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Novel models to study protein trafficking in rare genetic defects of severe congenital neutropenia

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Novel models to study protein trafficking in rare genetic defects of severe congenital neutropenia

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List of abbreviations

AP3B1	adaptor protein 3 complex
AD	autosomal dominant
AD-HIES	autosomal dominant Hyper IgE syndrome
AIN	autoimmune neutropenia
AmpR	ampicillin resistance gene
AR	autosomal recessive
BEN	benign ethnic neutropenia
BPI	bactericidal-increasing protein
BSA	bovine serum albumin
CGD	chronic granulomatous disease
CHS	Chediak–Higashi syndrome
CIN	chronic idiopathic neutropenia
CMV	human cytomegalovirus
COPI	coat protein complex I
COPII	coat protein complex II
DMEM	Dulbecco's modified Eeagle's medium
EGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
ERGIC	ER-Golgi-intermediate compartment
FCS	foetal calf serum
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
G6PD	glucose-6-phosphate dehydrogenase
HeLa	human cervical epitheloid carcinoma
HRP	horseradish peroxidase
HSCT	hematopoietic stem cell transplantation
ICAM	intercellular adhesion molecule
iPSc	induced pluripotent stem cells
IRES	internal ribosomal entry site
JAGN1	jagunal homolog 1
KO	knockout
LAD	leukocyte adhesion deficiency
LyzC	lysozyme C
M6P	mannose 6-phosphte
MAC1	macrophage-1 antigen
MCS	multiple cloning site
Mmp	matrix metalloprotease

MPO	myeloperoxidase
MPR	mannose 6-phosphte receptor
MS	mass spectrometry
NADPH	nicotinamide adenine dinucleotide phosphate
NE	neutrophil elastase
NETs	neutrophil extracellular traps
ori	replication origin
PBS	phosphate buffered salin
PFA	paraformaldehyde
PMA	phorbol myristate acetate
RUSH	retention using selective hooks
SBP	streptavidin-binding protein
SCN	severe congenital neutropenia
SDS	sodium dodecyl sulfate
SGD	neutrophil-specific granule deficiency
TBS	tris-buffered saline
TE	trypsin EDTA
TGN	trans-Golgi network
VPS13B	vacuolar protein sorting 13 homolog B
WAS	Wiskott-Aldrich syndrome
WT	wild type
XL	X-linked

1. Zusammenfassung

Mutationen im Gen *JAGN1*, das für das Protein jagunal homolog 1 (JAGN1) kodiert, verursachen eine schwere kongenitale Neutropenie (SCN). Patienten mit SCN leiden an lebensbedrohlichen Infektionen und weisen ein erhöhtes Leukämierisiko auf. Neutrophile Granulozyten von Patienten mit Mutationen in *JAGN1* zeigen einen Mangel an Granula, eine abnormale N-Glykosilierung von Proteinen und einen Sekretionsdefekt. Das Protein JAGN1 ist im endoplasmatischen Retikulum lokalisiert und ist durch seine Interaktion mit dem COPI-Komplex vermutlich an der Protein-Sekretion beteiligt. Die exakte Rolle von JAGN1 wurde bis heute nicht geklärt.

In dieser Arbeit wird die Rolle von JAGN1 beim Transport von Proteinen analysiert. Zuerst wurden Proteine identifiziert, die in Abwesenheit von JAGN1 unterschiedlich sezerniert werden. Dafür wurden von induzierten, pluripotenten Stammzellen (iPSc), die in vitro in bona fide neutrophile Granulozyten differenziert wurden, mittels Massenspektrometrie die Sekretome von Kontrollen und JAGN1-defizienten Zellen bestimmt. Anschließend wurde ein *JAGN1*-Knock-Out (KO) in HeLa-Zellen durchgeführt, um den intrazellulären Proteintransport zu untersuchen. Zur Visualisierung der Kinetik der Proteinsekretion verwendeten wir das Retention-Using-Selective-Hooks-System. Als Reporter dafür haben wir das Granulaprotein Lysozym C ausgewählt.

Wir konnten zeigen, dass lysosomale Hydrolasen und Granulaproteine, wie Myeloperoxidase oder Cathepsin D, in von *JAGN1*-KO iPSc abgeleiteten neutrophilen Zellen im Vergleich zu Kontrollklonen signifikant ($p<0,05$) häufiger sezerniert wurden. Interessanterweise waren in den *JAGN1*-KO-Klonen Histone signifikant ($p<0,05$) weniger im zellulären Überstand vorhanden, was möglicherweise mit einer gestörten NETosis (ein spezieller Mechanismus neutrophiler Granulozyten zur Freisetzung von Chromatin-Netzen) von JAGN1-defizienten Neutrophilen zusammenhängt. Der CRISPR/Cas9-vermittelte KO in HeLa-Zellen wurde effizient durchgeführt und durch Gelelektrophorese und Sequenzierung bestätigt. Darüber hinaus war die Sekretion des Proteins Lysozym C in HeLa JAGN1-defizienten Zellen im Vergleich zu HeLa-Kontrollzellen nach 40 Minuten ($p<0,01$) und 60 Minuten ($p<0,05$) signifikant verzögert.

Es konnte hiermit das erste Mal gezeigt werden, dass JAGN1 zu einem spezifischen Sekretionsdefekt in neutrophilen Granulozyten führt. JAGN1 beeinflusst die Kinetik der Sekretion von Lysozym C. Diese Beziehung zwischen Mutationen in *JAGN1* und dem Proteintransport eröffnet neue Wege zum besseren Verständnis von Krankheiten neutrophiler Granulozyten, wie der SCN.

1. Abstract

Mutations in the gene *JAGN1* encoding for the protein jagunal homolog 1 (JAGN1) cause severe congenital neutropenia (SCN). Patients with SCN suffer from life-threatening infections and have a predisposition to develop leukaemia. Neutrophil granulocytes from patients with mutations in *JAGN1* exhibit a paucity of granules, aberrant N-glycosylation of proteins and a secretion defect. The protein JAGN1 is located in the endoplasmic reticulum and is assumed to be involved in the secretory pathway by its interaction with the coat protein I complex (COP1). However, the detailed mechanism of action of how *JAGN1* participates in the secretory pathway has not been explained so far.

In this thesis, the role of JAGN1 in protein trafficking was analysed in greater detail. First, it was aimed to identify proteins that are differently secreted in the absence of JAGN1. To this end, differences in secreted proteins in *JAGN1* knockout induced pluripotent stem cell (iPSc) derived neutrophil-like cells were analysed using mass-spectrometry. Then, a *JAGN1* knockout (KO) in HeLa cells was performed to study intracellular protein trafficking. To monitor the kinetics of protein secretion the retention-using-selective hooks system was used. As a reporter the granule protein lysozyme C was chosen.

It was observed that lysosomal hydrolases and granule proteins, such as myeloperoxidase or cathepsin D were significantly ($p<0.05$) more secreted in JAGN1-deficient iPSc-derived neutrophil granulocytes compared to control clones. Interestingly, histones were significantly ($p<0.05$) less secreted in the *JAGN1* KO clones, which possibly is related to less efficient NETosis of JAGN1-deficient neutrophils. Additional studies in newly generated CRISPR/Cas9 mediated KO in HeLa cells showed that the secretion of the granule protein lysozyme C was significantly delayed compared to HeLa control cells after 40 minutes ($p<0.01$) and 60 minutes ($p<0.05$).

In summary, secretion of specific granule proteins, lysosomal hydrolases and histones is impaired in the absence of JAGN1. Thus JAGN1-deficiency is associated with a specific secretion defect. JAGN1 also influences the kinetics of the secretion of lysozyme C. These results provide new insights into functional defects of neutrophil granulocytes in SCN and may open new horizons for studies aiming to decipher the molecular machinery orchestrating protein trafficking.

2. Introduction

2.1. Neutrophil granulocytes

Neutrophil granulocytes are the most abundant white blood cells in the circulation. Representing approximately 50–70% of circulating leukocytes in humans (Mestas and Hughes 2004). The nucleus of mature neutrophils is segmented and their cytoplasm is enriched with granules and secretory vesicles. Neutrophils play a major role in host defence of the innate immune system (Borregaard 2010).

2.1.1. Origin and maturation of neutrophil granulocytes

Maturation of neutrophils takes place in the bone marrow where they are continuously generated from myeloid precursors. During this process of maturation, committed precursors of neutrophil granulocytes pass through several stages, namely myeloblast, promyelocyte, myelocyte, metamyelocyte, and band cell before they become polymorphonuclear (segmented) cells. The daily amount of neutrophil production in the bone marrow can culminate in up to 2×10^{11} cells (Borregaard 2010). The differentiation of neutrophils is controlled by transcription factors such as CCAAT/enhancer-binding protein α–ζ and PU.1 (Nerlov and Graf 1998, Dahl, Walsh et al. 2003). Granulocyte colony stimulating factor (G-CSF) is a critical cytokine driving granulocyte development, in particular in stress hematopoiesis (Lieschke, Grail et al. 1994). G-CSF production is regulated by an interaction of regulatory T cells, macrophages and dendritic cells involving several interleukins (e.g. IL-17A, IL-23) (Ley, Smith et al. 2006, Kolaczkowska and Kubes 2013). Peripheral neutrophils have a lifespan of approximately 5 days in circulation (Amulic, Cazalet et al. 2012).

2.1.2. Function of neutrophil granulocytes

Neutrophil granulocytes play a major role in the innate immune system and are essential to defend against bacterial and fungal pathogens (Kolaczkowska and Kubes 2013). To fulfil their function in host defence and returning tissue to a sterile state neutrophils can eliminate pathogens by various ways such as phagocytosis, release of granule proteins or release of neutrophil extracellular traps (NETs) (figure 1) (Borregaard and Cowland 1997, Brinkmann and Zychlinsky 2007, Khandagale, Lazzaretto et al. 2018).

Neutrophil recruitement

The recruitement of neutrophils to the site of infection is efficiently orchestrated in several steps: tethering, rolling, adhesion, crawling and transmigration (Kolaczkowska and Kubes 2013).

Initially tissue-resident leukocytes release inflammatory mediators, when they come into contact with pathogens. This triggers subtile changes in surface adhesion molecules on endothelial cells at the site of infection (Ley, Laudanna et al. 2007, Phillipson and Kubes 2011, Sadik, Kim et al. 2011). This activation of the endothelium can also be stimulated directly by pattern-recognition receptor-mediated detection of pathogens. It cumulates to an increased expression of adhesion molecules such as P-selectin and E-selectin (Ley, Laudanna et al. 2007, Petri, Phillipson et al. 2008). These two selectins maximize neutrophil recruitment by binding to their glyocosylated ligands, leading to tethering and rolling of neutrophils to the surface of endothelium (Ley, Laudanna et al. 2007, Zarbock, Ley et al. 2011). Because of the high shear stress in the vascular system, the formation and breakage of adhesive bonds is highly dynamic during the rolling of neutrophils (Ramachandran, Williams et al. 2004, Sundd, Pospieszalska et al. 2011, Sundd, Gutierrez et al. 2012).

