a peptide-MHC (pMHC) complex. In case of infected or tumor cells, structures recognized as potentially dangerous can activate an immune reaction, leading to targeted cell killing. MHC II, instead, is expressed only on the surface of professional

APCs, and present processed structures of engulfed bacteria and other extracellular material. Also presenting pMHC II complex can initiate immune reaction (Chaplin et al. 2010).

The B cells that bind their cognate antigen on BCRs internalize the antigen, and after that can act as APCs by processing the antigen and presenting that on the cell surface with their MHC II molecules. During that, these B cells also start to express CD80 and CD86 costimulatory molecules that activate, together with the antigen-MHC II complex, T helper (Th) cells with same antigen specificity. Th cells can then, in turn, signal via their CD40 ligand to CD40 on B cells, and induce the maturation of B cells via isotype switching of the Ig´s as well as activation of somatic mutations and affinity maturation. In addition to direct signaling, T cells also affect B cell functions by the cytokines they secrete during the crosstalk (Warrington et al. 2011). B cells can also function without the help of T cells, but the number of types of antigens they can recognize and the effectiveness of B cell responses are then more limited. The interaction between T and B cells during infection is crucial for the development of effective, regulated, and long-lived protection against pathogens (Chaplin 2010).

2.1.1 T cells in immune system

The most important feature of the adaptive immune system is its ability to increase the efficiency of its reactions against specific antigen after each encounter. This is enabled by the crosstalk between cells mediating cellular and humoral immunity that, after successful activation of adaptive immune cells, leads to the production of specific memory cells. *Table 2* shows the summary of differences between humoral and cellular immune system. Humoral adaptive immunity is mediated by widespread immunoglobulins, which can bind to foreign structures and inactivate them, or tag them for the recognition and responses of cellular immunity and innate immune cells. T-cell-mediated cellular immunity, instead, acts locally, and its function requires the direct contact of the immune cells to the antigen (Warrington et al. 2011). The contact site of effector T cell and APC is called immunological synapse, where TCR interacts with pMHC complex. Upon antigen binding, more TCRs, as well as several co-receptors, such as CD4, CD8, and CD28, are also recruited to this contact site to mediate the inhibitory and activating signals, which will define the nature of the T cell response. The availability and localization of these receptors, as well as several other cell surface glycoproteins, are, in part, regulated by the binding of galectins, and formation of galectin-glycoprotein lattice (Grigorian et al. 2009).

Table 2: Examples of characteristic differences between adaptive humoral and cellular immunity. *BCR = B cell receptor; TCR = T cell receptor; Th = helper T cell; Tc = cytotoxic T cells; Treg = regulatory T cells.*

	IMMUNE RESPONSE	
	HUMORAL	**CELLULAR**
Cells (place of development)	B (***b***one marrow)	T (***t***hymus)
Effector molecules	cytokines, antibodies	cytokines, degrative enzymes
Effector cells	plasma cells	Th, Tc, Treg
Antigen receptor	BCR	TCR
Antigen type	native	processed, linear
Co-receptors	CD40	CD4, CD8, CD28

T cell precursors, thymocytes, are produced in bone marrow from common lymphoid hematopoietic stem cells and maturated in thymus. For most of them, the maturation is followed by further antigen-mediated activation and differentiation in peripheral lymphoid organs, including spleen, lymph nodes, and the mucosa-associated lymphoid tissue (Luckheeram et al. 2012). Different T cell subtypes are summarized in *table 3*. Until encountering their cognate antigen, naïve T cells can be maintained in the immune system even for decades (Thome et al. 2016).

Table 3: T cell subtypes and functions.

T cell type	T cell subtypes	Immune response type	Biological function	Secreted cytokines
helper CD4+	Th1	inflammatory	activation of responses in intracellular infections, activation of macrophages	IFNγ
	Th2	anti-inflammatory	defence against helminths and other extracellular pathogens; allergic responses	IL-4, IL-5, IL-13
	Th17	inflammatory	defence in fungal and bacterial infections	IL-17, IL-21, IL- 22
	Treg	regulatory	attenuation of effector T cell functions	IL-10, TGFβ
cytotoxic CD8+	Tc1	inflammatory	cytolytic cell killing in intracellular infections	IFNγ
	Tc2	anti-inflammatory	allergic responses	IL-4, IL-5, IL-13
	Tc17	inflammatory	assisting Tc1 cells; recruiting neutrophils	IFNγ, IL-17, IL-21
	Treg	regulatory	attenuation of effector T cell functions	IL-10, TGFβ

During the maturation, thymocytes are in contact with thymic epithelial and dendritic cells that provide cues for the development. These cues include signals mediated by cytokines, and ligands for receptor-mediated signaling (Luckheeram et al. 2012). To produce an adequate adaptive immunity, T cells must be able to recognize pathogenic antigens and react accordingly and, on the other hand, they should be unreactive towards self-antigens to avoid autoimmunity. Major event during the T cell development is TCR gene segment rearrangement. A small portion of cells rearrange genes encoding γ and δ chains, giving rise to γδ T cells. A majority of cells, instead, rearrange α and β segments, and continue their development in thymus. During the first selective phase, positive selection, the TCRs are first tested for sufficient affinity for the MHC molecules expressed by thymic epithelial and dendritic cells. Cells with low-affinity TCRs don´t receive these signals for survival, and will be deleted. During the negative selection, instead, autoreactive cells that have too high affinity to self-peptide-MHC complexes are excluded. Only less than 5% of the developing thymocytes become mature T cells, while the rest die by apoptosis during the positive and negative selection process (Chaplin 2010). During the development, thymocytes also end up expressing either CD4 (T helper cells) or CD8 (cytotoxic T cells) co-receptors, depending on whether they interact with MHC II or MHC I (Luckheeram et al. 2012).

Of αβ T cells 60-70% are CD4+ Th cells that activate humoral, B cell mediated responses, and also cellular responses like delayed type hypersensitivity responses. For T cell-dependent B cell responses, the crosstalk between T cells and B cells with the same antigen specificity is needed to activate them and produce a full response to the encountered antigen. Crosstalk occurs with the direct contacts between cell types and cytokines secreted by Th cells, eventually leading to T and B cell activation and the formation of memory cells. Further differentiation into subtypes is determined by a combination of activating signals they receive during the first encounter with antigen (Chaplin 2010; Morel 2018), some of the signals being regulated by specific modifications of receptor glycoproteins (Grigorian et al. 2009). CD4+ T cells mainly differentiate into the Th type 1 (Th1), Th2, and Th17, and induced regulatory (iTreg) cells. In general, Th1 cells are responsible for the protection against intracellular infections, and they also help to activate the macrophages, increasing their ability to kill microbes. Th2 cells instead mediate the defense against helminths and other extracellular pathogens. The latter T cell population is also involved in allergies, and the former affects the development of some autoimmune diseases (Zhu and Paul 2008), together with the pro-inflammatory Th17 cells. Th17 cells promote the cell killing during fungal and bacterial infection by activating

neutrophils and macrophages (Waite and Skokos 2012). Tregs, instead, are anti-inflammatory subtype, as they respond weakly to received stimulus, and limit the effector T cell responses and proliferation by intercellular interaction (Grigorian et al. 2009).

30-40% of αβ T cells are CD8+ cytotoxic T (Tc) cells (Chaplin 2010). TCRs of CD8+ cytotoxic T cells recognize intracellularly produced and processed MHC type I-bound fragments of the antigens, that can be either normal cellular structures, or peptides produced during viral infections, and structures produced by tumor cells. In contrast to the MHC type II molecules that are expressed by the professional antigen presenting cells, MHC I molecules are expressed by all cells with nucleus. If the presented antigen is recognized as foreign or malignant, it can cause Tc cell activation and the killing of the cell presenting that antigen (Villani et al. 2018). Upon activation, Tc cells can differentiate into Tc1, Tc2, Tc17, and also regulatory subtypes. Tc1 effector cells are needed for the targeted cell killing in viral and other intracellular infections (Valentine and Hoyer 2019), and produce granzyme B and perforin for their cytolytic activity (Hamada et al. 2009). Tc2 instead react to specific allergens (Valentine and Hoyer 2019). Tc17 cells don´t produce granzyme B or express cytolytic activity, but instead assist Tc1 cells in viral infections by IFNγ and IL-17 signaling, also recruiting neutrophils to the site of infection (Hamada et al. 2009).

A small proportion of activated T cells become memory cells, which are able to respond more readily to recurring interaction with their cognate antigen. Memory cells can be divided into central and effector memory cells. The former expresses a set of receptors that allow homing to lymph nodes, while these homing receptors are lacking in the effector memory cells, which reside in the non-lymphatic tissues (Sallusto et al. 2004).

2.1.2 T cell activation

For the T cell activation and differentiation into effector cells, three types of signals need to be received. The first signal is mediated by interaction with TCR and antigenic pMHC. The second signal occurs via co-receptors in the immune synapse, and the third signal is mediated by secreted cytokines (Grigorian et al. 2009). For the first signal to occur, the antigen needs to be processed into peptides, which are then expressed on the cell surface together with MHC molecules. All cells present peptides produced inside the presenting cell, while extracellular antigens first need to be engulfed by professional APCs. Only the professional APCs present components of extracellular foreign pathogens on type II MHC and activate CD4+ helper T

cells. Instead, the intracellularly produced antigens, such as viral proteins, are presented with type I MHC, and activate the cytotoxic CD8+ T cells (Ademokun and Dunn-Walters 2010).

Before meeting their cognate antigen, the naïve peripheral T cells require basal signaling to survive. This occurs by the ligand-independent signaling via TCR and intracellular lymphocyte protein tyrosine kinase (Lck), the latter being associated with co-receptors CD4 and CD8 (Veillette et al. 1988). The recruitment of Lck-CD4 and other molecules, such as non-catalytic region of tyrosine kinase adaptor protein (Nck) and Wiskott–Aldrich Syndrome protein (WASp), to TCR leads to intracellular microfilament reorganization, causing the complex to be transferred to the glycosphingolipid-enriched microdomains (GEMs) and eventually leading to basal signaling (Demetriou et al. 2001; Grigorian et al. 2009).

A similar setup of components is involved also in the antigen-induced activation. For T cells being able to activate and proliferate, TCRs need to bind pMHC complex on APC with sufficient affinity and to be clustered at immunological synapse. The activation signaling requires high enough stability of the immunological synapse, enabled by threshold number of TCRs in the cluster (Demetriou et al. 2001). TCR clustering and antigen binding affinity, as well as lateral mobility and co-localization of co-stimulatory and inhibitory receptors is, in part, affected by their post-translational modification with carbohydrate chains during process called glycosylation (Grigorian et al. 2009).

The threshold number of TCRs that need to bind to pMHC for the T cells to activate can be reduced by T cell co-receptor signaling (Harding et al. 1992). Of co-stimulatory receptors, the main receptor is CD28, which is expressed in all naïve T cells (Howe et al. 2003). CD28 binds to CD80/CD86, which are upregulated in activated professional APCs and infected cells (Harding et al. 1992). Co-stimulation increases the effect of TCR activation, leading to the proliferation and differentiation into different T cell subpopulations (Howe et al. 2003). Without the co-stimulation, activating signals usually won´t be transduced, resulting in a prolonged unresponsive state of the T cell, called anergy (Chaplin 2010).

CD4+ T cell responses are regulated by interplay between inhibitory and stimulatory signals and mediate the balance between T cell tolerance and autoimmunity. For instance, CD28 and CTLA-4 both bind to CD80/CD86 co-stimulatory ligand, the former regulating T cell proliferation positively and the latter negatively. The positive regulation is constitutive, while rapid increase in surface expression of CTLA-4 is only induced a few days after T cell activation (Grigorian et al. 2009). Small amounts of CTLA-4 is expressed on the cell surface also in resting

state but is located mostly in endosomes and lysosomes due to constitutive endocytosis (Waterhouse et al. 1995). Even though its half-life on the cell surface is short, its affinity to CD80/CD86 is higher than that of CD28´s and is sufficient to induce growth arrest even with very small amounts (Waterhouse et al. 1995). In Tregs, CTLA-4 expression is constitutive and the overall membrane turnover is low, being one factor making them mostly unresponsive for external stimulus (Grigorian et al. 2009).

The receptor distribution and time spent on cell surface regulates T cell growth and differentiation upon activation. A few days after activation, CTLA-4 expression and signaling is increased, followed with growth arrest and differentiation into subtypes such as Th1, Th2, Th17, and induced Tregs (iTregs) (Grigorian et al. 2009). The inhibitory signals via CTLA-4, as well as other inhibitory receptors, promote growth arrest and differentiation, and inhibit autoimmune reactions (Gorelik and Flavell 2000; Waterhouse et al. 1995; Werner et al. 2019; Zhao et al. 2019).