The rolling of neutrophils enhances more intense exposure to activatin factors such as tumour necrosis factor-α and IL-1β and chemokines (such as IL-8) on the endothelial cells bearing activation of neutrophils (Summers, Rankin et al. 2010, Sadik, Kim et al. 2011, Sanz and Kubes 2012).

Chemokines are positively chareged molecules, which are bound to negatively charged heparan sulfates on the endothelium (Massena, Christoffersson et al. 2010). The sequestration of these chemokines formates an intravascular chemotactic gradient. The G-protein-coupled chemokine receptors on the surface of neutrophils are activated by chemokines and consequently lead to changes in the conformation of cell surface-expressed integrins, such as lymphocyte function-associated antigen 1 and macrophage-1 antigen (MAC1). After their conformational change these integrins bind to endothelial adhesion molecules, such as intercellular adhesion molecule (ICAM)1 and ICAM2 (Phillipson, Heit et al. 2006), inducing signaling pathways inside the neutrophil for adhesion and cell motility (Cicchetti, Allen et al. 2002, Ley, Laudanna et al. 2007).

After the firm adhesion of neutrophils to the endothelial cells, mature neutrophils must leave the blood circulation and transmigrate into the tissue. Therefor, neutrophils use endothelial cell–cell junctions, to which they actively crawl. Crawling is described in firmly adherent neutrophils as scanning the endothelial surface for the shortes distance to an endothelial junction. Neutrophils elongate perpendicularly und use their pseudopods in this process which is depending on the interaction of ICAM1 with neutrophil-expressed MAC1 (Phillipson, Heit et al. 2006).

The transmigration of neutrophils describes how neutrophils cross the endothelium and the basement membrane to reach the infected tissue (Ley, Laudanna et al. 2007). For this purpose neutrophils can cross the barrier either paracellularly or transcellularly. There is evidence that the paracellulary way is prefered by neutrophils due to its higher efficiency (Phillipson, Heit et al. 2006, Petri, Phillipson et al. 2008, Phillipson, Kaur et al. 2008, Woodfin, Voisin et al. 2011). The paracellular transmigration is a highly complex process which includes the release of junctional intercellular bonds between the endothelial cells, conformational changes of the cytoskeleton and rearrangement of the attachment to the extracellular matrix (Parsons, Sharma et al. 2012). Finaly, the neutrphils emigrate through the basement membrane using proteases such as matrix metalloproteinases (Mmps) or serine proteases (Kolaczkowska, Grzybek et al. 2009).
After the neutrophils have passed the endothelial barrier a subluminal chemokine gradient guides them to the infected tissue. Chemoattractand molecules, such as bacteria-derived N-formyl-methionyl-leucyl-phenylalanine or the complement component C5a (Foxman, Campbell et al. 1997) lead to the directional migration of neutrophils to the point of infection. It has been shown that chemotaxis is orchestrated by a several intracellular signalling pathways, including phosphatidylinositol 3-kinase signalling (Funamoto, Meili et al. 2002) and p38 mitogen-activated protein kinase (Heit, Robbins et al. 2008).

Phagocytosis

The major mechanism to remove pathogens, phagocytosis, was discovered by Elie Metchnikoff in the 19th century (Klein 2016). During phagocytosis the pathogen is internalized by the cell membrane into a vacuole, called the phagosome. The internalization depends on the interaction between the neutrophil and the pathogen mediated through recognition of pathogen-associated molecular patterns by pattern-recognition receptors, or opsonin mediated (Underhill and Ozinsky 2002). After encapsulation of the pathogens, preformed granules fuse with the phagosome to add antimicrobial molecules, such as cathepsins, lysozyme and defensins. Additionally nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent production of reactive oxygen species generates an acid and consequently toxic milieu for the pathogen (Lee, Harrison et al. 2003, Borregaard 2010, Hager, Cowland et al. 2010) (Kolaczkowska and Kubes 2013). Some pathogens like *Staphylococcus aureus, Helicobacter pylori, Francisella tularensis* and others bear strategies to survive inside neutrophils through interfering with engulfment, changing phagosome maturation or creating a more convenient intraphagosomal environment (Amulic, Cazalet et al. 2012).

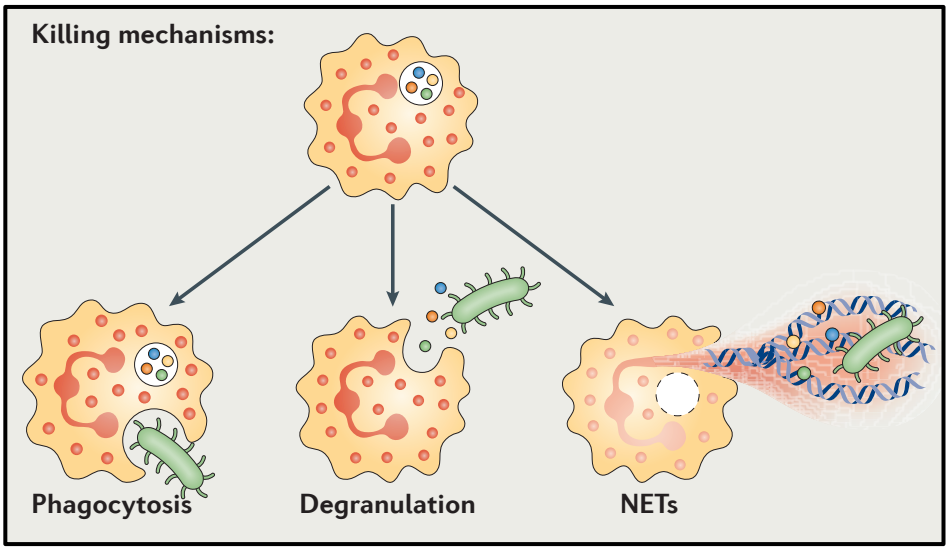


Figure 1: Killing mechanisms of neutrophils*. Neutrophils have several strategies to kill pathogens including phagocytosis, release of antimicrobial granule proteins or release of neutrophil extracellular traps (NETs)* (from Kolaczkowska E. et al., Nature Reviews Immunology, 2013).

Granules and secretory vesicles

In neutrophils a special storage organelle has been evolved: the granule. Neutrophils carry granules that are essential for their ability to fulfil their major role in innate immunity. Granules are packed with antimicrobial proteins. When these proteins are released to the phagosome (intracellular) or across the cell membrane (extracellular), they contribute to the killing of pathogens.

Even if neutrophil granules are highly heterogeneous regarding their content, they are traditionally classified into three types of granules (table 1): azurophilic (primary), specific (secondary) and gelatinase granules (tertiary).

Granules appear during the transition from the myeloblast stage of differentiation to the promyelocyte stage and continue to be formed until the segmented stage of the neutrophil (Borregaard 2010, Hager, Cowland et al. 2010, Galli, Borregaard et al. 2011, Kolaczkowska and Kubes 2013). It is not clear whether mature neutrophils maintain a capacity to produce granules, but most mRNAs for granule proteins are absent from peripheral neutrophils (Cowland and Borregaard 1999). It is also still not clear why different subtypes of granules exist. Cowland et al. hypothesized that some of the proteins can not exist together in one granule (Cowland and Borregaard 2016).

Due to their staining with the basic dye azure A in histological preparations granules were originally classified into peroxidase-positive (azurophilic) and peroxidase-negative (specific) granules (Bainton, Ullyot et al. 1971). Azurophilic granules (primary granules) are defined by the presence of myeloperoxidase (MPO), which is not found in other granules. Furthermore, only azurophilic granules contain the protease azurocidin, (Faurschou, Sorensen et al. 2002). Apart from MPO and azurocidin, proteins like serine proteases, proteinase 3, cathepsin G, neutrophil elastase (NE), neutrophil serine protease 4, defensins, bactericidal-increasing protein (BPI) and lysozyme are major cargos of primary granules (Ganz, Selsted et al. 1985, Pereira, Shafer et al. 1990, Perera, Wiesmuller et al. 2013, Cowland and Borregaard 2016). Primary granules are the largest granules (0.3 µM diameter) that can be visualized in neutrophils (Amulic, Cazalet et al. 2012).
Traditionally the peroxidase-negative granules are subdivided into secondary and tertiary granules. The secondary granules are also called specific granules and are smaller than primary granules, measuring approximately 0.1 µM in diameter. Secondary granules contain lactoferrin, lysozyme, pentraxin 3 and others. Cytochrome b558, that is crucial for the initiation of the NADPH oxidase machinery is also content of this subtype (Ramadass and Catz 2016).
The most important protein in tertiary granules (also called gelatinase granules) is Mmp 9 (also known as gelatinase B), which helps degrading the matrix during the process of neutrophil recruitment. Even though ultrastructure and more recently biochemical evidence provided a framework to categorize subset of granuloes in neutrophil granulocytes, there is an ongoing debate on the best classification of granules (Kjeldsen, Bainton et al. 1993, Borregaard 2010, Amulic, Cazalet et al. 2012, Cowland and Borregaard 2016).

In addition ot primary, secondary and tertiary granules, neutrophils also carry a fourth set of membranous structures: the secretory vesicles. In contrast to granules, secretory vesicles do not bud from the Golgi apparatus, but are generated by endocytosis during the final stages of neutrophil maturation (Borregaard, Sorensen et al. 2007). Accordingly, secretory vesicles contain predominantly plasma-derived proteins such as albumin. Proteins required for cell adhesion and migration, like β2 integrins, are rapidly transported to the cell surface of neutrophils (Amulic, Cazalet et al. 2012).

The granules are an essential reservoir in the killing mechanisms of pathogens (Amulic, Cazalet et al. 2012, Ramadass and Catz 2016). Antimicrobial proteins can be classified into three different types: a) cationic peptides and proteins that attach to microbial membranes, b) proteins that deprive microorganisms of essential nutrients and c) proteins with catalytic function (enzymes). Brogden and colleagues listed over 800 peptides that have antimicrobial functions. Most peptides are charged to facilitate their

primary interaction with microbial membranes. These peptides are thought to punch pores into the membrane or to disturb essential mechanisms such as replication or metabolism (Amulic, Cazalet et al. 2012).

Table 1: Neutrophil granules.

Primary Granules (azurophilic)	Secondary Granules (specific)	Tertiary Granules (gelatinase)
Lysozyme	Lysozyme	Lysozyme
Myeloperoxidase	Lactoferrin	Gelatinase
Elastse	Collagenase	Arginase-1
BPI	Pentraxin 3	…
Defensins	Defensin	
…	…	

Main differences in the content of the three subtypes of neutrophil granules. BPI: bactericidal-increasing protein (adapted from Amulic, 2012).

The second class of neutrophil antimicrobials acts by withdrawing nutrients from microbes, e.g. by chelating essential metals. Lactoferrin and calprotectin are examples for this mode of action (Weinberg 1975). Another method to fight against microbes is to use proteolytic enzymes such as neutrophil elastase (NE), cathepsin G, BPI or lysozyme. Lysozyme is one of the major enzymes to destroy the wall of pathogens. It has been shown that lysozyme is essential in the host defence in mice and rats (Markart, Korfhagen et al. 2004, Bu, Wang et al. 2006, Nash, Ballard et al. 2006). Lysozyme is generated throughout granulopoiesis from promyelocytes to band cells and is found in all classes of granules (Cowland and Borregaard 2016).
All these proteins are released from neutrophil granules into the extracellular milieu or into phagosomes.
Although there is accumulated evidence about the antimicrobial function of these proteins it is still challenging to clarify how all these factors interact *in vivo* to fulfil their combined role in host defence.

The degranulation of neutrophil granules is assumed to be triggered in different stages during the activation of neutrophils. The different subtypes of granules are mobilized in a hierarchical manner. Secretory vesicles seem to have the fastest kinetics followed by tertiary, specific granules and azurophilic granules.
Secretory vesicles are mobilized through selectins and chemokines during various steps of chemotaxis. Fusion of the secretory vesicles with the plasma membrane exposes key

factors (such as β2 integrins) to the surrounding environment for further activation of the neutrophils. Firm adhesion, mediated by β2 integrin mediated signaling, is followed by further activation to initiate the migration through the endothelium. Therefore, specific granules are mobilized to expose their enzymes such as Mmp 9 to degrade the extracellular matrix and helping neutrophils to traverse the basement membrane. The entire activation of neutrophils is provoked at the inflammatory site and leads to mobilization of azurophilic and specific granules to release their antimicrobial content to the phagosome or to the environment. The degranulation of all these granules eventually results in a highly toxic environment for the invading pathogens (Amulic, Cazalet et al. 2012).

Neutrophil extracellular traps

In addition to phagocytosis and granule proteins, neutrophil granulocytes exhibit a third mechanism to eliminate pathogens, the neutrophil extracellular traps (NETs). The NETs are a composition of uncondensed chromatin combined with histones and proteins. Enzymes, such as cathepsins and MPO, are released from neutrophil granules and attach to the core DNA. The fibrous NETs are spilled out over pathogens, when neutrophil granulocytes undergo an active form of cell death, so called NETosis (Brinkmann, Reichard et al. 2004, Brinkmann and Zychlinsky 2007, Fuchs, Abed et al. 2007). Recently however, the idea has been proposed that NETosis may not always be associated with cell death (Yipp, Petri et al. 2012).

The NETs not only support other killing strategies for pathogens through immobilization of the microorganisms, but also kill directly pathogens by antimicrobial histones and proteases (Hirsch 1958, Park, Yi et al. 2000, Kolaczkowska and Kubes 2013). Recent evidence emerges that NETosis is not only involved in intensifying the immune responses but also in inducing the coagulation system (Amulic, Cazalet et al. 2012, Sollberger, Tilley et al. 2018).

The pathways that lead to NETosis are not completely understood but studies indicate that the reactive oxygen pathway is involved, as the pharmacological agent, phorbol 12-myristate 13-acetate (PMA), which is a potent activator of the NADPH oxidase, can induce NET formation (Fuchs, Abed et al. 2007, Amulic, Cazalet et al. 2012, Metzler, Goosmann et al. 2014). Additional evidence suggests that MPO and NE are also necessary in NET formation (Papayannopoulos, Metzler et al. 2010, Metzler, Fuchs et al. 2011). A recent study also highlights the role of jagunal homolog (JAGN1) for fungal killing with NETs (Khandagale, Lazzaretto et al. 2018).

Hence, neutrophils are well equipped with a complex machinery for host defence that can be also toxic also for healthy tissue. It is obvious that defects in neutrophils can easily

disturb the balancing act between killing of invading pathogens and non-pathogenic conditions, such as autoimmune diseases (Thieblemont, Wright et al. 2016).

2.2. Rare genetic diseases of neutrophils as model system for cell biology

Conceptually, genetic disorders of neutrophils can be subclassified into four (partially overlapping) groups: a) disorders affecting neutrophil migration and chemotaxis, b) disorders affecting neutrophil granules, c) disorders affecting neutrophil killing and d), disorders associated with defective neutrophil differentiation. The focus will be on the last group, especially on severe congenital neutropenia (SCN).

2.2.1. Disorders of neutrophil chemotaxis

Disorders regarding neutrophil chemotaxis comprise three subtypes of leukocyte adhesion deficiency (LAD) and the autosomal dominant Hyper IgE syndrome (AD-HIES).
LAD-I is a syndrome with an autosomal recessive mode of inheritance that results from defects in CD18, the common β chain of the β2 integrin family. Clinical manifestations include early onset of bacterial infections of soft tissue, poor wound healing, periodontal disease, omphalitis, and neutrophilia (increased numbers of neutrophil granulocytes in the peripheral blood).
LAD-II also follows an autosomal recessive inheritance pattern and is caused by mutations in the guanosine diphosphate fucose transporter gene (*SLC35C1*) resulting in aberrant fucosylation of the neutrophil surface that impairs the rolling of neutrophils. Individuals suffering from LAD-II present with an increased susceptibility of infections, intellectual disability, abnormal facies, microcephaly, and cortical atrophy as well as the rare Bombay blood phenotype.
The third subtype of LAD (LAD-III, equally autosomal recessive) results from from mutations in *FERMT3*. The corresponding protein, named kindlin 3, is an adaptor that binds the integrin cytoplasmic tail to activate integrin-mediated signaling. The clinical phenotype resembles that of mild LAD-I. In contrast to LAD-I, the major source of morbidity is increased bleeding severity due to impaired platelet aggregation.
Likewise, monoallelic mutations in STAT3 which are associated with Hyper-IgE-syndrome lead to a defect in neutrophil chemotaxis. The syndrome is a multi-system disorder including elevated serum levels of IgE, recurrent pulmonary and cutaneous bacterial and fungal infections, pneumatoceles, dermatitis, and skeletal as well as dental abnormalities. In addition to impaired chemotaxis of neutrophils numerous other immune-defects, such as loss in production of Th17 cells, are observed (Leiding 2017).

2.2.2. Disorders of neutrophil killing

Chronic granulomatous disease (CGD) resulting from functional defects in the NADPH oxidase, a protein complex comprised of five subunits. Thus, killing of microbes is directly affected in these patients. Children before the age five suffer from recurrent infections in the lungs, on the skin, in lymph nodes and in the liver. Of note, the clinical phenotype is not restricted to immunodeficiency but also involves dysregulation of inflammatory response, resulting in granuloma formation and inflammatory bowel disease. Five genes, each affecting a subunit of the NADPH oxidase system, have been linked to CGD: CYBA, NCF1, NCF2, NCF4 and CYBB. The prevalence of CGD is approximately 1:200,000 (Winkelstein, Marino et al. 2000) and the inheritence of CGD is AR, besides the most common mutations in CYBB, which have been described as X-linked (Leiding 2017). Some subunits of the NADPH oxidase system are membrane bound ($gp91^{phox}$ and $p22^{phox}$) and others are located in the cytoplasm($p47^{phox}$, $p67^{phox}$, and $p40^{phox}$). Not before intracellular ingestion of microbes, the components in the different compartements come together in an oxidative burst leading to activation of the NADPH oxidase. Consequently superoxide is produced and directly kills the microbes (Leiding and Holland 1993). In patients suffering from CGD the reduced superoxide production can be directly measured using the dihydrorhodamine assay (Kuhns, Alvord et al. 2010).

Similar symptoms as in CGD have been described in some patients with mutations in glucose-6-phosphate dehydrogenase (G6PD). This enzyme participates in a shunt pathway in forming NADPH. The major symptom of patients with G6PD deficiency is red cell haemolysis resulting from oxidative stress.
MPO deficiency also affects the neutrophil killing because of mutations in the gene, encoding for MPO. Although it has been shown, that neutrophil killing was impaired in vitro, patients with MPO deficiency present with no specific clinical symptoms (Leiding 2017) and therefore MPO deficiency has been eliminated from the list of primary immunodeficiency diseases (Picard, Bobby Gaspar et al. 2018).

2.2.3. Disorders of neutrophil granules

While there is certainly an overlap to defects of differentiation, at least two rare genetic diseases are primarily characterized by a defect of neutrophil granules: Chediak–Higashi syndrome (CHS) and specific granule deficiency.
CHS is a complex syndrome, inherited in an autosomal recessive mode, that associates (partial) albinism, central neurologic symptoms and progressive white matter changes,

increased bleeding tendency and immunodeficiency with a high risk of bacterial infections. The defects, mostly caused by mutations in the gene *LYST,* lead to disturbances in granule morphogenesis, such as giant azurophilic granules with delayed and insufficient degranulation. The giant granules are derived from azurophilic granules (Kjeldsen, Calafat et al. 1998). Also the platelets show aberrant morphology. Complications in CHS patients include the lymphoproliferative infiltration of the bone marrow and other reticuloendothelial system organs (Karim, Suzuki et al. 2002, Schaffer and Klein 2007).

Neutrophil-specific granule deficiency (SGD) is a rare AR disorder that affects the terminal differentiation of neutrophial granulocytes and lactoferrin production (Lomax, Gallin et al. 1989). As a consequently, specific granules are scarce, and occasionally patients present with neutropenia. Bilobed nuclei can be seen in peripheral blood smears. The disease is caused by biallelic mutations in *CEBPE,* encoding the transcription factor CCAT/enhancer binding protein ε (Gombart and Koeffler 2002). Patients with SGD have a higher risk to severe invasive pyogenic infections and present mostly in the first few years with severe infections. Impaired disaggregation and low bactericidal activity also affects chemotaxis of neutrophils in SGD patients (Leiding 2017).

2.2.4. Disorders of neutrophil quantity

Neutropenia is defined as a condition with a reduction in the absolute number of neutrophils circulating in the blood below 1500/µl. The neutropenia is said to be severe with absolute neutrophil count below 500/µl. An exception of these defined lower limits are the first weeks of life, during which the absolute number of peripheral neutrophils physiologically increased. In the first 72 h neutrophils are elevated and then gradually decrease. Consequently, neutropenia in newborns is defined with an ANC of 2500/µl (Schmutz, Henry et al. 2008).
It is known that the absolute neutrophil count (ANC) fluctuates under physiological und pathological conditions. Therefore, it is obligatory to determine the ANC in several samples (minimum 3) over a 3-month period. Permanent neutropenia is diagnosed when all samples show an ANC below 1500/µl. Intermittent neutropenia is determined when normal levels of neutrophils alternate with neutropenia (Donadieu, Beaupain et al. 2017).