In addition, the cytokine signals guide the direction of differentiation (Morel 2018). A similar setup of cytokines induce both Th and Tc cell subtype differentiation. Th1 and Tc1 is induced by IFNγ and IL-12 signaling, Th2 and Tc2 differentiation is induced by IL-4 signaling, TGFβ and IL-6 induce differentiation towards Th17 and Tc17, and CD4+ and CD8+ Tregs are induced by TGFβ (Valentine and Hoyer 2019). In the case of CD4+ T cells, TCR signal strength also affects the direction of differentiation. A stronger TCR signal tends to initiate intracellular signaling pathways leading to differentiation to Th1 and Th17, while the pathways induced by weaker signal are more likely to lead to Th2 differentiation (Morel 2018).

2.2 Proprotein convertases

The proprotein convertase subtilisin-kexin (PCSK) is an enzyme family consisting of nine members, PC1/3, PC2, FURIN, PC4, PC5/6, PC7, PACE4, SKI-1/S1P, and PCSK9, encoded by genes *PCSK1-9* (Seidah 2011a). The first seven PCSKs, or PCs, are structurally more closely related, and cleave after single or paired basic amino acid residues, while the last two, more distant members, don't have this requirement (Seidah et al. 2013). The domain structure characteristics are described in *figure 1a*. Also, the cleavage consensus sequence differs between PC1-7, SKI-1/S1P, and PCSK9, as seen in *figure 1a*. Some PCs are ubiquitously expressed, like FURIN, SKI-1/S1P, and PC7, while some are more tissue-specific like PC4,

which is expressed in reproductive organs. The functions of PCs can be overlapping, complementary, or even opposite (Seidah et al. 2013). Due to shared cleavage consensus sequence, some of the PCs have functional redundancy and are capable to activate the same target precursors. Their transport and secretion can be either constitutive, or strictly regulated, occurring only as a result of specific signaling, and they also differ with their localization in cell, as described in *figure 1b*.

The activity of PCs is inhibited with their prosegment, which needs to be cleaved off for the PC to mature. In addition to this, the PC activity requires presence of Ca^{2+} (Turpeinen et al. 2013b). PCs have a role in regulation of cell signaling and immunological responses, and they are also involved with development of diseases like cancer (Jaaks & Bernasconi 2017) and autoimmunity, as well as infectivity of viruses (Izaguirre 2019; Lenz et al. 2001; Vincent et al. 2003), which makes them interesting targets for drug development. Many of them, including PC5/6 (Essalmani et al. 2008; Hoac et al. 2018; Zheng et al. 1997), PACE4 (Bessonnard et al. 2015), PC7 (Turpeinen et al. 2013a), SKI-/S1P (Yang et al. 2001), and FURIN (Roebroek et al. 1998), are vital for a variety of processes during different phases of embryo development. These regulatory tasks are carried out by producing mature, biologically active proteins, such as enzymes, signaling molecules, adhesion molecules, and receptors, from their available but inactive pro-forms. Some of them are summarized in *figure 2*. By these activities, PCs provide a mechanism for switch-like regulation for the activation of several biological processes.

PC1/3 and PC2 are mainly expressed in the cells of neural and endocrine tissues, and are responsible for regulated secretion of many neuropeptides and hormones, many of them produced by complementary processing from precursor proteins (Benjannet et al. 1991; Malide et al. 1995; Rehfeld et al. 2008). In addition to neural and endocrine cells, PC1/3 and PC2 are expressed in the cells of both adaptive and innate immune system, at least in lymphocytes and macrophages, and differentially expressed during the immune reaction (Lansac et al. 2006). In mice the lack of PC1/3 has seen to cause uncontrolled cytokine secretion, major systemic uncontrolled inflammatory response also called cytokine storm (Refaie et al. 2012). In macrophages, PC1/3 plays part in promoting tumor growth by polarization towards anti-inflammatory M2 phenotype, which protects the tumor from attacks of immune system. Conversely, inhibiting of PC1/3 decreases tumor survival and viability in breast and ovarian cancer cells by enhancing cytotoxic responses towards tumor cells (Duhamel et al. 2015).

PC4 is known of its role in reproductive functions in both sexes and it´s vital for the production of male (Iamsaard et al. 2011; Li et al. 2000) and female germ cells (Li et al. 2000; Tadros et al. 2001), and also for normal fetoplacental growth (Qiu et al. 2005). PC5/6, instead, is vital for embryo implantation (Nicholls et al. 2011; Paule et al. 2012). PC5/6 has two splicing isoforms, soluble PC5/6A and membrane-bound PC5/6, which are differentially expressed in different organs (Essalmani et al. 2006; Nour et al 2005). PACE4 has a role in the maintenance of normal blood pressure (Chen et al. 2015), insulin-mediated signaling (Kara et al. 2015), and tumor progression (D'Anjou et al. 2011; Panet et al. 2017; Longuespée et al. 2015). PC7 is vital in iron homeostasis (Guillemot et al. 2013), and is needed also for normal cognitive functions (Wetsel et al. 2013). SKI-1/S1P (subtilisin/kexin-isozyme-1/site 1 protease) is expressed in most tissues (Seidah et al. 1999) as one secreted form and as two different membrane-bound forms, depending on its N-glycosylations (Seidah et al. 1999). SKI-1/S1P as well as PCSK9 have role in nutrient homeostasis. SKI-1/S1P is involved in cholesterol and fatty acid synthesis, and also in carbohydrate and amino acid metabolism (De Windt et al. 2007), while PCSK9 is responsible for blood LDL-cholesterol upregulation via non-enzymatic mechanism (McNutt et al. 2007; Seidah and Prat 2012; Zhang et al. 2007).

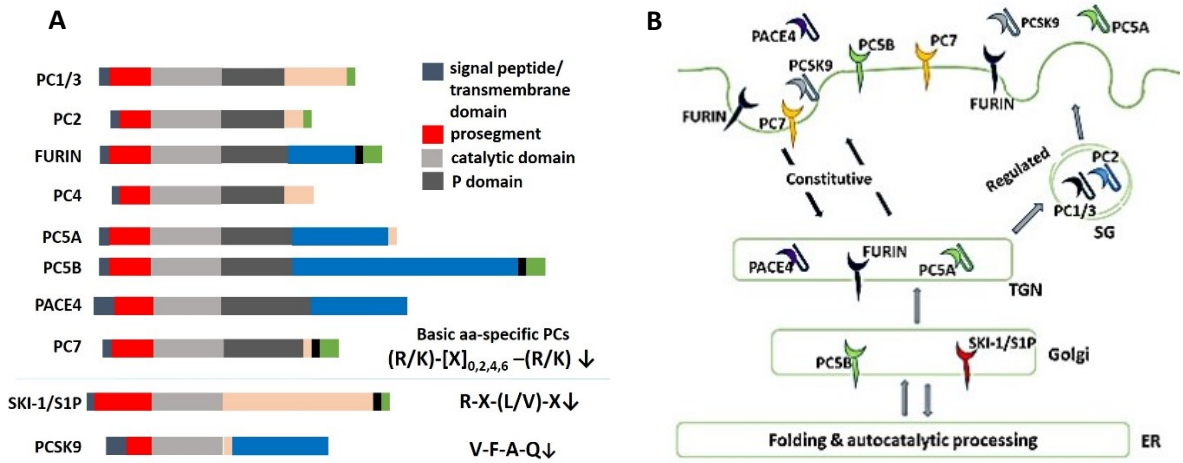

Figure 1: Schematic illustration of domain structure similarities (1a) and cellular localizations (1b) of PCs*. 1a: PC 1-7 share the same domain structure, all of them having signal sequence, prosegment, catalytic domain, and P domain. 1b: After their production and autocatalytic processing in ER, PCs can localize in Golgi, trans-Golgi network, or on the cell surface and cycling endosomes. They can also be secreted out of the cell. PC = proprotein convertase; ER = endoplasmic reticulum; SG = secretory granule; TGN = trans-Golgi network. Images adapted from Seidah 2011b.*

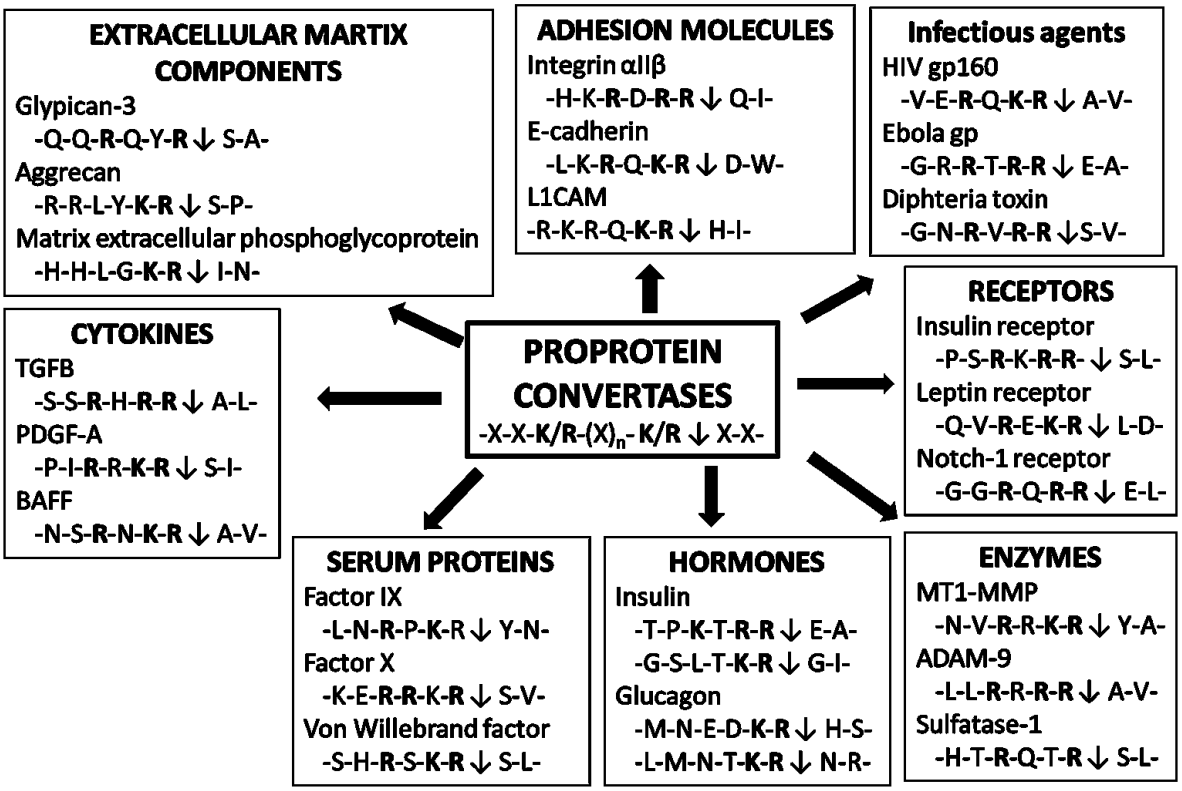

Figure 2: Targets and cleavage sites of proprotein convertases*. Image shows some of PC target molecules and their cleavage sites inside squares, and general consensus sequence in the middle. Bolded amino acids are critical for cleavage, and arrows indicate the site of cleavage. Image adapted from Turpeinen et al. 2013b.*

2.2.1. PCSK enzyme FURIN

FURIN is the third member of proprotein convertases, which belong to subtilisin superfamily of serine endoproteases. FURIN is a type I transmembrane protein, its N-terminal extracellular/luminal domain regions sharing homology with other PCs. Catalytic domain has highly conserved aspartate, histidine and serine residues, catalytic triad, that is found in other PCs. Signal peptide guides pro-FURIN to correct location in cell, and the pro-segment takes part in proper folding and regulation of its activity (Thomas 2002). The propeptide might also have a role in inhibiting tumor progression (Scamuffa et al. 2014). P-domain is essential for enzyme activity, and cytoplasmic domain instead is needed for sorting and localization in trans-Golgi network (TGN) and endosomes (Thomas 2002).

The molecular mass of full-sized, mature FURIN is about 98 kDa, and with N-glycan bound sialylations about 104 kDa. The synthesis and signal peptide cleavage, as well as autocleavage of propeptide at N-terminus, occurs in ER, following the transport to Golgi apparatus, where

the addition of sialyl groups takes place. Most of FURIN´s catalytic activity occurs in TGN and cycling endosomes, but also at the cell surface. FURIN also has a C-terminally truncated, soluble form, which can be secreted to extracellular space (Vey et al. 1994).

FURIN is ubiquitously expressed in tissues and cell types of all vertebrates (Thomas 2002). It has numerous target proproteins that it cleaves after the preferred cleavage sequence –Arg–X–Lys/Arg–Arg↓– (X = any amino acid), but it can also cleave after –Arg–X–X–Arg↓– sequence (Thomas 2002). Expression is vital for embryo development, and full knockout of *FURIN* is embryonically lethal. FURIN-deficient embryos fail to produce cardiac and vascular structures, showing it is needed already during the early stages of development (Roebrok et al. 1998). Conditional, tissue-specific knock-out animal models have showed that in some tissues there is redundancy between different PCs; liver-specific *FURIN* deletion in mice doesn´t seem to cause any major effect in developing embryos (Roebroek et al. 2004), and conditional knock-out of *FURIN* in T cells doesn´t seem to cause any effect until in adulthood, when its needed for normal functions of immune system (Cordova et al. 2016; Oksanen et al. 2014; Pesu et al. 2008).