2.2.4.1. Chronic neutropenia

Neutropenia is defined to be chronic if it lasts more than 3 month. On a global scheme, the most common condition associated with low neutrophil counts is benign ethnic

neutropenia (BEN). In contrast to the diseases discussed later, BEN does not predispose to infections. BEN has been linked to a polymorphism in the gene encoding the Duffy antigen receptor for chemokines (Reich, Nalls et al. 2009, Klein 2011). The pathomechanism of this benign variant of congenital neutropenia is still not solved. Recently, Palmblad and Hoglund have proposed that the condition may be explained by an increased egress of granulocytes to the tissues (Palmblad and Hoglund 2018).
From a clinical point of view, another frequent cause of low neutrophil counts are acquired conditions, such as destruction of peripheral neutrophils by autoantibodies (AIN, autoimmune neutropenia). In particular in children, isolated AIN is a common and usually self-resolving condition (Afzal, Owlia et al. 2017) (Dale and Bolyard 2017).

2.2.4.2. Congenital neutropenia associated with syndromatic disorders

More than 400 monogenic diseases affecting the innate and adaptive immune system have been discovered in humans (Yu, Orange et al. 2018). Among them, many include transitory or constant reductions in the numbers of neutrophil granulocytes. While these disorders are not necessarily grouped as defects of neutrophil granulocytes, many have dysfunctional intracellular membrane-bound structures and therefore are briefly reviewed here.

Hermansky-Pudlak syndrome type 2, caused by mutations in the beta 1 subunit of the adaptor protein 3 complex (AP3B1), is a syndromic disorder including albinism, bleeding and pulmonary fibrosis and neutropenia (Dell'Angelica, Shotelersuk et al. 1999, Jung, Bohn et al. 2006, Vicary, Vergne et al. 2016). Most of the symptoms can be related to abnormalities in formation and trafficking of lysosome-related organelles. Benson and colleagues suggested that NE is a transported by the adaptor protein 3 complex (Schaffer and Klein 2007). A related disorder, caused by a mutation in the gene *AP3D1,* has been described that destabilizes the adaptor protein 3 complex. In this case neurological symptoms and impaired hearing were also clinical manifestations (Ammann, Schulz et al. 2016).

Griscelli syndrome type 2 is caused by mutations in the gene *RAB27A* (Jung, Bohn et al. 2006). The syndrome is characterized by albinism, thrombocytopenia and neutropenia. As cytotoxic lymphocytes are also defect, the susceptibility to infections is high for these patients (Klein, Philippe et al. 1994, Schaffer and Klein 2007). RAB27A is involved in the regulation of granule exocytosis (Ostrowski, Carmo et al. 2010, Matsunaga, Taoka et al. 2017), including the release of MPO in neutrophils (Munafo, Johnson et al. 2007).

p14/LAMTOR2 deficiency represents a related defect in intracellular trafficking and aberrant lysosomal organelles. Patients with LAMTOR2 deficiency suffer from a primary immunodeficiency, including oculocutaneous albinism, growth failure, and congenital neutropenia. Neutrophils exhibit abnormal elongated shape of azurophilic granules (Bohn, Allroth et al. 2007, Hauck and Klein 2013).

I Cohen syndrome is caused by biallelic mutations in the *gene vacuolar protein sorting 13 homolog B (VPS13B).* This gene encodes for a *cis* Golgi membrane protein which is crucial for the integrity of the Golgi ribbon and is thought to be related to protein sorting and vesicular trafficking (Rodrigues, Fernandes et al. 2018). Mutations in *VPS13B* are associated with glycosylation defects. Characteristic clinical manifestations associate, intellectual disability and developmental delay, and craniofacial abnormalities, abnormal truncal fat distribution in teenage years, visual impairment and leukopenia, especially SCN (Seifert, Kuhnisch et al. 2011, Rodrigues, Fernandes et al. 2018).

Shwachman-Diamond syndrome is a rare syndrome that is characterized by exocrine pancreatic dysfunction, bone marrow failure and a predisposition to malignant transformation to MDS and leukaemia. 90 % of the patients carry mutations in the *SBDS* gene that encodes for a protein that is involved in ribosomal maturation. The vast majority of patients have either intermittent or persistent congenital neutropenia. (Donadieu, Fenneteau et al. 2012, Myers, Davies et al. 2013).

WHIM syndrome patients typically present with warts, hypogammaglobulinaemia, infections, and myelokathexis. The term “myelokathexis” describes the presence of senescent and apoptotic neutrophil granulocytes in the bone marrow, resulting from an inability of neutrophils to egress physiologically to the peripheral blood stream. Myelokathexis thus describes hyperplasia and accumulation of apoptotic neutrophils in the bone marrow whereas in the peripheral blood neutrophils are only present in reduced numbers. WHIM syndrome is a result of monoallelic gain of function mutations in the chemokine receptor CXCR4. These mutations cause constitutive activation of CXCR4-mediated signalling. It has been observed that delayed trafficking in the endocyte pathway may play a role in the pathogenesis of WHIM syndrome (McCormick, Segarra et al. 2009).

In a clinical perspective, the differential diagnoses of congenital neutropenia must also include a variety of additional diseases, such as methylmalonic aciduria, Pearson syndrome, Barth syndrome, Glycogen-storage disease Ib, Bruton agammaglobulinaemia, AK2 deficiency, STK4 deficiency, poikiloderma with neutropenia, Cartilage-hair hypoplasia, CD40L deficiency, GATA1 deficiency, large granular lymphocyte syndrome and some variants of severe combined immunodeficiency (Zeidler, Boxer et al. 2000,

Hauck and Klein 2013). Whereas these defects will not be considered here in greater detail, I will now focus on severe congenital neutropenia (SCN).

2.2.4.3. Severe congenital neutropenia

SCN encompasses a heterogeneous group of rare diseases typically characterized by an an ANC below 0.5 G/l and the pathognomonic feature of myeloid maturation arrest in the bone marrow. The incidence proportion of SCN is estimated at 1 case per 2 000,000 persons (Skokowa and Welte 2007). For diagnosis, differential blood counts (at least 3 times within 3 months) should be repeatedly tested, indicating persistent ANC below this level. Furthermore, an evaluation of the bone marrow is recommended to visualize the characteristic early myeloid arrest at the promyelocyte or myelocyte stage (figure 2) including atypical nuclei and cytoplasmic vacuolization (figure 3). It is also recommended to perform molecular testing to define the genetic etiology of SCN (Klein 2011, Klein 2017).

Prior to the development of therapeutic strategies (first symptomatic antibiotic therapies, then the advent of recombinant human GCSF (Bonilla, Gillio et al. 1989) and finally definitive cures by allogeneic hematopoietic stem cell transplantation (Zeidler, Welte et al. 2000), all patients died during the first years of life.

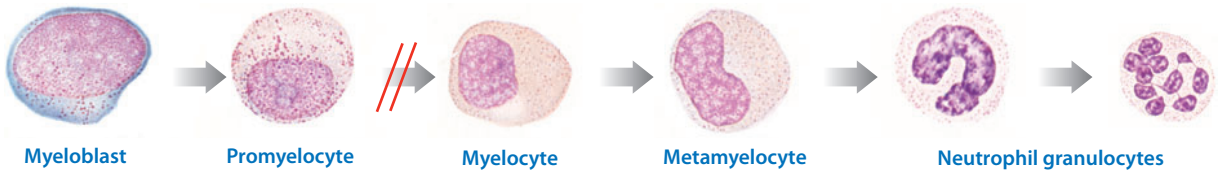


Figure 2: Myeloid maturation arrest in the bone marrow. *Neutrophils from patients with severe congenital neutropenia typically stop the differentiation from the promyelocyte stage to the myelocyte stage (from Klein, C., Annu. Rev. Immunol. 2011).*

Heterogeneity of SCN

Since the first description of inherited neutropenia as infantile genetic agranulocytosis by the Swedish paediatrician Rolf Kostmann in 1956 (Kostmann 1956), the list of monogenetic diseases causing congenital neutropenia has evolved, in particular over the last decade, numerous genetic defects have been discovered (table 2) (Schaffer and Klein 2007, Hauck and Klein 2013, Klein 2016).

The most common genetic etiology in European/North American patients are heterozygous mutations in *ELANE* (SCN1). *ELANE* encodes Neutrophil Elastase (NE), a specific protease that cleaves a large array of proteins, including enterobacterial virulence

factors. When *ELANE* is mutated neutrophil granulocytes undergo premature apoptosis (Amulic, Cazalet et al. 2012, Klein 2016).

SCN2, another subtype of SCN, is caused by monoallelic mutations in Growth-factor-independent 1 (*GFI1),* a transcription factor controlling hematopoiesis and in particular the differentiation of neutrophil granulocytes. In addition to granulopoiesis, defects in GFI1 affect also lymphoid and other myeloid cells. Patients with mutations in GFI1 can also suffer from osteoporosis (Klein 2011, Geissler, Textor et al. 2018), which is however also seen in many other genetic variants of SCN.

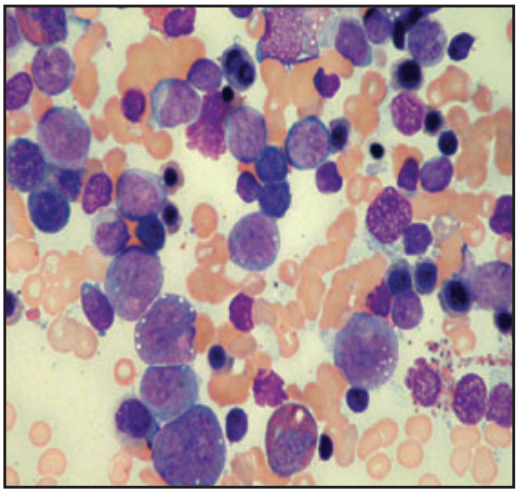


Figure 3: Representative bone marrow smear of patients suffering from sever congenital neutropenia. *Visualization of the characteristic early myeloid arrest at the promyelocyte stage including atypical nuclei and cytoplasmic vacuolization (from Klein, C., Annu. Rev. Immunol. 2011).*

SCN3 is caused by mutations in the gene *HAX1*, encoding for HCLS1 associated protein X-1. HAX1-deficiency has also been shown to be the underlying genetic cause in the patients originally described by Dr Kostmann. *HAX1* mutations are particularly frequent in Sweden and in Turkey. HAX1 is a mitochondrial protein and controls the mitochondrial membrane potential. HAX1-deficiency leads to a higher rate of apoptosis in neutrophil granulocytes but the detailed mechanism is still not elucidated (Klein, Grudzien et al. 2007, Klein 2017).