FURIN expression is associated with many cancers (Jaaks and Bernasconi 2017), including brain (Mercapide et al. 2002) and ovarian tumors (Chen et al. 2020), and tumors with epithelial origin (Farhat et al. 2020; Fu et al. 2012). Instead, when expressed by T cells, FURIN restricts epidermal tumor development (Vähätupa et al. 2016). FURIN also seems to have a role in the development of atherosclerosis (Yakala et al. 2019) and cholesterol homeostasis (Essalmani et al. 2011). In addition, FURIN has a role in the proliferative functions (Yang et al. 2013; Yin et al. 2020), and activation of multiple viral glycoproteins (Izaguirre 2019), potentially including membrane proteins of novel SARS-CoV-2 coronavirus (Coutard et al. 2020). Because of its various roles in many diseases, it’s a promising drug candidate.

For T cell development FURIN is not necessary, but is needed for normal functions of memory and effector T cells (Pesu et al. 2008). TCR and IL-12 signaling initiates differentiation towards CD4+ Th1 cells (Jacobson et al. 1995), and induces FURIN expression via the activation of transcription factor STAT4 (Pesu et al. 2006). FURIN, in turn, is needed for the production of several mature cytokines that are vital for Th1-type responses. FURIN regulates three key T cell transcription factors NF-κB, NFAT, AP-1, that control the IL-2 cytokine production (Ortutay et al. 2015). FURIN also activates regulators of cytokine production, such as tumor necrosis factor-alpha converting enzyme (TACE) that activates TNFα (Endres et al. 2003), and

regulates expression of cytokines, such as IFN-γ, that are needed for T cell differentiation (Pesu et al. 2006). FURIN is also needed for maintaining balance between inflammatory and anti-inflammatory functions. Interaction of Tregs and effector cells is needed for the maintenance of peripheral tolerance. This process is, in part, mediated by cytokine signaling, FURIN´s role in this being cleavage and activation of cytokines such as anti-inflammatory TGF-β1. Instead, FURIN-deficient T cells are overactive and do not respond to suppressive signals of normal Tregs (Pesu et al. 2008). In addition to T cells, FURIN inhibits the expression of several pro-inflammatory genes in myeloid cells. Similar to T cells, also polarization of macrophages is affected by FURIN, as it has a role in guiding macrophages to develop towards inflammatory M1 cells, instead of anti-inflammatory M2 type cells (Cordova et al. 2016).

2.3 Glycans and glycosylation process

Glycosylations are a type of post-translational modifications that affect the folding and degradation of cellular proteins, and also modify interaction between receptors and ligands, affecting cell signaling and signal transduction (Lyons et al. 2015). Glycosylation patterns themselves are not gene encoded, but a result of expression of genes encoding glycosylation enzymes and activity of these enzymes, target proteins, their co-localization, and availability of substrates (Lyons et al. 2015; Ohtsubo and Marth 2006). As adding carbohydrate side chains can occur only at certain amino acid residues, serine in case of O-glycosylations and asparagine in N-glycosylations, mutations at target protein-encoding genes can affect production of these modifications. Mutations can result in the misfolding or premature degradation of proteins, or affect protein-protein interactions (Lyons et al. 2015). Approximately 2% of the genome encodes glycan-related proteins, such as those responsible for the addition, removal, and recognition of carbohydrates (Lyons et al. 2015), and mutations in these genes can cause congenital disorder of glycosylations (CDGs) (Chang et al. 2018), with disease phenotypes ranging from mild (Giurgea et al. 2005) to even lethal (Metzler et al. 1994). Several immune system-related diseases are associated with dysregulated glycosylations, including multiple sclerosis (MS) (Grigorian et al. 2012b) and type 1 diabetes (Yu et al. 2014). Altered glycosylation patterning has also seen to help tumor cells to escape immune surveillance (Dusoswa et al. 2020). In addition, lymphocyte trafficking is affected by the strength of receptor-ligand affinities between immune cells and vascular endothelial cells. Via altered affinities and selectin-ligand interactions, the changes in glycosylation patterns of selectin

ligands affect, in addition to leukocyte recruiting, also homing of leukocytes to secondary lymphoid organs (Sperandio et al. 2009). Glycan structures regulate effector functions by enabling the optimal binding of immunoglobulin (Ig) Fc regions to Fc receptors. Additions of glycan structures also affect the Ig transport and secretion, as well as the solubility and conformation (Arnold et al. 2007).

The N-glycan core structure is produced outside ER and added to asparagine residues at consensus sequence N-X-S/T (X=any amino acid except P) of target protein in ER. In ER and cis- and medial Golgi the core structure is trimmed by removing glucose and galactose residues by glucosidases and mannosidases. N-glycosyltransferase family Gnt mediates transfer from high-energy UDP-GlcNAc donor, which takes place in medial and trans-Golgi. Capping, a further addition of galactose, sialic acid, or fucose, can also occur to some of the Gnt-modified glycans in Golgi (Nagae et al. 2020). *Figure 3* shows a schematic representation of N-glycan processing pathway. Gnt-mediated processing is needed for many further modifications, as each of those end products act as specific substrates for subsequently modifying enzymes. For example, additions of galactose to Gnt-V modified branches produce the minimal binding structure for galectins, N-acetyllactosamine, which is needed for formation of galectin-glycoprotein lattice on cell surface (Grigorian 2009). In T cells, this lattice is an important part of the regulation of several T cell functions.

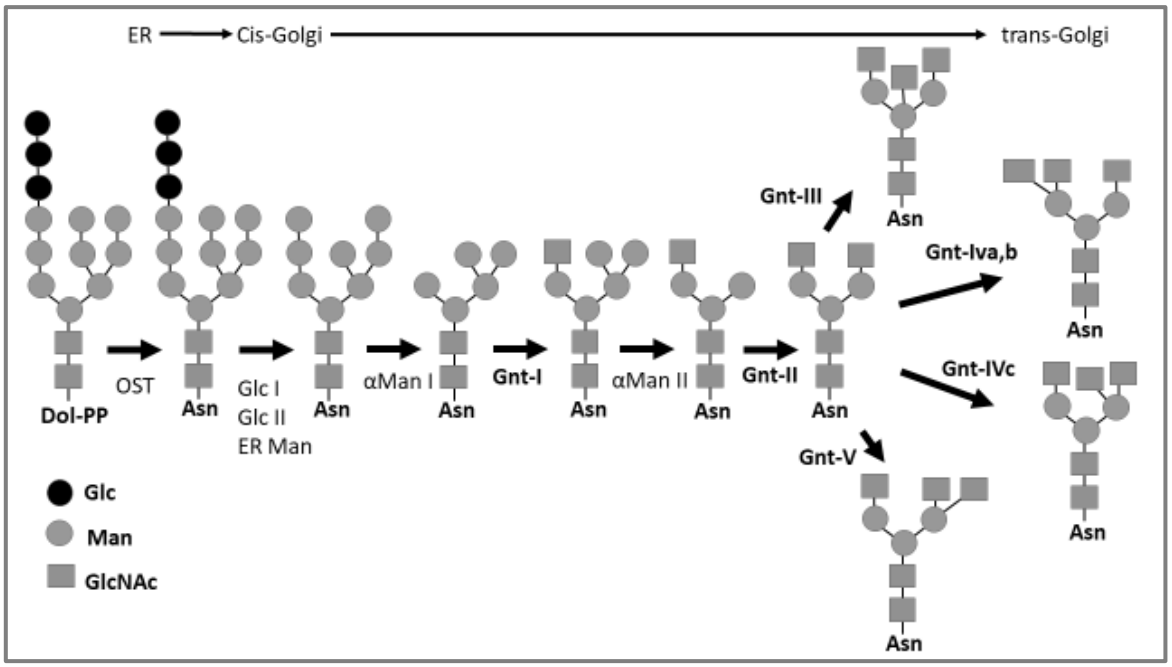

Figure 3: sequential processing of N-glycans in ER and Golgi. *14-mer glycan core is produced in cytoplasm, and transferred from dolichol donor (Dol-PP) to cytoplasmic asparagine (Asn) residue bound in ER membrane by oligosaccharyltransferase (OST). In ER and Golgi, excess glucose (Glc) and mannose (Man) residues are removed by glucosidases and mannosidases (Glc I & II, ER Man, αMan I & II). Gnt family members of N-glycosyltransferases, instead, modify glycan structure by adding N-Acetylglucosamine (GlcNAc) residues at specific positions, either enabling or restricting further modifications. Image adapted from Nagae et al. 2020.*

2.3.1 Glycosylations in T cell biology

Many immune cell functions are affected by the post-translational glycosylation of surface proteins and intracellular and secreted factors. Glycosylation processes play important roles in the T cell development (Zhou et al. 2014), differentiation (Grigorian et al. 2009; Zhou et al. 2014), trafficking (Rossi et al. 2005; Sperandio et al. 2009), antigen binding and T cell signaling (Grigorian et al. 2009), and activation (Grigorian et al. 2009; Pereira et al. 2018).

The development and differentiation into CD4+ and CD8+ T cells are affected by receptor-ligand interactions between the developing T cells and thymic cell types. The strength of these interactions, and following signal transduction is, in part, dependent on carbohydrate chains on interacting molecules (Zhou et al. 2014). Increased branching and number of glycans decrease the surface receptor endocytosis, increasing the number of especially the inhibitory receptors expressed on the cell surface, but also affects CD4 and CD8 co-receptors. This further increases the sensitivity of TCR to low-affinity peptide-MHC (pMHC) and lowers the affinity threshold

for survival during positive selection, widening the affinity repertoire of developing thymocytes. Negative selection of the high-affinity pMHCs is also affected: N-glycan branching increases the upper affinity threshold for exclusion, including greater proportion of thymocytes in the future T cell population (Zhou et al. 2014).

TCR signaling and T cell activation and differentiation is affected by specific glycans on various cell membrane proteins. The glycan structures on glycoproteins act as galectin-binding sites and regulate formation of a glycoprotein-galectin lattice. Galectin molecules can restrict protein clustering by binding and acting as spacers between them, guide co-localization of specific proteins to provide regulatory enzymatic activity, and restrict endocytosis. By regulating lattice formation and, further, lateral mobility and rate of endocytosis of surface proteins, glycosylation is important part of regulation of T cell functions (Grigorian et al. 2009).

For direct regulation of activity to occur, an enzyme and its target need to be in contact with each other. Receptor protein tyrosine phosphatase CD45 can inactivate CD4- or CD8-associated intracellular Lck, which is needed in its active form for TCR signaling. With sufficient lattice strength, Lck and CD45 are co-localized on cell membrane, allowing Lck to be inactivated. By restricting its mobility, Lck is also kept unavailable for TCR and immune synapse (Chen et al. 2007; Grigorian et al. 2009).

TCR signaling is dependent on sufficient relative affinity between pMHC and TCR, compared to the galectin-glycoprotein lattice strength. In case of cognate antigen, pMHC-TCR affinity is strong enough to break the lattice, and TCRs, co-receptors, and other assisting molecules can cluster on the immune synapse, initiating TCR signaling. Insufficiency for lattice formation enables spontaneous clustering of TCRs and recruiting of CD4-Lck (Grigorian et al. 2009), and TCR signaling can then occur even in the absence of antigen, leading to loss of self-tolerance (Demetriou et al. 2001).

As TCR signal strength is affected by galectin-glycoprotein lattice strength, surface receptor glycosylation can affect T cell differentiation into subtypes (Grigorian et al. 2009). Combination of antigen dose, received cytokine signals, and TCR signal strength induce differential intracellular signaling pathways, leading to different outcomes during T cell differentiation into subtypes and affecting also the generation of effective memory cells (Morel 2018). In general, number and complexity of glycan structures is proportional to the lattice strength and negatively regulate the TCR signal strength. Therefore, a higher number of these structures induce weaker signal and skew the differentiation towards Th2, while low level of

glycans tend to result in higher proportion of pro-inflammatory Th1 and Th17 cells (Grigorian et al. 2009). In addition, in Tregs, expression of branched N-glycan structures is highly upregulated compared to other subtypes. As a consequence, in Tregs TCR stimulation causes only low level of proliferation, and they can suppress responses of effector T cells (Sakaguchi 2004).

Activation and signal strength of naïve T cells depend on the sum of inhibiting and activating signals. Surface retention of several inhibitory receptors is increased by the addition of galectin-binding glycan structures. For instance, inhibitory CTLA-4 and activating co-receptor CD28 compete for binding to CD80/CD86 on APC (Grigorian et al. 2009). Upon activation, the number of CTLA-4 on the cell surface is increased in part due to the enhanced production of N-glycans (Alegre et al. 2001; Grigorian and Demetriou 2011). Also, a higher percentage of TCRs are isolated within lattices. These together with other inhibitory effects of glycosylation decrease the probability of immediately recurring activation (Grigorian et al. 2009).