SCN4 is caused by defects in glucose-6-phosphate catalytic subunit 3 (G6PC3), a ubiquitously expressed paralog of glucose-6-phosphatase. In addition to congenital neutropenia, G6PC3 deficiency is characterized various congenital defects, such as cardiac and genital malformations, inner ear hearing loss, facial dysmorphism, increased visibility of superficial veins, myopathy, or endocrine abnormalities. There have also been reported disrupted bone remodelling and abnormalities in the integument (Gatti, Boztug et al. 2011, Boztug, Rosenberg et al. 2012, Banka, Wynn et al. 2013).

Table 2: Monogenetic disorders causing severe congenital neutropenia

Disease	Gene	Mode of Inheritance
SCN1	*ELANE*	AD
SCN2	*GFI1*	AD
SCN3	*HAX1*	AR
SCN4	*G6PC3*	AR
XLN	*WAS*	XL
SCN5	*VPS45A*	AR
SCN6	*JAGN1*	AR
SCN7	*CSF3R*	AR

SCN: severe congenital neutropenia, AD: autosomal dominant; AR: autosomal recessive; XL: X-linked; adapted from Klein C. (Klein 2016).

Thierry Vilboux et al. characterized a new immunodeficiency syndrome involving congenital neutropenia in seven pedigrees in 2013 (Vilboux, Lev et al. 2013). In addition to neutropenia, patients had nephromegaly with extramedullary hematopoiesis and bone marrow fibrosis. The authors described homozygous mutations in VPS45, which encodes a protein that participates in the regulation of membrane trafficking via the endosomal system. In VPS45-deficient cells, the level of β1 integrin cell surface expression and cellular motility is reduced. Several other patients have been reported (Meerschaut, Bordon et al. 2015, Shah, Munson et al. 2017). The exact role of VPS45 in orchestrating endosomal function is under active investigation.

Inherited loss-of-function mutations in *CSF3R*, encoding the G-CSF receptor, have been defined as SCN type 7 (Triot, Jarvinen et al. 2014). Homozygous missense mutations leading to impaired N-glycosylation and aberrant localization of G-CSF-receptor have been reported. Not surprisingly, patients with inherited mutations in *CSF3* fail to respond to treatment with recombinant G-CSF (Triot, Jarvinen et al. 2014). Of note, this disease

must be differentiated from acquired somatic mutations in the G-CSF receptor gene, a frequent phenomenon preceding the onset of acute myeloid leukemia in SCN.

X-linked neutropenia (XLN) results from mutations in the Wiskott-Aldrich syndrome (WAS) gene (Massaad, Ramesh et al. 2013). In contrast to loss of function mutation in WAS causing Wiskott Aldrich syndrome, XLN is caused by mutations in WAS that prevent auto-inhibitory conformations. Thus, XLN is associated with a constitutively active WAS allele.

Loss-of-function mutations in the gene *JAGN1*, encoding for the protein jagunal homolog 1 (JAGN1) are the cause of SCN7. Originally, nine distinct homozygous mutations were identified in 15 individuals suffering from SCN (Boztug, Jarvinen et al. 2014, Baris, Karakoc-Aydiner et al. 2015, Cifaldi, Serafinelli et al. 2018). The protein JAGN1 is located in the endoplasmic reticulum and has four transmembrane domains. The N- and the C-terminus are located in the cytosol. The amino acid sequence of JAGN1 contains a dilysine motif (KKLI on position 37 to 41) that is a retention signal to the ER. The protein is 183 amino acids long; the molecular weight is 21,125 Daltons (Lee and Cooley 2007).

Lee et al. showed that JAGN1 is involved in ER clustering and loss of JAGN1 reduces vesicular traffic during vitellogenesis in Drosophila melanogaster (Lee and Cooley 2007).
JAGN1-mutated neutrophil granulocytes exhibit ultra-structural ER-defects, a paucity of granules, aberrant N-glycosylation of several proteins, including the G-CSF receptor GSF3R. Similar to other genetic variants, JAGN1-deficiency is associated with an increased susceptibility to apoptosis in neutrophils (Boztug, Jarvinen et al. 2014). Khandagale et al. reported that JAGN1 may be involved in formation of NETs. Neutrophils from a patient with homozygous *JAGN1* mutations were stimulated with PMA to induce NET formation. Whereas MPO expression was reduced in NETs, overall NET release was not affected. Killing of *Candida albicans* in NETs was dependant on JAGN1 expression (Khandagale, Lazzaretto et al. 2018).
Patients with mutated *JAGN1* are refractory to the treatment with G-CSF. This may be due to abnormal expression of CSF3R on the cell surface. In conditional knockout mice in which expression of JAGN1 is delected in the hematopoietic system, the capacity of granulocytes to kill Candida albicans is impaired. Interestingly, treatment with GM-CSF could restore this defect in vitro and in vivo (Wirnsberger, Zwolanek et al. 2014).
Interestingly, in mice the pattern of N-glycosylation was severely perturbed in murine neutrophil granulocytes, including glycoproteins involved in cell adhesion and cytotoxicity (Wirnsberger, Zwolanek et al. 2014).

Symptoms of severe congenital neutropenia

Due to the lack of mature neutrophils in the peripheral bloodstream, individuals with SCN suffer from life-threatening infections, which can be fatal (Klein 2016, Donadieu, Beaupain et al. 2017, Khandagale, Lazzaretto et al. 2018). For example, patients have bacterial upper and lower respiratory tract infections, tonsillitis, skin abscesses, and otitis media. Gingivitis is very common. The lack of pus at sites of infections is a characteristic sign of SCN. SCN is a life-threatening disease, since patients are prone to develop sepsis at any time.

Investigations of the bone mineral density have revealed that SCN patients can develop osteopenia or osteoporosis that can lead to symptoms, such as bone pain and pathological fractures. It remains unclear how SCN can lead to osteopenia or osteoporosis. Some patients with SCN have reportedly developed vasculitis, including renal involvement. It is unclear whether this may have been triggered by recombinant G-CSF, as the symptoms abated in all patients after the therapy with G-CSF was temporary interrupted. Some patients developed splenomegaly upon treatment with G-CSF (Zeidler, Boxer et al. 2000).

The most severe complication of patients with SCN is the development of clonal hematopoietic disorders such as myelodysplasia or overt leukemia. In case leukemia evolves, it is usually a manifestation of acute myeloid leukaemia rather than acute lymphoblastic leukemia. Interestingly, the molecular make-up of these leukemias is quite distinct from leukemia in patients without underlying SCN.

It is not clear at this point, to what extent G-CSF therapy contributes to the onset of leukemia. Most likely, G-CSF is an additive factor on the basis of an underlying premalignant condition (Link 2018).

Regular haematological follow-ups including bone marrow evaluation are highly recommended to allow early discovery of leukemia. Once a clonal disorder is diagnosed, allogeneic hematopoietic stem cell transplantation must strongly be considered.

Therapy of severe congenital neutropenia

The treatment of patients suffering from SCN is based on antibiotics and on daily subcutaneous injections of recombinant G-CSF that has fundamentally improved the survival. Ca. 90 % of SCN patients respond to treatment with recombinant G-CSF with an ANC greater than 1.0 G/l. Partial responders achieving ANC levels of 0.5 to 1.0 G/l may still suffer from bacterial infections (Zeidler, Boxer et al. 2000). Therapeutically, ANC counts of more than 1.0 G/l are targeted. However, some patients are completely refractory, even to G-CSF therapy at very high doses (up to 100 microgram/kg/d). These paients are candidates to allo-HSCT.

Even if HSCT is a curative therapy, the procedure remains associated with morbidity and even mortality. In a recent study from Fioredda and colleagues, the transplant-related

mortality was 17 % (Fioredda, Iacobelli et al. 2015). 82 % of the transplanted patients survived after three years. Death occurred because of graft-versus-host disease, infection, and EBV post-transplant lymphoproliferative disease. Fully matched HLA donors can improve the outcome. If HSCT is successful, no further admission of G-CSF is necessary. Thus, selection of HSCT candidates in patients that do not respond to G-CSF should be undertaken (Fioredda, Iacobelli et al. 2015, Klein 2016).
In view of these inherent challenges, new therapeutic approaches are under research. A recent in vitro study from Pittermann and colleagues demonstrated that gene correction of neutrophils from SCN patients using CRISPR/Cas9 system is possibly a new therapeutic approach in the future (Pittermann, Lachmann et al. 2017). However, more preclinical studies are needed before these new methods can be clinically implemented in the therapy of SCN patients (Klein 2016).

Even though the list of the monogenetic disorders leading to severe neutropenia is constantly growing, the epistatic relationships of distinct genetic defects remain to be unravelled. Here, I propose that deficient protein trafficking could be an underlying theme. I focus on JAGN1 deficiency as a paradigmatic defect to she light on the mechanisms orchestrating differentiation and function of neutrophil granulocytes.

2.3. Cell biology of vesicular trafficking

Eukaryotic cells are highly organized with respect to compartimentalisation of cytoplasmic organelles. The communication is mediated by intracellular vesicular trafficking of molecules between these different intracellular compartments. Vesicular trafficking is of central importance for nutrient transport, organelle fusion and fission, receptor recycling and secretion. Thus, vesicular trafficking is crucial in maintaining the homeostasis of the cell (figure 4). Intracellular trafficking of molecules plays also a crucial role in cell-cell interaction. The interaction between cells is based on the secretion of proteins, such as antibodies, hormones, or extracellular proteases. To enable the secretion of proteins, specialized secretory pathways are necessary (Guo, Sirkis et al. 2014).

2.3.1. Early secretory pathway

The secretory pathway is a complex mechanism to transport the proteins in a correct amount to the destined cellular compartment or to the extracellular space. First, proteins are synthesized and folded in the endoplasmic reticulum (ER). Many of these proteins undergo post-translational modifications, such as glycosylation or phosphorylation.

Proteins that are not ER resident are routed from the ER via the ER-Golgi-intermediate compartment (ERGIC) to the *cis* face of the Golgi apparatus. In the Golgi apparatus proteins are sorted – mainly at the trans-Golgi network (TGN) by specific sorting machineries. Then, they are packed into vesicles and routed to their final destinations, such as endosomal compartments, apical and basolateral membrane, and specialized compartments, such as granules in neutrophils (Guo, Sirkis et al. 2014).
The ER is part of the secretory pathway. Proteins that are resident in the ER are retained through a retention motif. This motif consists of four amino acids (KDEL for lumen located proteins and KKXX for transmembrane located proteins; K, Lys; D, Asp; E, Glu; L, Leu; X any amino acid) and functions as a cargo-motif binding site (Stornaiuolo, Lotti et al. 2003, Dodonova, Diestelkoetter-Bachert et al. 2015).
Proteins destined to be transported throughout the cell are marked with a signal peptide at the N-terminus. These proteins are designated for the secretory pathway (Owji, Nezafat et al. 2018).