2.3.2 N-acetylglycosylaminyltransferase family Gnt

Gnt family of glycosyltransferases, encoded by genes *Mgat1-5*, modify N-glycans by adding N-acetylglucosamine (GlcNAc) residues to trimmed $Glc_3Man_9GlcNAc_2$ core structure that is attached to target protein. These modifications occur mainly in the Golgi apparatus, and include branch formation, elongation and capping in sequential manner. Subsequent addition and removal of sugars to specific positions at cis-, medial, and trans-Golgi network provides elongation and branching points for the next enzymes to process (Nagae et al. 2020).

In production of branched N-glycan structures, Gnt family members have complementary, redundant, and inhibitory functions. The activity of Gnt-I and -II initiate formation of complex-type glycans by addition of GlcNAc to recipient mannose residue at each arm of the glycan. Other Gnt family members can recognize these structures, and further increase branching by GlcNAc additions to different positions in same mannoses (Nagae et al. 2020). Of the five Gnt IV homologs, Gnt-IVa and Gnt-IVb produce the same structure, the latter having higher substrate affinity than the latter (Oguri et al. 2006). Function of Gnt-IVc is less known. In mouse there is also an active homolog Gnt-IVe, that isn´t expressed in human but exists only as a pseudogene (NCBI Gene database, available at *https://www.ncbi.nlm.nih.gov/gene/71001#gene-expression*; 13.4.2020). Gnt-IVd and Gnt-III are inhibitory to other members, the former known as Gnt-I inhibitory protein (Gnt-1IP) with no enzymatic activity (Huang et al. 2015). The latter instead produces bisecting branch structure

that cannot be processed further by Gnt-V (Nagae et al. 2020). Gnt-Vb, also known as Gnt-IX, is exceptional member in Gnt family, as it processes O-linked glycans instead of N-glycans (Nagae et al. 2020).

2.3.3. Gnt-V

N-acetylglucosaminyltransferase-V (Gnt-V), sometimes referred to as Gnt-Va to distinguish it to its paralog Gnt-Vb, is encoded by gene *Mgat5(A)*. In literature the protein is also often referred to as MGAT5; here, for clarity, we will use the name Gnt-V. Gnt-V transfers GlcNAc to α1-6-linked mannose of N-glycans via a β1-6 linkage. It is 741 amino acids long type II membrane protein with N-terminal transmembrane part and N-terminal helical domain that is needed for its localization to Golgi (Nagae et al. 2020). The catalytic domain localizes in its C-terminal half. Gnt-V shares 42% sequence similarity with its paralog Gnt-Vb, but otherwise it´s amino acid sequence doesn´t resemble that of the other members of Gnt family (Inamori et al. 2003) and is, instead, included to Gnt family due to similar functionality. However, its amino acid sequence among species is highly conserved (Nagae et al. 2020). Gnt-V is expressed in almost all tissues, with exception of skeletal muscle cells, and only very low levels in brain (Kaneko et al. 2003). When its paralog Gnt-Vb is absent, Gnt-V can partially process also O-linked branched glycans in brain (Lee et al. 2012).

Gnt-V is a vital enzyme for modifying membrane proteins with branched N-glycans with high binding affinity to galectins. Galectin-glycoprotein lattice limits the lateral mobility and endocytosis of cell surface glycoproteins, such as TCRs, that tend to cluster without galectins that act as spacers. Number of Gnt-V modified glycans increase the threshold number of TCRs that need to cluster for initiation of TCR signaling upon antigen binding (Grigorian et al. 2009). TCR activation increases Gnt-V activity and production of β1,6GlcNAc-branched N-glycans within 2 days of naïve T cell activation (Chen et al. 2009), which in turn negatively regulates TCR signaling and enhances Th2 development and polarization, producing less inflammatory phenotype (Morgan et al. 2004). Gnt-V can change the glycosylation pattern on many important proteins, such as TCR and CTLA-4, as well as other components involved in attenuation of T cell activation (Grigorian et al. 2009). Its proper function is vital for the regulation of T cell signaling, and Gnt-V deficiency, which causes decreased galectin binding and galectin-glycoprotein lattice formation, increases TCR clustering. This in turn causes hyperactive TCR signaling and excess T cell proliferation and increases the susceptibility to autoimmune disorders (Demetriou et al. 2001; Grigorian et al. 2009).

Gnt-V is upregulated in gastric cancer (Huang et al. 2014), and its expression has been associated with increased gastric carcinogenesis and cancer cell invasiveness (Carvalho et al. 2016), as well as with other cancers with epithelial origin (Guo et al. 2010; Terao et al. 2011; Yamamoto et al. 2007). The overexpression of Gnt-V also results in increased number of cancer stem cells (Guo et al. 2010) and increased epithelial mesenchymal transition (EMT) and keranocyte migration, although also in faster wound healing *in vivo* (Terao et al. 2011). Gnt-V overexpression-associated cell migration, instead, is decreased by Gnt-III-mediated inhibition of β1,6 branching (Zhao et al. 2006). Gnt-V also has tumor-promoting functions not related to its enzymatic activity. Shed and secreted Gnt-V can interact with extracellular molecules, releasing angiogenesis-inducing FGF-2 from heparin-sulphate proteoglycans on extracellular matrix (ECM) and cell surface. Basic amino acids (264-269) also stimulate endothelial cell proliferation (Saito et al. 2002). Cleavage and secretion seem to be regulated by γ-secretase, which can cut proteins at their transmembrane region when co-localized in TGN (Nakahara et al. 2006). Another enzyme shown to cause this cleavage-mediated regulation of Gnt-V activity and secretion is signal peptide peptidase-like 3 (SPPL3) (Voss et al. 2014).

3 AIMS OF THE STUDY

FURIN and Gnt-V are localized in the same subcellular compartment, their expression is induced upon T cell activation, and they both are vital for normal T cell functions, but their interrelatedness hasn´t yet been studied. This thesis aims to investigate if FURIN affects the activity of Gnt-V, either by affecting the activation of the protein by direct cleavage, or indirectly by regulating its gene expression. The study was executed in three phases:

I. The effect of FURIN on Gnt-V activity is first studied by analyzing the differences of Gnt-V-modified N-glycosylation on cell surface proteins on mouse splenocytes with and without conditional FURIN knockout.
II. Direct FURIN cleavage activity on Gnt-V is investigated using transfected hamster cell lines and western blot analysis.
III. The effect of FURIN on gene expression level of Gnt enzyme family members is studied by analyzing RNA sequencing data on mouse thymocytes with and without conditional FURIN knockout.

4 MATERIALS & METHODS

4.1 Primary mouse T cells with conditional FURIN knockout

Mice with T cell-specific *FURIN* knockout (Fur KO)(Pesu et al. 2008) were maintained according to regulation, with a permit from the National Animal Experiment Board of Finland (ESAVI/2267/ 04.10.03/212). Fur KO mice differ from FURIN wild type (Fur WT) animals in that their T cells have conditional deletion in exon 2 of *FURIN* gene, and thus are unable to produce FURIN protein. For glycosylation analysis, 8 animals (4 Fur KO and 4 Fur WT) were euthanized, and splenocytes were extracted and purified for lectin staining and flow cytometry (FACS) analysis. For gene expression level analysis with RNA sequencing, 4 age and sex-matched Fur KO and 4 Fur WT mice were euthanized and T cells from spleens and lymph nodes were collected. Naïve CD8+ T cell fraction was purified using magnetic-activated cell sorting (MACS) with T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany).

4.2 Cell lines & cell culturing

4.2.1 Chinese hamster ovary (CHO) fibroblast cell line

To study the effect of FURIN on production and size of transfected human Gnt-V, CHO-derived FURIN-deficient RPE.40 cells (Spence et al. 1995) and CHO-K1 hamster fibroblasts were cultured in Ham´s F-12 culture medium (Lonza, Basel, Switzerland) supplemented with 10% FBS (Thermo Fischer Scientific, Waltham, MA, USA) and 100 ug/ml of Streptomycin (Lonza) and 100U/ml of Penicillin (Lonza). To keep confluence between 60 and 80%, every 2-3 days cells were washed with PBS (Biotop, Uppsala, Sweden), detached using 0.25% Trypsin-EDTA (Lonza) and split 1:4 or 1:5, depending on current growth rate. For transfections, cells were split into concentration of $1.5*10^5$/2ml/well on 6-well plate one day prior transfections.

4.2.2 Jurkat human T cell line

Three Jurkat E6.1 cells cell lines (ATCC, Manassas, VA, USA), stable transfected with empty pcDNA3.1-Strep III plasmid, pcDNA3.1-Strep III plasmid with loss-of-function-*FURIN* sequence and pcDNA3.1-Strep III plasmid with wild type *FURIN* sequence (Ortutay et al. 2015), were used. Cells were cultured in RPMI-1640 (Lonza) medium supplemented with 1% L-glutamine (Lonza), 100 ug/ml of Streptomycin (Lonza) and 100U/ml of Penicillin (Lonza),

and 10% FBS (Thermo Fischer Scientific) in 6-well plates. Cells were split every 2-3 days into concentration of 3 *10^5/ml, 2ml/well, and collected and lysed for further western blot protein analysis.

4.2.3 U937 human lymphoma cell line

For the optimization of FURIN detection with western blotting, U937 human promonocytes (ATCC) were grown and differentiated, and half of them were stimulated while half of them were left unstimulated. Cells were seeded into concentration of 2* 10^6/2ml and cultured for 3 days with complete RPMI-1640 medium (RPMI-1640 (Lonza) supplemented with 10% FBS (Gibco/Thermo Fischer Scientific), 2 mM L-glutamine (Lonza), 1% Penicillin-streptomycin 10 000 IU/10mg (Lonza). Cells were differentiated by growing cells 48h in differentiation medium (complete RPMI-1640 (Lonza) with 100 ng/ml of PMA (Sigma, St. Louis, MO, USA)). After that, differentiation medium was changed to complete RPMI-1640 for 24h. The cells were stimulated with LPS (2 µg/ml; Invitrogen, Carlsbad, CA, USA) for 24h. Both stimulated and unstimulated cells were then lysed as described below.

4.3 FACS

To study if FURIN affects the degree of T cell glycoprotein N-glycosylations, Fur KO and WT spleens were harvested from euthanized animals, and from all the splenocytes, T cells and different T cell subpopulations were distinguished by surface staining and fluorescence-activated cell sorting (FACS). The naïve and memory subpopulations were studied for their phytohemagglutinin-L (L-PHA) binding with flow cytometry. The activity of Gnt-V was assumed to be proportional to the amount of branched N-glycosylations on the surface of T cells, which could be detected using fluorescent stain-bound L-PHA that selectively binds to these glycosylation products (Cummings & Cornfeld 1982; Demetriou et al. 2001).

Homogenization of the spleens was carried out by passing cells through the 40µm filters and treated with ACK lysis buffer (Lonza) for red blood cell lysis. To stop the lysis, ice cold PBS was added up to 50 ml and cell lysates were centrifuged (400x g, 5 min, +4 ºC). Supernatant was removed and cells were suspended to 10 ml of fresh PBS. After that, the cells were washed twice with 2 ml staining buffer containing PBS and 1 % FBS (Gibco), and then stained with mixture of fluorescent antibodies listed in *table 4*.

Table 4: Surface marker stains and dilutions used for FACS. *Dilutions were made into staining buffer containing 1% FBS in PBS.*

Cell surface marker-stain	Dilution	Purpose	Company
CD3e-APC	1:100	Detection of T cells; exclusion of non-T cells	Thermo Fisher Scientific
CD62L-PE	1:100	Detection of naive T cells	Thermo Fisher Scientific
CD4-APC-eFluor780	1:100	Detection of CD4+ T cells	AH Diagnostic (eBioscience)
CD8-PerCP-Cy5.5	1:100	Detection of CD8+ T cells	Thermo Fisher Scientific
L-PHA – FITC	1:500	Detection of Gnt-V modified glycans	Vectorlab

The stain dilution was added to cells and then incubated for 20 min on ice protected from light. Cells were washed with 2 x 2 ml staining buffer and fixed by adding 100 µl Fixation buffer (FoxP3 Kit, eBioscience, San Diego, CA, USA) per tube while mixing with tube shaker. The cells were incubated in the dark for 20 min in room temperature, then washed 1 x 2 ml staining buffer, resuspended and stored in +4ºC overnight. The next day the cells were washed with 2 x 2 ml staining buffer and resuspended in 500 µl staining buffer. 50 000 events were analyzed in FACSCanto II flow cytometer, and different T cell populations were defined based on their surface markers (CD3, CD4(+/-)CD62L, CD8(+/-)CD62L) and analyzed by their L-PHA binding, seen by the intensity of fluorescence signal of L-PHA-bound FITC, with FlowJo software (TreeStar, Ashland, OR, USA). The image panel below (*figure 4*) shows defining of T cells into subpopulations of CD4+ and CD8+ T cells with FlowJo software. The cells were further defined into subpopulations of naïve (CD62L+) and memory (CD62L-) CD4+ helper (first row) and CD8+ cytotoxic (second row) T cells.

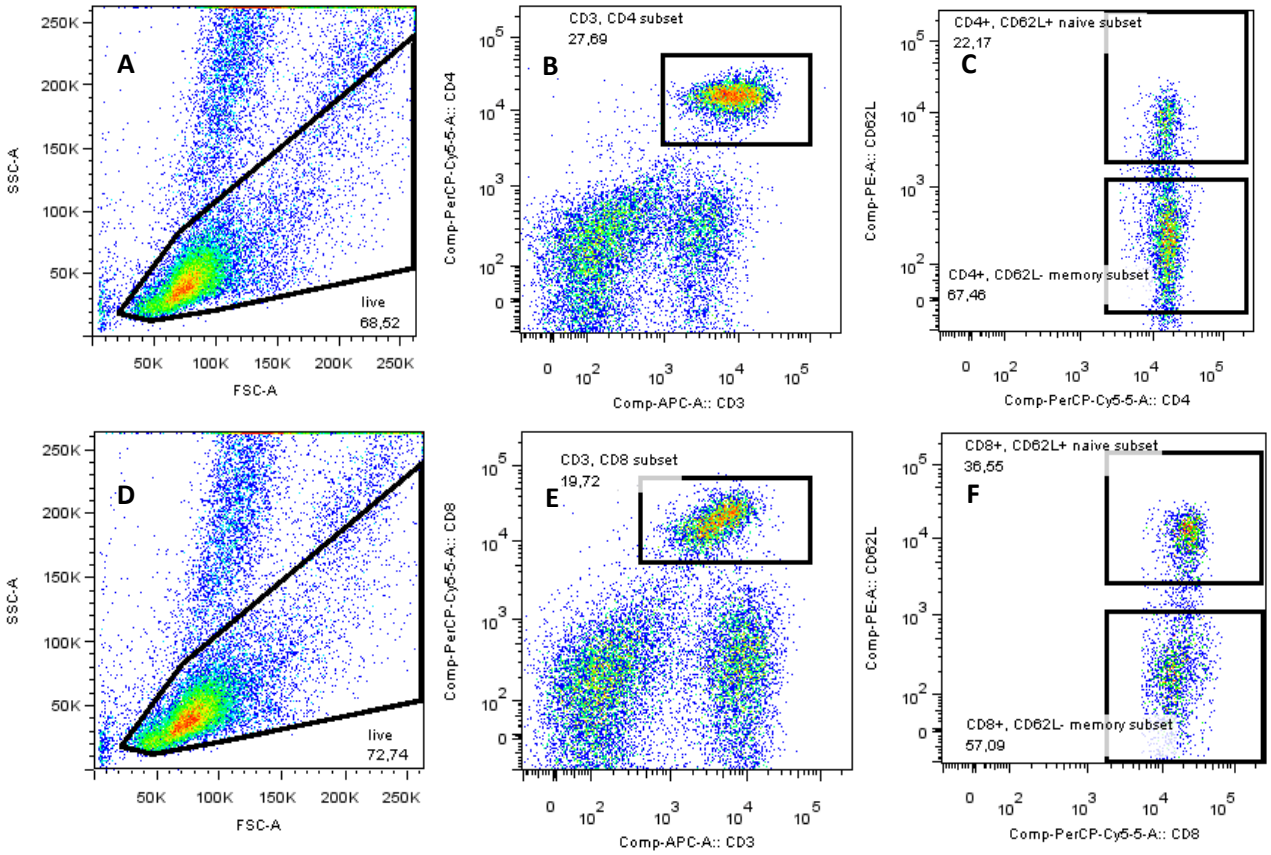

Figure 4: Definition of subpopulations of mouse T cells. *Of the whole cell population (A) T cells were defined based on their CD3 marker, and further defined based on their CD4 marker (B). Of those, CD4 positive T cells were further defined as naïve (CD62L+) and memory (CD62L-) subpopulations. Naïve and memory CD8+ T cells were defined in similar manner (D-F).*

4.4 DNA constructs and transient transfections

4.4.1 Production and purification of DNA constructs

To produce *Mgat5* plasmids for transfections, competent bacteria (JM109 *E.coli* (Promega, Madison, WI, USA)) were transformed with *pcDNA3.1-Flag-huMgat5-V5* construct (a present from professor Regina Fluhrer, Ludwig-Maximilians University Munich, Munich, Germany; see Voss et al. 2014). A mixture of 10µl of plasmid and 100µl of *E.coli* were incubated on ice for 30 min, following 45 s heat shock (42ºC), and 2 min cooling on ice. The bacteria were incubated with 200µl of LB (Sigma) for 1.5h in +37ºC, and transferred to agar plates overnight in room temperature. One clone was selected for further plasmid production and handled as described below. To produce other plasmids (*pcDNA3.1-StrepIII* (a gift from Prof. Jukka Westermarck, Turku Centre for Biotechnology, Finland; Oksanen et al. 2014), *pSVL_hu_FURIN_WT* (ATCC, Manassas, VA, USA), *pcDNA3.1(-)hTGFβ_MycHis* (AddGene,

Cambridge, MA, USA), *pCMV-β-galactosidase* (AddGene)) for transfections, previously transformed stocks of bacteria were used. 2ml of LB with 50µg/ml Ampicillin (Merck, Calbiochem, Darmstadt, Germany) was inoculated with bacteria from glycerol stocks, as well as those transformed with *Mgat5* construct, and incubated 6h on shaker in +37ºC. The bacteria were transferred to 200ml LB with 50µg/ml Ampicillin to grow overnight on shaker, +37ºC. Plasmid DNA was purified using NucleoBond Xtra Midi Kit (Macherey-Nagel, Düren, Germany) according to manufacturer´s instructions.

4.4.2 Optimizing the FuGENE:DNA ratio

As suggested in FuGENE (Promega) transfection reagent user manual, the FuGENE:DNA ratio was optimized with RPE.40 cells using ratios 6:1, 4:1, 2:1 and 1.5:1, with 650ng of plasmid DNA in each transfection, including 150ng of *pCMV-beta-galactosidase* construct and 500ng *pcDNA3.1-Flag-huMgat5-V5* construct.

4.4.3 Optimizing the amount of DNA

To evaluate the effect of total amount of transfected DNA/transfection on cell viability and protein yield, *pcDNA3.1-Flag-huMgat5-V5* construct was transfected to RPE.40 and CHO-K1 cells with 0, 500, 1000 and 5000ng of DNA/transfection with 6:1 FuGENE:DNA ratio. Viability was visually evaluated by microscopy before lysis.

4.4.4 Transfections for western blot analysis

To study the cleavage activity of human FURIN in RPE.40 hamster cell line (CHO cell line with inactive FURIN; described by Moehring et al. 1992), DNA expression vectors of *FURIN*, *TGFβ*, and Gnt-V encoding *Mgat5* were transfected into the cells in different combinations. TGFβ is known to be processed by FURIN (Dubois et al. 1995) and was thus used in transfections to control that transfected FURIN is active. CHO-K1 cell line with functional *FURIN* gene was for same purpose. *β-galactosidase* vector was used in all transfection conditions as a reporter gene for evaluation of the transfection efficiency with β-galactosidase assay. RPE.40 and CHO-K1 cells were split to 6-well plates into concentration of $1.5*10^5$/well 24h prior transfections. FuGENE transfection reagent was added to transfection medium (Ham´s F-12 (Lonza) without FBS and antibiotics), and incubated in room temperature for 5 min. The following conditions were used on RPE.40 and CHO-K1 cells:

1. Non-transfected control

2. pcDNA3.1 -Flag -huMgat5 -V5 (500 ng) and pCMV- β-galactosidase (150 ng)

3. pcDNA3.1 -Flag -huMgat5 -V5 (500 ng) and pSVL_hu_FURIN_WT (500 ng) and pCMV- β-galactosidase (150 ng)

4. pcDNA3.1(-)-hTGFb-MycHis (500 ng) and pCMV-beta galactosidase (150 ng)

5. pcDNA3.1(-)-hTGFb-MycHis (500 ng) and pSVL_hu_FURIN_WT (500 ng) and pCMV-β-galactosidase (150 ng)

6. pcDNA3.1-Strep III (150 ng)

Plasmid DNA was added to transfection medium, which was then added to cells within 15 min and mixed immediately. After that the cells were transferred to incubator (37 ^{o}C, 5% CO2) for 48h. After a 48h incubation, the transfected and control cells were washed with PBS, detached with 0.25% Trypsin-EDTA (Lonza), and split in two for downstream examinations (β-galactosidase assay and western blot).

4.4.5 Cell lysis of fractions for β-galactosidase assay and western blot

Fractioned cells were centrifuged to separate supernatant and then the cells were washed once with PBS. The fraction for western blot analysis was lysed using lysis buffer containing 20mM Tris-HCl (pH 8.0) (VWR), 300mM NaCl (VWR), 20% glycerol (Sigma), 0.1% Triton X-100 (Sigma), 1mM EDTA (Sigma), and 50mM NaF (Sigma-Aldrich) in dH_2O. Lysis buffer was completed by adding 1 mM TCEP (tris(2-carboxyethyl)phosphine hydrochloride; Sigma) and one Complete Mini pill protease inhibitor (Roche Diagnostics, Indianapolis, IN, USA) per 10ml of lysis buffer 1h prior lysis. Lysis buffer was added to cells (100ul/1 well of 6-well plate), mixed thoroughly, incubated on ice for 30min, and centrifuged (+4^{o}C, 10min, full speed). Fraction for β-galactosidase assay was lysed using 5X CCLR lysis buffer (Promega) diluted with sterile water. The same volume of lysis buffer was used as above, with 10min incubation on ice after thorough mixing, followed by centrifuging as above. Supernatants were carefully separated and stored in -20^{o}C.

The transfection efficiency was measured from β-galactosidase fractions using colorimetric β-galactosidase assay. β-galactosidase assay master mix (10% 10X LacZ buffer (Sigma), 50% o-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma), 20% dH20) and lysates were mixed (4:1), incubated 20min in RT, and the absorbances were measured with ELISA plate reader with 420nm. The absorbance values were used to exclude cells with low transfection efficiency from further experiments. Protein concentrations of lysates for western blot were measured

colorimetrically using 3µl cell lysate and 150µl Bradford reagent (QuickStart™ Bradford protein assay; BIO RAD). After 20 min incubation (RT) the absorbances were measured with ELISA plate reader with 595nm.

4.5 Western Blot

Lysates with equal amounts of protein were mixed with 4x Laemmli sample buffer (Bio-Rad) and boiled for 10min in 98ºC. Lysates were run in SDS-PAGE with 9% gel, running buffer containing 25 mM Tris (LabChem), 192 mM glycine (Sigma-Aldrich), and 0.1 % SDS (Sigma) (100V 20min + 160V 50min). The gel was washed with transfer buffer containing 20% methanol (Sigma-Aldrich), 24.6 mM Tris (LabChem), and 0.192 M glycine (Sigma-Aldrich). The samples were transferred (15V 50min 250mA) to nitrocellulose membranes (GE Healthcare) and washed with TBST before blocking for 1h in room temperature (RT). The membranes were washed 3x5min with TBST and incubated overnight in primary antibody dilutions (*table5*) in +4ºC, washed 3 times with TBST and incubated 1h, RT with anti-actin antibody. After that membranes were washed 3x10min with TBST and incubated 1h, RT in 1:5000 secondary antibody dilution (table 1), washed once with TBST, twice with PBST, and once with PBS before developing the blot with Odyssey Infrared Imaging System (Li-Cor Biosciences UK, Cambridge, United Kingdom).

4.5.1 Optimization of FURIN detection

Prior to analyzing transfected cell lysates, detection of FURIN was optimized using 4 different FURIN antibodies. FURIN-deficient RPE.40 cells were used as negative control. To ensure sufficient amount of FURIN for detection, human U937 promonocytes were differentiated into macrophage-like cells with PMA, and half of them were then stimulated with LPS, as described above (*4.2.3 U937 human lymphoma cell line*). Lysates with equal amount of protein were used for optimization. Information about the antibodies, dilutions, and blocking reagents used are listed below in *table 5*. Default protocol was used in first four-antibody setup and further modified after that.