Whereas initial glycosylation of proteins takes place in the ER, subsequent modifications of the glycan structures occurs in the Golgi apparatus. Interestingly, glycosylation of proteins is also involved in the secretory pathway. Most of the transmembrane proteins and secretory proteins are glycoproteins (Ohtsubo and Marth 2006). It has been shown that N-linked glycans play a major role in folding and quality control of newly synthesized glycoproteins. Only properly folded proteins are delivered from the ER to the Golgi apparatus. Galectins, proteins that bind to carbohydrate play a role in the regulation of apical sorting of cargo molecules that bear N-or O-glycans (Guo, Sirkis et al. 2014). It has also been shown that the lack of N-glycans could result in ER retention (Vagin, Kraut et al. 2009).
If proteins are not properly folded they are not transported from the ER to the next compartment. These unfolded proteins cause ER stress and the so called “unfolded protein response” (UPR). ER stress can be based on perturbations in calcium homeostasis, redox regulation, deprivation of glucose, viral infection, or increased synthesis of secretory proteins (Ma and Hendershot 2001, Kaufman, Scheuner et al. 2002).

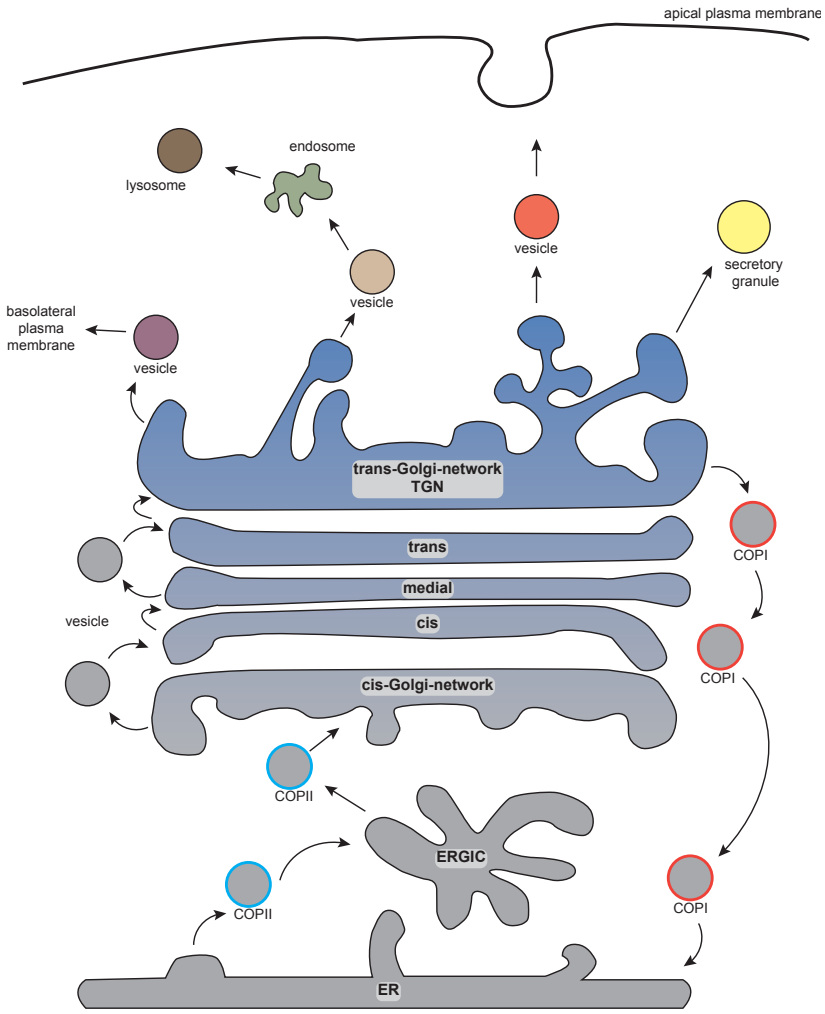


***Figure 4: Schematic depiction of the secretory pathway**. After cotranslational insertion into the endoplasmic reticulum (ER) lumen, correctly folded proteins are transported in COPII coated vesicles (blue ring) across the ER-Golgi intermediate compartment (ERGIC) to the Golgi network (blue). After traversing the different Golgi cisternae, proteins are sorted at the trans Golgi Network (TGN) by complex sorting machineries in a broad variety of transport carriers. These vesicles deliver their cargo to their destined destinations including secretory granules (yellow), the apical and basolateral membrane (orange and purple) and the endosome (beige) (adapted from Tran, Mai Ly, Max-Planck-Institute of Biochemie, Martinsried).*

The early secretory pathway combines the trafficking from the ER via the ERGIC to the Golgi apparatus, from the Golgi apparatus to the ER, and trafficking within the Golgi stacks. This pathway is mediated by the coat protein complexes I and II (COPI and COPII)

(Duden 2003). Proteins that are properly folded and glycosylated in the ER are packed into budding COPII vesicles at the ER exit sites to be transported to the ERGIC and consequently the Golgi apparatus (Kondylis, Tang et al. 2011). Proteins that are marked as ER resident but delivered to the Golgi apparatus are transported back from the Golgi apparatus to the ER via COPI (Duden 2003). The COPI complex has seven subunits (Dodonova, Diestelkoetter-Bachert et al. 2015) and provides different binding sites for the sorting cargo-motif KKXX of cargo proteins (Popoff, Adolf et al. 2011). The process to retain proteins in the ER or to deliver them back from the Golgi apparatus after they have escaped is essential for key cellular processes, such as protein quality control (Moon, Han et al. 2018).

Beside the function between the trafficking between the ER and the Golgi apparatus there are some reports that underline a role of COPI in endosomal transport and function, mRNA transport, involvement in the homeostasis of lipid droplet and the segmentation of the nuclear envelope (Popoff, Adolf et al. 2011).

Regarding its essential and various function, COPI dysfunction causes disease such as Alzheimer's disease (Bettayeb, Hooli et al. 2016), a craniofacial syndrome (Izumi, Brett et al. 2016) dilated cardiomyopathy (Hamada, Suzuki et al. 2004) or lung disease and arthirits (Watkin, Jessen et al. 2015).

2.3.2. Sorting at the Trans Golgi Network

Proteins that have correctly entered the Golgi apparatus at the *cis* face pass through the stacks and exit the complex at the TGN. The TGN is responsible for sorting and packing of proteins into specific constitutive secretory vesicles for the intracellular transport to their final destination (Guo, Sirkis et al. 2014).

Sorting and export of transmembrane cargoes and lysosomal hydrolases at the TGN are well understood. Kornfeld and colleagues (Kornfeld and Mellman 1989) described the first mechanism how acid hydrolases are sorted from the TGN to the endolysosomes: the mannose 6-phosphte (M6P) dependant sorting mechanism. Acid hydrolases are labelled by M6P moieties on their N-linked oligosaccharide chains. This labelling is recognized by the mannose 6-phosphte receptor (MPR), which is a type I integral membrane protein at the TGN. After binding of the receptor to the luminal hydrolases and adaptors, clathrin coats are recruited on the cytosolic part of the TGN membrane. The lysosomal hydrolases are being sent to late endosomes via early endosomes. The high pH of endosomes enhances release of lysosomal enzymes from MPR. Rab9, a retromer induces the recycling of MPR back from endosomes to the TGN (Braulke and Bonifacino 2009). There has been shown that MPR dysfunction leads to more secretion of lysosomal enzymes (Koster, Saftig et al. 1993, Ludwig, Ovitt et al. 1993).

Sorting mechanisms that are mannose 6-phosphate-independent (including the protein sortilin and lysosomal integral membrane protein type 2) have been reported (Reczek, Schwake et al. 2007, Canuel, Korkidakis et al. 2008, Braulke and Bonifacino 2009, Gonzalez, Valeiras et al. 2014), but molecular details remain to be investigated (Guo, Sirkis et al. 2014, Pakdel and von Blume 2018).

Very little is known about sorting of soluble proteins. Recently, a sorting machinery for soluble proteins at the TGN has been described (Blank and von Blume 2017). In HeLa cells the granule protein lysozyme C and other proteins are sorted and packed into vesicles by a sorting machinery including the cytoplasmic actin cytoskeleton, the protein cofilin, the soluble TGN-resident protein Cab45, and the TGN calcium pump SPCA1. The sphingomyelin lipid metabolism has been added to this complex sorting machinery at the TGN (Deng, Pakdel et al. 2018). The complexity of the secretory pathway, including the relevance of kinetics of vesicular transport between the compartments, poses a remarkable challenge for cell biology research.

2.3.3. Regulated secretory pathway in neutrophil granulocytes

The complex mechanism of sorting proteins at the TGN is especially important to professional secretory cells, such as pancreatic cells, neuronal cells or neutrophil granulocytes. In contrast to cells that perform secretion in a constitutive manner, professional secretory cells need additionally a regulated secretory pathway to secrete proteins in a stimulus-dependent manner. Proteins that are designated to storage granules are sorted in parallel to the consecutive secretion at the TGN. It remains unclear how this separation between the consecutive and the regulated secretory pathway is mediated (Guo, Sirkis et al. 2014).
Especially in neutrophils, very little is known about the mechanism how proteins, which are designated to neutrophil granules, are routed away from the consecutive secretory pathway (Cowland and Borregaard 2016). However, neutrophils are unique in their diversity of granules that need to be filled with proteins and mobilized in an accurate and timely manner to fulfil their function in host defence and beyond. Granule-specific trafficking is a highly dynamic process and has to be regulated in a strict technique to achieve the balance between host defence and tissue damage (Ramadass and Catz 2016).

Bulow and colleagues investigated MPO sorting in myeloid cells and hypothesized that the propeptide of MPO delays the exit independently from ER chaperones calreticulin and calnexin (Bulow, Nauseef et al. 2002). Several members of the RAB family of proteins (e.g.

Rab27a and Rab11) have been involved in vesicular trafficking in neutrophils (Munafo, Johnson et al. 2007, Johnson, He et al. 2016). Rab proteins are ras-like monomeric GTPases to regulate membrane identity, sorting, and fusion in human cells by recruitment of specific effectors. It has been suggested that the identification and sorting of granules occurs based on the Rab protein on the surface of the granule (Ramadass and Catz 2016). While this hypothesis may explains how granules are transported to the membrane in response to specific stimuli, there is no evidence how proteins are separated from the constitutive secretory pathway into the distinct granules.

N-glycosylation of serglycin may also play a role in sorting of proteins to granules (Niemann, Abrink et al. 2007, Glenthoj, Cowland et al. 2011). Serglycin is the major intracellular proteoglycan of neutrophils and the localization of elastase in azurophilic granules depends on serglycin (Niemann, Abrink et al. 2007). Furthermore, routing defensins to granules relies on serglycin in a murine model (Glenthoj, Cowland et al. 2011). However, it is not known whether a lack of serglycin leads to more degradation rather than misrouting or retention of granule proteins. The nature of posttranslational glycosylation may have a role to regulate the secretory pathway. The mature granule protein MPO containing complex oligosaccharides is being sorted to azurophilic granules, whereas non-properly glycosylated MPO is not stored in these granules (Olsson, Bulow et al. 2004).