Table 5: Used antibodies, dilutions, blocking reagents and incubation times. *BSA = bovine serum albumin; OBB-PBS = 1:1 mixture of Odyssey blocking buffer and phosphate buffered saline; TBST = Tris-base salin with 0,1% Tween; o/n = overnight. Abbreviations for companies: SC = Santa Cruz (Santa Cruz Biotechnology, Heidelberg, Germany); ELS = Enzo Life Sciences (Farmingdale, NY, US); LT = Life Technologies (Carlsbad, CA, USA); S = Sigma (St. Louis, MO, USA); RD = R&D Systems (Minneapolis, MN, USA); L = LI-COR (Li-Cor Biosciences UK, Cambridge, United Kingdom). Anti-Mgat5 = antibody against Gnt-V protein, named after gene encoding Gnt-V.*

Primary antibody	Host	Dilution	Dilute, incubation time	Company	Blocking solution, incubation time
FURIN detection					
H220 anti-FURIN, SC-20801	rabbit, polyclonal	1:200	5% BSA in PBS + 0.01% sodium azide, o/n	SC	5% BSA in TBST, 1h/2h
MON-139 anti-FURIN	mouse, monoclonal	1:1000	5% BSA in PBS + 0.01% sodium azide, o/n	ELS	5% BSA in TBST, 1h/2h
MON-148 anti-FURIN	mouse, monoclonal	1:1000	5% BSA in PBS + 0.01% sodium azide, o/n	ELS	5% BSA in TBST, 1h/2h
MON-152 anti-FURIN	mouse, monoclonal	1:1000	5% BSA in PBS + 0.01% sodium azide, o/n	ELS	5% BSA in TBST, 1h/2h
GntV detection					
anti-V5, R960-25	mouse, monoclonal	1:5000	OBB-PBS, o/n	LT	OBB-PBS, 1h
anti-Flag, F3040	mouse, monoclonal	1:500	0.05M TBS + 1mM $CaCl_2$, o/n	S	5% BSA in TBST, o/n
anti-Mgat5, 706824	mouse, monoclonal	1:750	0.1% TBS-Tween (TBST), o/n	RD	OBB-PBS, 1h
Control (actin)					
anti-actin, MABI50IR	mouse, monoclonal	1:1000	5% BSA in PBS + 0.01% sodium azide, 1h	S	(according to first Ab)
anti-actin, 20-33	rabbit, monoclonal	1:250	OBB-PBS, 1h	S	(according to first Ab)
Secondary antibody					
anti-rabbit, IRDye 680 LT	goat	1:5000/ 1:10000	TBST, 1h	L	
anti-mouse, IRDye 800 CW	goat	1:5000/ 1:10000	TBST, 1h	L	

Based on the result of the first set of experiments, H220 and MON-152 FURIN antibodies were used for further optimization. Lysates were loaded into the gel and run according to standard

protocol. The blocking period was extended to 2h and secondary antibodies were diluted 1:10000 instead of 1:5000, rest of the protocol remaining the same as previously.

4.5.2 Lysates from optimization of transfection with Mgat5 construct

To see a quantitative effect of different FuGENE:DNA ratios, equal cell lysate volumes instead equal amounts of protein were used. 15µl of lysates from RPE.40 cells transfected with *pcDNA3.1-Flag-huMgat5-V5* construct were handled according to standard protocol using anti-Mgat5 antibody.

4.5.3 Evaluation of the effect of different amounts of transfected DNA on cell viability

Cell lysates of RPE.40 and CHO-K1 cells transfected with 0, 0.5 and 1.0 µg/ml of *pcDNA3.1-Flag-huMgat5-V5* plasmid DNA were measured for their protein concentration, and samples with 22µg of protein were then handled according to standard protocol, using anti-Flag, anti-V5, and anti-Mgat5 (antibody detecting Gnt-V protein, named after gene *Mgat5* that encodes the protein) antibodies. Based on the first antibody, either mouse or rabbit anti-actin dilution was used for detection of actin. Secondary antibodies (anti-mouse and anti-rabbit with different fluorescence labels) were used with 1:10 000 (anti-Flag membrane) and 1:5000 (other membranes) dilutions (see *table 5*).

4.5.4 Transfection samples

RPE.40 and CHO-K1 cell lysates with 30µg of protein were handled according to standard protocol using anti-V5, anti-Flag, anti-Mgat5, and H220 anti-FURIN antibody dilutions. Mouse or rabbit anti-actin dilutions, and secondary antibody dilutions (1:5000 for anti-V5 and anti-Mgat5 membranes, 1:10000 for anti-Flag and anti-FURIN blots), were chosen based on the first antibody (see *table 5*).

4.6 Gene expression analysis with RNA sequencing

To investigate if the gene expression levels of Golgi N-glycosylation pathway enzymes are affected by presence of FURIN, previously achieved RNA sequencing data (unpublished data) was used to analyze possible differences in gene expression levels among Mgat family in CD8+ T cells. To achieve the data, CD3+CD8+CD62L+CD44^{low} cells were enriched from total T cell populations using MACS kit & columns (Miltenyi) and sorted with FACS (FACSaria, Turku). The cells were lysed using RLT buffer from RNeasy Kit (Qiagen, Dusseldorf, Germany). To

maximize RNA yield, RNA was isolated without DNase I treatment. The RNA library was prepared and RNA was sequenced by Finnish Microarray and Sequencing Centre with HiSeq, followed by a bioinformatics analysis by bioinformatics group in Tampere University. *Mgat1*, *Mgat2*, *Mgat3*, *Mgat4a-e*, *Mgat5a* and *Mgat5b* gene expression levels were compared between Fur KO and WT T cells.

4.7 Statistics

We wanted to see if there are significant differences in L-PHA staining between Fur KO and WT T cells. Of the L-PHA staining data from FACS, geometric means were first calculated for four parallel samples. Then we calculated the average of geometric means of each T cell subgroup and compared the averages between Fur KO and Fur WT using R software. Because the number of parallel samples is insufficient for assuming normal distribution, non-parametric Mann-Whitney Test was used for statistical analysis, with two-tailed p-values to describe statistical significance.

5 RESULTS

5.1 FURIN affects T cell surface protein N-glycosylations

To investigate FURIN´s effect on Gnt-V activity, we stained mouse Fur WT and Fur KO splenocytes with fluorescent L-PHA-FITC stain. L-PHA selectively binds to Gnt-V-modified branched complex N-glycans at glycoproteins on cell surface (Cummings & Cornfeld 1982; Demetriou et al. 2001). The signal strength from L-PHA stained splenocytes is proportional with the activity of glycosyltransferase Gnt-V (Zhou et al. 2014). *Figure 5* shows the lectin binding comparison between Fur KO and Fur WT T cells as fluorescence intensity of L-PHA -FITC stained cells (lectin binding) in each cell subgroups. In both CD4+ and CD8+ T cell subpopulations, the geometric means of fluorescence intensity of L-PHA-stained Fur WT cells are significantly higher on naïve compared to the memory cells, while in Fur KO subpopulations, the differences are remarkably smaller. Differences between the Fur WT and Fur KO CD4+ T cell subpopulations were statistically significant ($p<0.05$). Also the CD8+ naïve T cell subpopulations had a significant difference between Fur WT and Fur KO ($p<0.05$), while this wasn´t seen in memory cells.

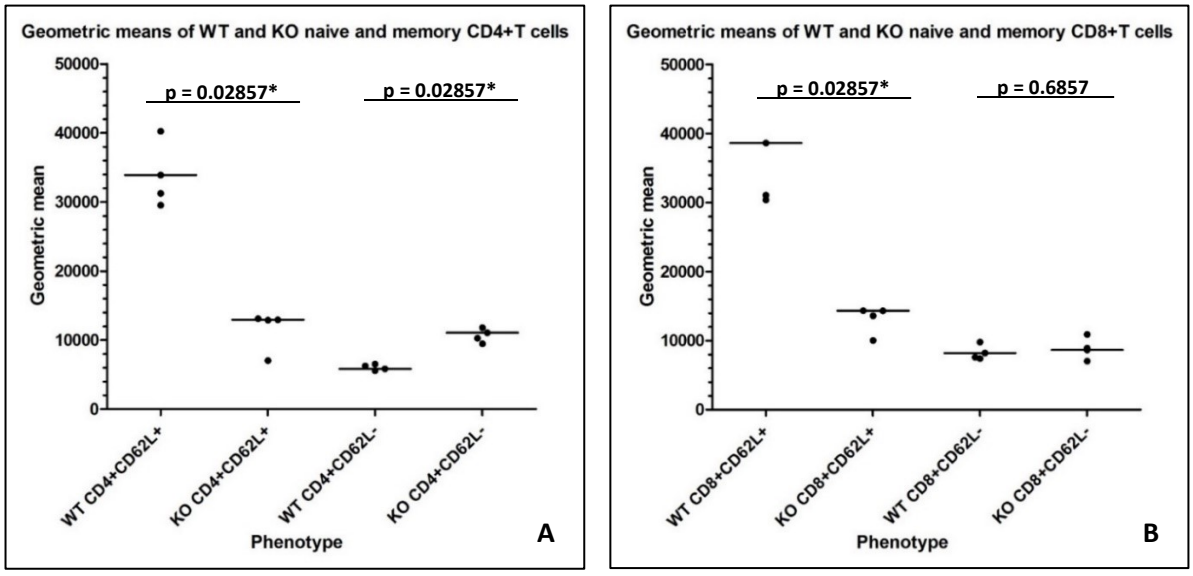

Figure 5: Comparison of L-PHA binding of CD4+ (A) and CD8+(B) FURIN-expressing (WT) and FURIN-deficient (KO) T cells. *Dot plots show geometric means of fluorescence intensities of Fur KO and WT mice naïve (CD62L+) and memory (CD62L-) T cells in CD4+CD62L+ and CD4+CD62L-, and CD8+CD62L+ and CD8+CD62L- subgroups. Dots indicate geometric means of each sample, and lines indicate medians of values. N=4/genotype. 2-tailed p-values in figures are from Mann-Whitney nonparametric t-test, calculated using R software.*

5.2 Gnt-V has potential FURIN cleavage site

The differences seen in L-PHA staining between subpopulations of mouse Fur KO and WT T cells indicated that FURIN affects the activity of Gnt-V. To investigate if Gnt-V is a potential target of direct cleavage by FURIN, a protein cleavage site prediction tool at ProP 1.0 server (Duckert et al. 2004) was used to find potential cleavage sites. The prediction scores indicate a probability that the target protein is cleaved at specific position of amino acid sequence, 0 indicating a 0% probability and 1 indicating a 100% probability. In addition to predicted signal peptide cleavage site between positions 26 and 27, one FURIN target site was found after human Gnt-V amino acid sequence position 244, with prediction score 0.719. The same score for FURIN cleavage was found also in position 243 of Chinese hamster Gnt-V sequence. For human Gnt-V protein, the two other potential cleavage sites were found to be possible targets of cleavage activity of other PCs after positions 56 and 445, with score of 0.896 and 0.606, respectively. For the Chinese hamster Gnt-V, positions 56 and 444 were predicted, with same scores. Comparison between mouse, human, rat and hamster amino acid sequences shows high level of conservation among Gnt-V protein produced in these species, shown in *figure 6* below. Gnt-V in figure is named as MGAT5A, according to the gene encoding Gnt-V protein.

If cleaved by FURIN after position 244, Gnt-V fragments should be sized approximately 30 kDa and 60 kDa, calculated using Protein Molecular Weight calculation tool (*https://www.bioinformatics.org/sms/prot_mw.html*).

Figure 6: Alignment of amino acid sequences on mouse, human, rat, and hamster Gnt-V protein (here labeled MGAT5A). *Alignment shows high degree of evolutionary conservation of sequences of this protein between species. Highlighted: Dark grey= identical; light grey=difference in one or more sequence; yellow=transmembrane region; orange=site of N-glycosylation. Black line indicates predicted FURIN cleavage site and blue lines indicate predicted PC cleavage sites. Image acquired using UniProt database.*

5.3 FURIN does not process Gnt-V

As discussed, we saw a significant difference in L-PHA binding between Fur KO and WT mouse T cells, suggesting differential Gnt-V activity in the absence of FURIN. We also predicted that FURIN cleaves Gnt-V with high probability, and wanted to investigate whether or not Gnt-V is direct target of FURIN's proteolytic activity. Cleavage at the predicted site of amino acid sequence would result in products sized approximately 30 kDa (N-terminal fragment) and 60 kDa (C-terminal fragment), instead of non-cleaved, approximately 90 kDa protein. For a differential detection of these fragments in western blot, we used *pcDNA3.1-Flag-huMgat5-V5* DNA construct with N-terminal Flag and C-terminal V5 sequences, which could be detected using anti-Flag and anti-V5 antibodies. We also used anti-Mgat5 antibody to detect the whole protein.

To validate the assumed FURIN cleavage on Gnt-V, we also aimed to show the availability of FURIN in RPE.40 and CHO-K1 cells transfected with *Mgat5* vector. Since the detection of endogenous FURIN by western blotting has been found to be particularly challenging, the detection was first optimized using 4 different FURIN antibodies: polyclonal H220, and monoclonal antibodies MON-139, MON-148, and MON-152. Of those 4 antibodies, MON-152 and H220 gave the most intense FURIN bands, and were thus used for secondary optimization. For that, we modified the western blot protocol by extending the blocking period to decrease unspecific antibody binding, and changed the secondary antibody dilutions from 1:5000 to 1:10 000 to decrease background staining. Of the two antibodies, polyclonal H220 FURIN antibody was chosen to be used for further FURIN detection. Results of these experiments can be seen in *figure 7*.