The protein neutrophil gelatinase-associated lipocalin, stored in azurophilic granules, is not any more stored but constitutively secreted when undifferentiated HL-60 cells were differentiated into granulocytes (Le Cabec, Calafat et al. 1997). This suggests that sorting into granules is depending on the stage of differentiation of myeloid cells. Cowland and colleagues hypothesized that timing of biosynthesis determines the localization of the individual granule proteins. MPO, the granule protein that is only found in azurophilic granules, was mainly synthesized in myeloblasts and promyelocytes whereas lysozyme was synthesized during all stages of neutrophil development with a maximum in myelocytes and metamyelocytes (Cowland and Borregaard 2016).
In rabbits, primary granules seem to be formed at the *cis*-Golgi, while granules appearing at later stages of granulopoiesis appear to be generated from the *trans*-Golgi (Bainton and Farquhar 1966). Furthermore, primary granule proteins are glycosylated by granule-specific β–hexosaminidase A. These so called paucimannosidic N-linked carbohydrate structures are not found in peroxidase negative granules (Thaysen-Andersen, Venkatakrishnan et al. 2015). Since N-linked carbohydrates are added in the ER and its modification is performed in the Golgi apparatus (Hang, Lin et al. 2015), this would be in line with the hypothesis of timing of biogenesis by adding glycosylation as the major factor (Thaysen-Andersen, Venkatakrishnan et al. 2015).

Interestingly, most of the newly formed protein lysozyme is spilled out of the cell and not stored into granules (from early promyelocyte stage up to band cells (Arnljots, Sorensen et al. 1998). In general, lysozyme appears to be sorted with lower efficiency granules (around 40%), when compared to MPO (Cowland and Borregaard 2016). In contrast, defensins are efficiently routed into granules up to the stage of promyelocytes and are mostly spilled out, when formed in mature neutrophils (Arnljots, Sorensen et al. 1998). This suggests some selectivity for granule proteins between the regulated and the constitutive secretory pathway, respectively.

Nevertheless, many open questions remain on how the decision is made regarding sorting of proteins to storage granules or to the constitutive pathway in neutrophils.

2.4. JAGN1 deficiency as a model to study cellular trafficking

Several characteristics of *JAGN1*-mutant neutrophils indicate an involvement of JAGN1 in the early secretory pathway of vesicle-mediated transport. Lee et al. observed that loss of *JAGN1* impairs vesicular trafficking during vitellogenesis in drosophila suggesting that JAGN1 protein is orchestrating ER organization (Lee and Cooley 2007).

JAGN1-deficient myelocytes exhibit enlarged ER structures (figure 5, A) and staurosporine induced ER stress in *JAGN1* mutated neutrophils lead to enhanced apoptosis (Boztug, Jarvinen et al. 2014). The N-terminus of the protein JAGN1 interacts with three (COPA, COPB2, COPG2) out of seven subunits of COPI, indicating an involvement of JAGN1 in the sorting and/or packaging of proteins from the Golgi apparatus back to the ER (Boztug, Jarvinen et al. 2014). Recently, that mutations in COPA have been reported to cause ER-stress and impaired trafficking of proteins from the Golgi apparatus back to the ER (Watkin, Jessen et al. 2015). Thus, in JAGN1 deficiency the filling of granules could be affected in a COPA-dependent manner, explaining the phenotype of paucity of granules in neutrophil granulocytes. JAGN1 may have a role as a part of the sorting machinery in the ER, which helps packing cargoes into COPI. Increased ER stress response markers (such as BIP) and the enlargement of the ER seen in electron microscopy (Boztug, Jarvinen et al. 2014) may therefore be consistent with the idea that JAGN1-deficiency is associated with a secretion defect.

Several proteins in JAGN1-deficient neutrophils showed aberrant N-glycomic profiles, underscoring the relationship of glycosylation of proteins in the ER and the secretory pathway (Wirnsberger, Zwolanek et al. 2014). One of these affected proteins was the G-CSF receptor. Furthermore, the secretion of *Gaussia* luciferase was reduced in *JAGN1* siRNA-mediated knockdown HeLa cells, indicating that the involvement of JAGN1 in the secretion of proteins is not specific for neutrophils, even if JAGN1 deficiency in humans appears to affect only neutrophils (Boztug, Jarvinen et al. 2014). Interestingly, granulocytes of *JAGN1*- patients showed less granules compared to healthy donors (figure 5, A). This striking reduction of granularity could also be confirmed in hematopoietic-specific *JAGN1* KO mice (figure 5, B) (Boztug, Jarvinen et al. 2014, Wirnsberger, Zwolanek et al. 2014).

Additionally, there were detected changes of N-glycans of proteins in these hematopoietic-specific *JAGN1* KO mice. The affected proteins are stored in granules and participate in migration and toxicity of neutrophils, indicating that JAGN1 takes part in the glycosylation process and protein trafficking between the ER and the Golgi apparatus. The granule protein MPO as well as Mmp8 and Mmp9 were reduced, suggesting that all three subtypes of granules are affected (Wirnsberger, Zwolanek et al. 2014).

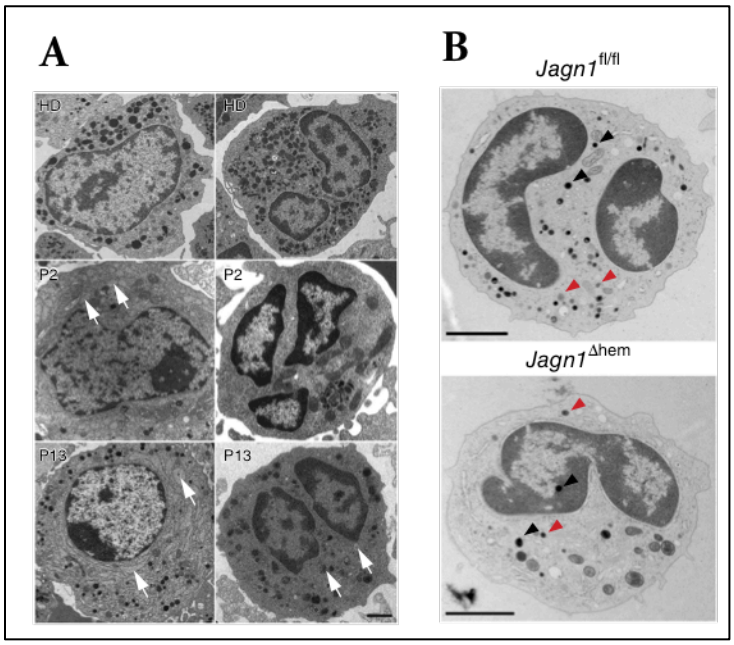


Figure 5: Enlarged ER structures and paucity of primary granules in JAGN1 deficiency ***(A)*** *Transmission electron microscopy of the bone marrow. Myelocytes (left) from affected patients exhibited abnormal, enlarged ER structures (arrows) in contrast to myelocytes from a healthy donor (HD). Differentiated granulocytes (right) from affected subjects showed a paucity of typical granules (arrows). Scale bar, 1 µ m (adapted from Boztug et al., 2014).* ***(B)*** *Representative electron micrographs of segmented neutrophils from the bone marrow of a Jagn1 fl/fl and a hematopoietic-specific* JAGN1 *KO mice. Scale bar, 2 µ m). Black arrowheads indicate primary granules, and red arrowheads indicate secondary granules (from Wirnsberger et al., 2014).*

A recent study showed that upon knocking-down JAGN1 in pancreatic β-cells, more glucose-stimulated insulin is secreted (Nosak, Silva et al. 2016). It is not obvious at this point, why there is more secretion of insulin, but generally, this is in line with the idea JAGN1 is involved in the secretory pathway.

3. Aim of this thesis

The orchestration of protein transport is crucial for cellular homeostasis. In neutrophil granulocytes, the formation of granules is highly dependent on a balanced and regulated secretory pathway to fulfil an important role in host defence. However, the mechanism how granules are formed is poorly understood so far.
JAGN1 deficiency was chosen as a model to shed more light into protein sorting and vesicular trafficking in neutrophils.

Therefore, the specific aims of this thesis were:

1. Establishing cellular models to analyse the role of JAGN1 in secretion
2. Analysis of intracellular protein transport using new biochemical tools

4. Material and methods

4.1. Secretome studies with mass spectrometry

Induced pluripotent stem cells (iPSc) were differentiated into mature neutrophil-like cells (Live/CD49d/Cd11b/CD33) by Dr. Yoko Mizoguchi and were sorted by fluorescence-activated cell sorting aria in each two clones of wild type (WT) and *JAGN1* knockout (KO) on day 32 after differentiation. 250,000 cells per 250 µl Hank's Balanced Salt Solution were stimulated with 1,5 µM of phorbol myristate acetate (PMA) or let unstimulated as a control. Cells were incubated for 25 min. at 37 °C. Supernatants were collected and concentrated with 10 kD cut-off membrane amicon filters to a final volume of 30 µl and stored at -80 °C. To the concentrated supernatant equal volume of a buffer containing 1 % sodium deoxycholate, 10 mM Tris(2-carboxyethyl)phosphine and 40 mM choloracetamide in 25 mM Tris pH 8.5 was added. The sample was mixed thoroughly and incubated at 37 °C for 20 minutes. The whole sample was diluted once with equal volume of water. The diluted samples were then digested with about 1 µg of trypsin overnight at 37 °C.
The digested peptides were then purified and concentrated using a 3 plug SCX Stage Tip. The peptides were loaded on a 15 cm long reversed phase column (75 µm inner diameter, packed with 1.9 µm C18 beads) and eluted using a binary gradient of 0.1 % formic acid (buffer A) and 80 % acetonitrile in 0.1 % formic acid (buffer B). Eluted peptides were directly sprayed in to a bench top Orbitrap instrument (Q Exactive HF) (Nagaraj, Kulak et al. 2012) via a nano-electrospray ionization interface. Peptides were analysed using a regular top N data dependent acquisition scheme using HCD fragmentation (Olsen, Macek et al. 2007). Raw data were processed using MaxQuant computation proteomics (Cox and Mann 2008) platform and peak lists were searched against a Uniprot human proteome database. All peptide and protein identifications were filtered at 1 % false discovery rate. Proteins across different samples were quantified using MaxLFQ (Cox, Hein et al. 2014) label free quantification algorithm.

4.2. CRISPR/Cas9 knock-out cell lines

For the retention using selective hook-system human cervical epitheloid carcinoma (HeLa) C and HeLa C CRISPR/Cas9 mediated *JAGN1* KO cells were generated.