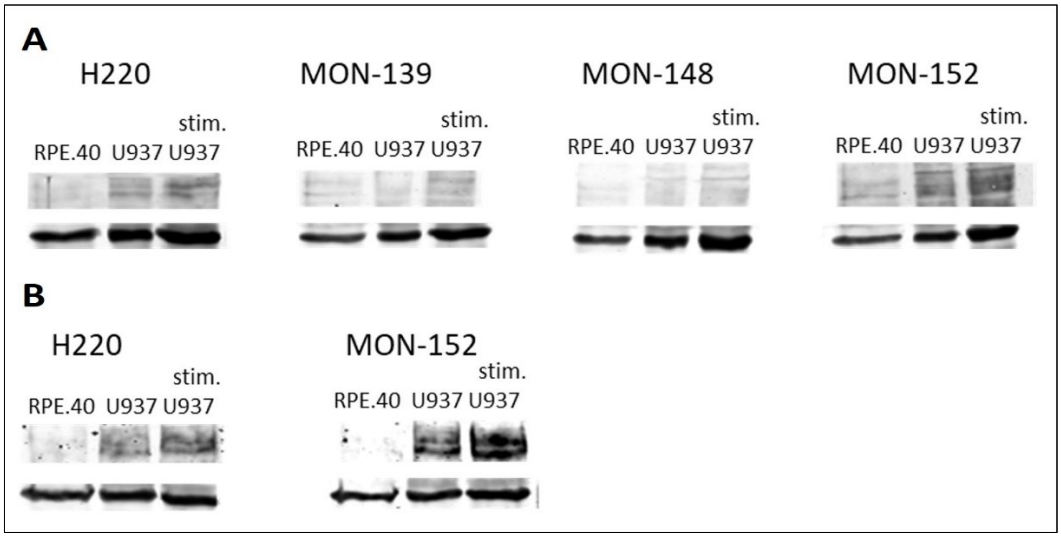

Figure 7: Optimization of western blot analysis for FURIN detection*. A) Blots resulting from first set of FURIN antibody optimization experiments are seen on top of the panel. B) Two blots below them were gained from further optimization with modified protocol. RPE.40 cell lysates were used as negative control, while unstimulated and stimulated U937 cells were used to produce large amount of FURIN, the latter noticeably more than the former. Actin, seen on the lower bands of each blot was used as loading control.*

To define the optimal conditions for transfections, we first tested 4 different FuGENE:DNA ratios using *Mgat5* vector. The western blot image, acquired from the ratio optimization experiment, is seen in *figure 8.* The optimization was done using same amount of DNA and different amounts of FuGENE reagent, the FuGENE:DNA ratio in the image decreasing from left to right. The uppermost band (white arrow) is assumed to represent the native hamster Gnt-V, as it isn´t affected by change of transfection conditions, while the rest of the bands below faint as the ratio decreases. The second band seen in blot (grey arrow) is likely to represent the transfected, non-cleaved, 90 kDa human Gnt-V, and we assume bands in the lower part of the blot (black arrows) are its digested fractions. Of different ratios, 6:1 showed the brightest bands and was used for further experiments.

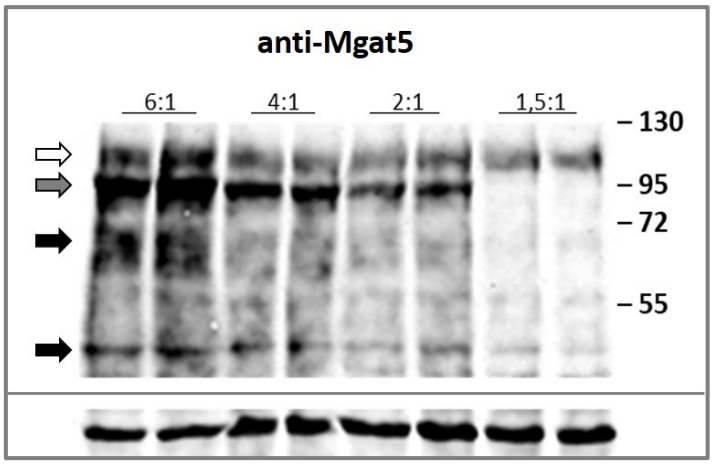

Figure 8: Optimization of transfection with pcDNA3.1-Flag-huMgat5-V5 plasmid. *Numbers above each of the two biological parallels represent FuGENE:DNA ratios used for transfections. Full-sized, 90 kDa Gnt-V bands are marked by grey arrow, and its approximately 65 kDa and 45 kDa fragments are marked by black arrows. Full-sized native hamster Gnt-V, sized approximately 110 kDa, is marked by white arrow. Actin, bands seen on the bottom of the blot, was used as loading control.*

To define the optimal amount of DNA to be transfected, 0 µg, 0.5 µg, 1.0 µg, and 5.0 µg of *Mgat5* vector were transfected into CHO-K1 cells and FURIN-deficient RPE.40 cells with 6:1 ratio of FuGENE and DNA. In addition, we wanted to see if CHO-K1 cell lysates produce specific bands, that wouldn´t be present in RPE.40 cells. These bands would indicate FURIN-mediated processing on Gnt-V. Due to excessive cell death, the cells with 5.0µg transfection were excluded from the blot. *Figure 9* shows cell lysates blotted using antibodies for tags V5 and Flag as well as the whole protein. Anti-V5, which binds to C-terminal tag, would show a C-terminal cleavage product, and anti-Flag, instead, the N-terminal product. Both would also show the full-sized protein. Specific bands using these antibodies are shown at approximately 90 kDa (grey arrows), and represent the whole Gnt-V protein. With the anti-Mgat5 antibody, also several additional specific bands, pointed with black arrows, were seen below 72 kDa marker, and two separate specific bands could be seen, with size of approximately 45 and 15 kDa. The latter two bands were also seen using anti-Flag antibody.

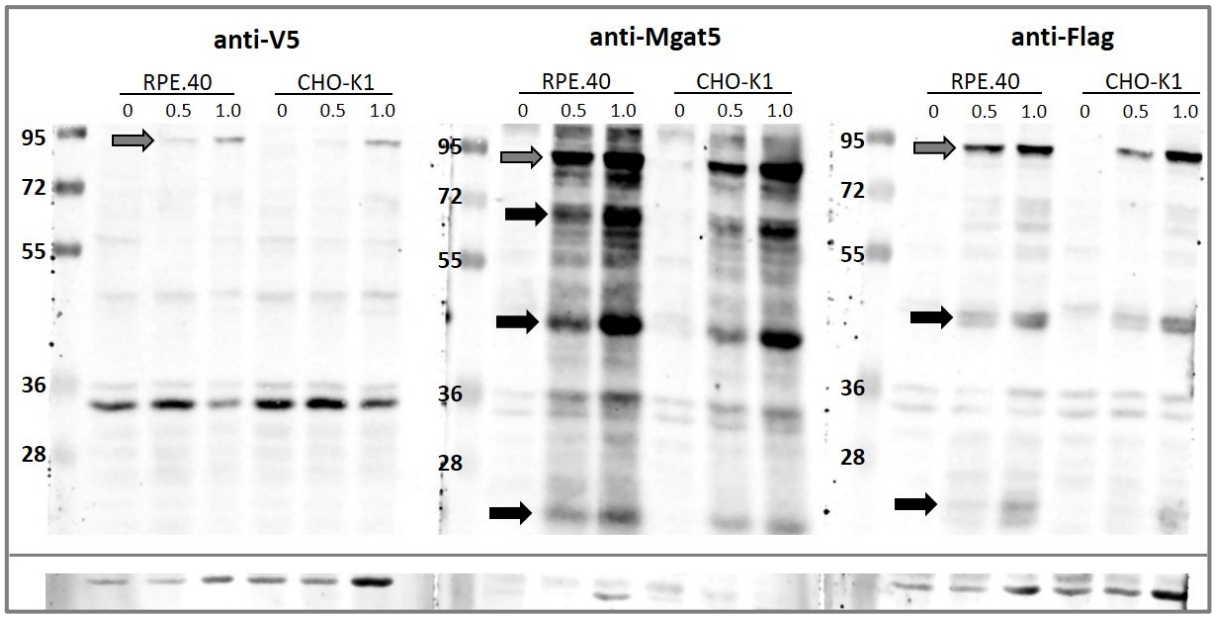

Figure 9: Comparison between RPE.40 and CHO-K1 cells transfected with 0, 0.5, and 1.0µg of pcDNA3.1-Flag-huMgat5-V5 plasmid. *Grey arrows show the whole Gnt-V protein sized 90 kDa, and black arrows show fragments of Gnt-V, sized approximately 70, 45, and 15 kDa. Actin, seen on the lower bands of each blot, was used as loading control.*

FURIN-deficient RPE.40 cells were transfected with *Mgat5*, and *Mgat5* with *FURIN*, as well as with *TGFβ*, and lysates were blotted using anti-Mgat5 and anti-V5 antibodies. If transfected human FURIN would have an effect on transfected Gnt-V protein, the band sizes would differ between cells transfected with *Mgat5* and *Mgat5* together with *FURIN*. We also wanted to see if there are C-terminal fractions that anti-Mgat5 can´t detect, or C-terminally truncated fractions not detectable with anti-V5. This would be seen in different band sizes between the anti-V5 and anti-Mgat5 blots. Results didn´t show any band size differences between these antibodies, but, instead, lysates with *Mgat5*, co-transfected with *FURIN*, showed higher band intensity at smaller bands, while those transfected with only *Mgat5* had higher band intensity at larger bands, as seen in *figure 10*. This indicated that FURIN might have an effect on Gnt-V processing into fragments.

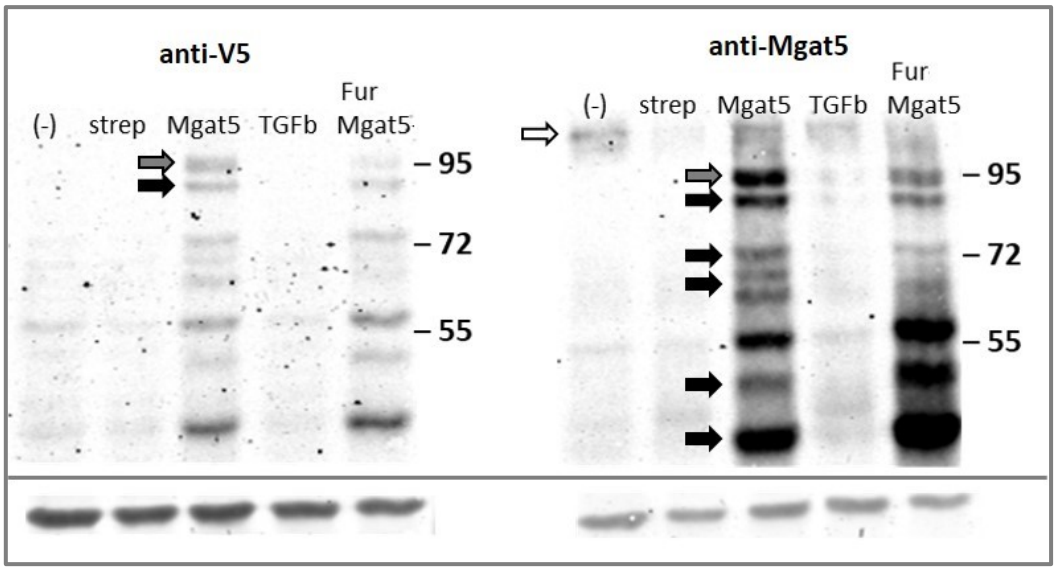

Figure 10: Lysates of transfected FURIN-deficient RPE.40 cells. *Anti-V5 and anti-Mgat5 blots show no difference in band size. Same bands appear in Mgat5 and Mgat5+FURIN transfected samples, with band intensities of the latter being higher in smaller fragments, and lower in larger bands. White arrow shows the native hamster Gnt-V, grey arrows the transfected protein, and black arrows the fragments of the protein. Actin, seen in the lower panel, was used as a loading control.*

To see if FURIN has some kind of a chaperon (Pavlaki et al. 2011) or a quantitative effect on production of Gnt-V protein, Jurkat T cell lines with stable transfections of mutated, proteolytically inactive FURIN (FurMUT), and WT FURIN (FurWT), as well as control line with *pcDNA3.1* vector (ctrl), were tested. If the amount of FURIN, either in enzymatically active, or in inactive form, would affect Gnt-V production or processing, this could be seen as difference in band intensity or fragment size between different cell lines. Instead, western blot of these cell line lysates in *figure 11* shows no clear differences between the cell lines in Gnt-V processing.

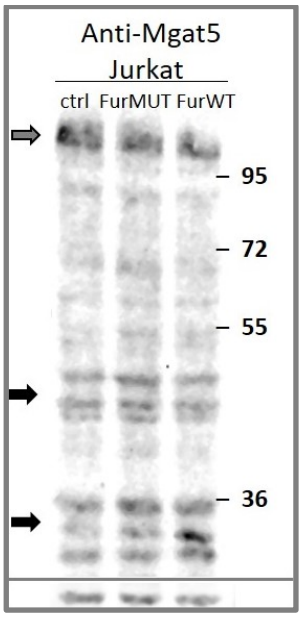

Figure 11: Western blot of Jurkat cell lines stable transfected with inactive mutant FURIN (FurMUT), wild type FURIN (FurWT), and control line (ctrl). *No clear differences can be seen in band size or intensity between Jurkat cell lysates with different FURIN constructs. Grey arrow shows the whole Gnt-V protein and black arrows show its fragments. Actin (shown as the lowermost band on blot) was used as loading control.*

5.4 FURIN does not influence the expression of *Mgat* family

The L-PHA binding of naïve CD4+ and CD8+ T cells, as well as memory CD4+ T cells, was significantly lower in Fur KO cells compared to Fur WT cells, and higher in naïve than in memory cells. This indicates that in Fur WT cells Gnt-V is more active than in Fur KO cells, and also more active in naïve than in memory cells. This could be the consequence of either increased gene expression, or by altered protein processing. To test the former idea, we used previously acquired RNA sequencing data (unpublished) to compare gene expression levels of Fur KO and WT CD8+62L+44^{low} naïve T cells, isolated from 8 mice in total. This comparison, represented in *figure 12*, didn´t show significant differences in mRNA levels of any *Mgat* family members.

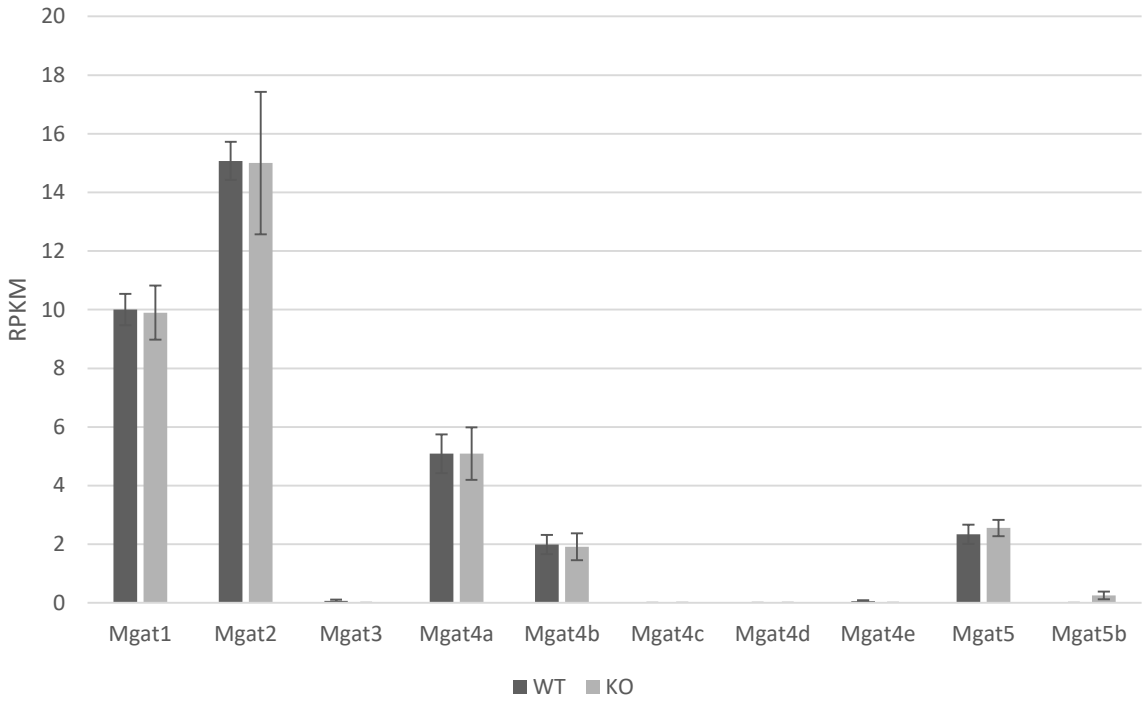

Figure 12: Comparison of gene expression levels of Mgat family in the naïve mouse CD8+62L+44l^{ow} Fur KO and WT T cells. *Figure shows the means of normalized number of reads of different members of Mgat family mRNA in mouse Fur KO and WT naïve CD8+ T cells. Error bars show standard deviation. RPKM = Reads Per Kilobase of transcript per Million mapped reads.*

6 DISCUSSION

The connection of Gnt-V-modified glycans and TCR activation in the context of autoimmunity has been previously studied. Production of *Mgat5* mRNA and Gnt-V-modified branched N-glycans is rapidly increased in activated T cells (Chen et al. 2009), and an insufficient production of these glycans is associated in the development of autoimmune diseases and T cell hyperreactivity (Demetriou et al 2001; Grigorian and Demetriou 2011). The Gnt-V-modified glycans limit lateral mobility of T cell surface proteins, decrease clustering of TCRs on immune synapse, and increase the activation threshold or naïve T cells (Demetriou et al. 2001).

Also, the role of proprotein convertase FURIN in T cell has been investigated, and it has been found to be vital for maintenance of immune tolerance, at least in part by activation of anti-inflammatory TGFβ (Pesu et al. 2008). FURIN is upregulated upon TCR signaling (Pesu et al. 2006), and has a role in T cell activation by regulating the activation of transcription of regulatory cytokine IL-2 (Ortutay et al. 2015).

Both FURIN and Gnt-V are upregulated upon T cell activation, have the same subcellular localization, and are vital for normal functions of T cells. Despite these similarities in T cell biology, their connection hasn´t previously been investigated. Because FURIN has a wide variety of target proteins, we wanted to study if Gnt-V is one of its targets, either being activated or inactivated by it.

L-PHA binds to Gnt-V-modified glycans on cell surface proteins. By using L-PHA with fluorescent FITC label, the amount of L-PHA binding, and further, the level of Gnt-V-processed cell surface N-glycosylations, can be detected. By using this label combined with markers defining different T cell subpopulations in FACS, we could define levels of Gnt-V activity in Fur KO and WT T cells, and, further, the effect of FURIN in Gnt-V activity. The L-PHA staining experiment showed that deletion of FURIN affects the expression of Gnt-V-processed branched N-glycans on the surface proteins of the mouse CD4+ T cells. The level of these N-glycosylations was significantly higher on naïve CD4+ and CD8+ WT T cells than in Fur KO cells, and, in contrast, lower in WT than Fur KO memory T cells. The level of N-glycosylation in naïve Fur KO T cells were closer to WT memory cell phenotype and was only slightly lower in Fur KO memory T cells. This phenomenon was seen in both CD4+ and CD8+ T cells. Due to these differences between the Fur KO and WT T cells, it was reasonable to presume that FURIN has either direct or indirect effect on the Gnt-V-mediated N-glycosylations on the

surface proteins. Compared to naïve cells, memory cells are more sensitive for antigenic stimulus and can be rapidly activated. Previously it has been seen that Fur KO T cells express pre-activated phenotype in their initial response to stimulus and IL-2 and IFNγ production, even though they are naïve in their CD62L expression (Ortutay et al. 2015). A high reactivity and lowered activation threshold are also seen in Gnt-V-deficient T cells (Demetriou et al. 2001). Due to the decrease in galectin-binding glycans and formation of galectin-glycoprotein lattices, TCRs have an increased mobility and tendency to clustering, as well as higher ratio of co-receptors transmitting activating and inhibitory signals. Because these features cause them to more readily activate, which makes them memory cell-like, and naïve FURIN-deficient, as well as WT memory T cells, were shown to have lowered Gnt-V activity, N-glycosylation by Gnt-V could be a part of the mechanism how memory cells are sensitized to antigenic stimulus.

The western blot analysis showed that FURIN is not required for proteolytic degradation of Gnt-V, although the blots showed increased band intensity on fractioned Gnt-V protein on cells transfected with both *FURIN* and *Mgat5* vectors compared with those transfected with only *Mgat5*, suggesting that ectopic FURIN enhances Gnt-V proteolysis. This was seen in both CHO-K1 line and FURIN-deficient RPE.40 line when cells were co-transfected with *Mgat5* and *FURIN* expression vectors. Also, during the optimization of transfection conditions in RPE.40 cells with *Mgat5* construct, it was seen that transfected human Gnt-V resulted as two clear bands below the native hamster Gnt-V band in western blot. Since non-transfected RPE.40 cells didn´t show more than just one band, it seems that native Gnt-V is not processed in these cells, even though transfected Gnt-V is, although it´s possible that used antibody can´t recognize the cleaved native Gnt-V. This doesn´t seem to be likely since according to sequence alignment there is high level of conservation (96.4%) between hamster and human Gnt-V. Similarly, in CHO-K1 cells, which express functional FURIN, no processed native Gnt-V was seen, while the same two clear bands appeared when transfected with *Mgat5* construct. Bands with increasing intensity while amount of transfected DNA increases could be due to cytotoxic effect on the cells: as more DNA is transfected, more of the cells die and break down their constituents; this could be, and indeed was, seen as multiple bands of different sizes. Western blots of Jurkat cell lysates showed no clear difference on band size or intensity between cells with WT FURIN construct, inactive FURIN construct, or control cells. This implies that at least in resting state T cells FURIN doesn´t have quantitative, or chaperon-like or other non-enzymatic effect on Gnt-V processing. This result can be applied only to resting T cells, and in further studies it could be interesting to see if stimulating Jurkat cells would produce different

outcome, as activation has previously been seen to affect *Mgat5* mRNA levels (Chen et al. 2009), as well as FURIN production (Pesu et al. 2008), in T cells.

Despite the optimization of FURIN detection, FURIN couldn´t be detected in transfected cell lysates. Therefore it can´t be reliably said that FURIN transfections have been successful, and that differences seen between *Mgat5*-transfected cells with and without FURIN are really the consequence of FURIN processing, although based on the β-galactosidase assay results overall transfections worked well in cell lysates that were used in western blot. The used cell lines may not produce enough FURIN in resting state for detection. Considering this difficulty, using cell lines that could be stimulated, or that inherently produce more FURIN would have been more useful choice.

Because Gnt-V has multiple predicted cleavage sites, one by FURIN and two by PCs in general, it´s possible that Gnt-V is processed by other PCs. For example, surface protein gp160 is normally FURIN-processed, but in FURIN-deficient RPE.40 cell line it is still efficiently processed into gp120 and gp41, by FURIN homolog PACE4 (Inocencio et al. 1997). Also, Gnt-V activity is known to be regulated by γ-secretase (Nakahara et al. 2006) and SPPL3 (Voss et al. 2014), that sheds it from Golgi membrane, causing it to be secreted out of the cell as soluble, enzymatically inactive form. As the regulation mechanisms of processed (and processing) proteins differ between cell types, it´s possible that in mouse T cells FURIN has an effect on activity of SPPL3. Also γ-secretase might be target of FURIN-mediated regulation, as it is known to regulate α-secretase activity (Hwang et al. 2006). This could be indirectly affecting the cleavage activity on Gnt-V, and further, changing the glycosylation pattern on cell surface proteins, as seen between naïve and memory T cells, as well as between FURIN-deficient and WT mouse primary T cells. To study this further, one could analyse the secretome of primary T cells or cultured cell lines with and without functional FURIN, and also before and after activation. In addition to this, SPPL3 and γ-secretase should be immunoblotted from same cells.

Gnt-III activity is antagonistic to Gnt-V activity (Zhao et al. 2006), and it´s upregulation could explain how production of Gnt-V-modified glycans might be regulated. RNA sequencing analysis on Fur KO and WT naïve CD8+ T cells suggested that FURIN doesn´t affect expression of *Mgat5*, or other genes in *Mgat* family, at mRNA level. This analysis was done only with naïve CD8+ T cell population, and even though they seemed to have a similar connection in Gnt-V activity compared to CD4+ T cells in lectin staining experiment, this finding about non-affected mRNA levels doesn´t necessarily apply for other T cell states or

subpopulations. It´s also possible that the activity and lifetime of these glycosylation enzymes can be modulated during or after translation, which wouldn´t be seen in their mRNA levels.

Mgat5 mRNA has a short half-life and it is assumed to be rapidly up- and downregulated upon TCR signaling (Chen et al. 2009). Half-life has been shown to be prolonged by the stabilizing effect of TGFβ (Miyoshi et al. 1995), which is cleaved and activated by FURIN. A decreased stability of Gnt-V mRNA in the absence of biologically active TGFβ could thus be one mechanism for FURIN-mediated regulation of Gnt-V activity. In addition to this, IL-2 increases *Mgat5* expression in activated, but not in resting, T cells (Grigorian et al. 2012a). Upon TCR activation, FURIN induces upregulation of IL-2, and activation of transcription factor AP-1 (Ortutay et al. 2015). TCR activation is also followed by Ras/Raf/Ets signaling, involved in upregulation of IL-2 (Avots et al. 1997) and *Mgat5* transcription (Chen et al. 2009). FURIN-mediated activation of transcription factors AP-1 and Ets, which are known to bind to *Mgat5* promoter and activate *Mgat5* gene expression (Chen et al. 1998; Ko et al. 1999), could be part of the molecular mechanisms how FURIN affects the Gnt-V function in T cells.

7 CONCLUSION

This study investigated the effect of proprotein convertase FURIN on N-glycosyltransferase Gnt-V gene expression, protein processing, and the level of Gnt-V-processed glycosylations on cell surface proteins. While the gene expression was shown to not be affected, ectopic FURIN seemed to enhance Gnt-V protein processing in hamster cells, although in Jurkat cells results were inconclusive. Instead, FURIN had a significant effect on Gnt-V mediated N-glycosylation on membrane proteins, suggesting an indirect role in Gnt-V activity.

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