Cell culture
To wash the cells Gibco® DPBS (1X) Dulbecco's Phosphate Buffered Salin (PBS) was used. To trypsinate HeLa cells 45 ml PBS and 5 ml Trypsin EDTA (TE) were mixed 1:10 (45 ml PBS and 5 ml TE). In 6-wells 2ml, in 12-wells 1ml, in 100 mm dishes 10 ml medium and in

150 mm 18 ml growth medium was used to culture the cells. For HeLa Gibco® (life technologies™) Dulbecco's Modified Eagle's Medium (DMEM) (1X) + GlutaMAX™-I Pen Strep (penicillin, streptomycin) and 10 % foetal calf serum (FCS) was added. To count the cells Life Technologies Countess 2 was used with Countess™ Cell counting chamber slides. 15 µl of Trypan Blue stain 0,4 % (Invitrogen™, Thermo Fisher Scientific) and 15 µl oft cells in media were counted.

JAGN1 knockout in HeLa cells

The guide sequence of 20 nucleotides targeting human *JAGN1* was designed using the CRISPR design tool at www.genome-engineering.org/crispr (Hsu, Scott et al. 2013) and cloned into a mammalian expression vector (pX458) bearing the Cas9 coding sequence, the sequences encoding the RNA components and a puromycin selection cassette (plasmid 48139; Addgene; (Cong, Ran et al. 2013). The guide sequences used to target exon 1 of human *JAGN1* were 5′-ggcacaatggcgtctcgagc-3′ (guide 1), 5′-gtctcgagcaggcccgcgag -3′ (guide 2) and 5′- ccgcgagcggccggcaccga-3′ (guide 3). All three different gRNA were used to transfect against *JAGN1.*

The backbone was digested at 37 °C for 2 hours with 30 µl of backbone d37_pCas9 (pX458), 5 µl of 2.1. NEB buffer, 2 µl of Bbs I enzyme and 13 µl of ddH_2O. The resulting fragments were ligated at room temperature for one hour in the mix of 1 µl backbone, 1,5 µl of the insert (1:200), 2 µl of ligase buffer, 1 µl of T4 ligase and 14,5 µl of ddH_2O.
A total of 4 µg of the pX458 vector containing the sequence of the sgRNA was transfected into HeLa cells. 24 h after transfection, cells were selected for 48 h in 2 mg/ml puromycin. Then, 1000 or 2000 or 4000 cells were seeded in 15 cm culture dishes and cultured until single cell colonies were large enough to be manually scrap them off the dish and transferred to 96-well plates. Single clones were then expanded and screened for JAGN1 by gel electrophoresis and Western blot. Clones were confirmed using sequencing with JAGN1 primers.

Sequencing

Constructs were verified by sequencing procedures. Mix2Seq Kits (Eurofins Genomics) was used for sequencing according to manufactures instructions. For DNA extraction purification Metabion PCR purification KIT was used according to manufactures instructions. For gel extraction Metabion *mi*-Gel extraction KIT was used according to manufactures protocol. 1g agarose was diluted in 100ml TAE and heated in a microwave and cooled down afterwards. Ethidium bromide was added in a concentration of 1:1000. Samples were diluted 1:10 with Orange G Loader. TAE buffer was added. Gel

electrophoresis was performed for 20 min. at 100 Volt. As marker 5 µl of Gene Ruler low range (< 700kb) was used. Following PCR programs were used (table 3):

Table 3: PCR program for genome preparation

5 min.	95°C	1 cycle
30 sec.	95°C	35 cycles
30 sec.	58°C	
3 min.	72°C	
5 min.	72°C	1 cycle

To determine correct knock-out following primers were used for the sequencing (table 4).

Table 4: Used Primers (Metabion)

Primer	**Sequence 5' > 3'**
hJAGN1_fwd.	CGCAAATAGGGTCAGTGGGC
hJAGN1_rev.	CGCAAATAGGGTCAGTGGGC

Gel electrophoresis

To determine the *JAGN1* KO in HeLa cells gel electrophoresis and Western blotting were used according to following protocols. 15 % sodium dodecyl sulfate (SDS)-Gels and 5 % stocking gels were used prepared according to following mixtures (table 5 and 6):

Table 5: Mix for two 15% SDS gels

4.8 ml	H_2O
10 ml	30 % Acrylamide mix
5 ml	1.5 M Tris (pH 8.8)
0.2 ml	10 % SDS
0.2 ml	10 % Ammonium Persulfate
32 µl	Tetramethylethylendiamin

Table 6: Mix for two 5% stacking gels

3,4 ml	H_2O
830 µl	30 % Acrylamide mix
630 µl	0.5 M Tris (pH 6.8)
50 µl	10 % SDS
50 µl	10 % Ammonium Persulfate
5 µl	Tetramethylethylendiamin

Samples were diluted 1:4 with lemli for 5 min. at 95 °C. Gel electrophoresis was performed for 120 min. at 120 volt and blotted in transfer buffer for 70 min. at 100 volt.

To evaluate *JAGN1* KO in HeLa cells, blot was blocked in in 5 % tris-buffered saline (TBS) RT for 1 hour. Afterwards the blot was incubated in 10 ml of 1:2000 JAGN1 polyclonal rabbit antibody in 2,5 % bovine serum albumin (BSA) in TBS at +4 °C. The Western blot was washed 2 times for 15 min. with TBS/Tween at room temperature and incubated for

30 min. in 10 ml of 1:5000 secondary anti-rabbit horseradish peroxidase (HRP) antibody in 2,5 % BSA in TBS at 4 °C. Western blot was washed 4 times for 15 min. with TBS/Tween at room temperature.

For stripping the Western blot was incubated with Restore™ PLUS Western Blot stripping buffer (Thermo Scientific) for 15 min. at room temperature. To evaluate loading of the samples actin was used. Blot was washed 2 times for 15 min. with TBS/Tween at room temperature. The blot was blocked in in 5 % TBS at room temperature for 1 hour. Afterwards the blot was incubated in 10 ml of 1:10000 actin mouse antibody in 2,5 % BSA in TBS at +4 °C. The Western blot was washed two times for 15 min. with TBS/Tween at room temperature and incubated for 30 min. in 10 ml of 1:5000 secondary anti-mouse HRP antibody in 2,5 % BSA in TBS at 4 °C. Western blot was washed 4 times for 15 min. with TBS/Tween at room temperature.
For Imaging Western blots were incubated in Immobilon™ Western Chemiluminescent HRP Substrate (Millipore) and the ChemiDoc Touch Imaging System (Bio-Rad Laboratories Gmbh) was used according to manufactures instructions.

4.3. Retention using selective hooks-system

On day one 2 sterile cover slips were placed into each 6-well. 55.000 HeLa control cells and HeLa *JAGN1* KO cells were seeded into each 6-well with 2 ml DMEM and 10 % FCS. On day 2 HeLa cells were washed with PBS once and fresh 2 ml DMEM and 10 % FCS was added. Cells were transfected with the RUSH plasmid pIRESneo3-Str-KDEL_LyzC-SBP-EGFP (figure 6).
The RUSH plasmid Str-KDEL_ST-SBP-EGFP was a gift from Franck Perez (Addgene plasmid # 65264). The pIRESneo3-Str-KDEL_LyzC-SBP-EGFP plasmid used for HeLa RUSH transfection was a gift from M. Pakdel (Max-Planck-Institute of biochemistry, Martinsried, Germany), (Deng, Pakdel et al. 2018).

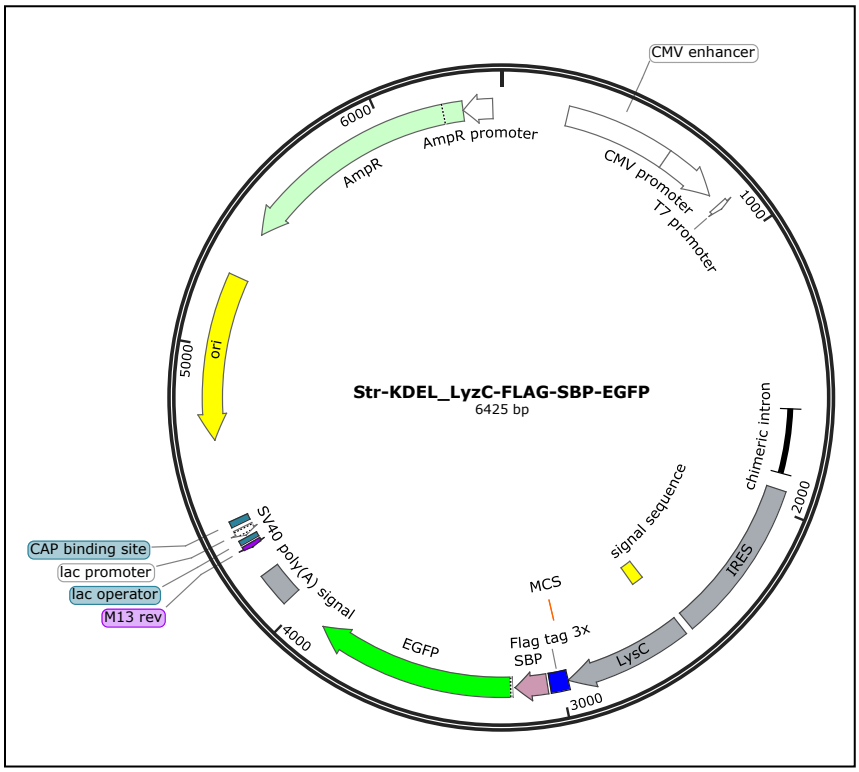


***Figure 6**: **Vector map of RUSH-plasmid pIRESneo3-Str-KDEL_LyzC-SBP-EGFP.** The plasmid contains the human cytomegalovirus (CMV) promoter/enhancer, the ampicillin resistance gene (AmpR) including the promoter, the internal ribosomal entry site (IRES), the signal sequence KDEL, lysozyme C (LyzC) as the reporter, the streptavidin binding protein (SBP), the gene of enhanced green fluorescent protein (EGFP), and a multiple cloning site (MCS), FLAG-tag and the E. coli replication origin (ori). The plasmid was provided by M. Pakdel (Max-Planck-Institute of biochemistry, Martinsried, Germany).*

Plasmid was amplified using thermo competent E. coli. Thermo competent E. coli was defrosted on ice. 1 µl of the plasmid was added and incubated for 20 min. on ice. After 90 sec. of transformation at 42 °C cells were recovered with 1 ml LB medium for 40 min. at 37 °C. For Maxi preparation cells were incubated in 400 ml LB and 600 µl ampicillin overnight at 37 °C. For mini-preparation cells were incubated in 4 ml LB medium and 6 µl ampicillin overnight at 37 °C. For Maxi preparation Nucleo Spin® Plasmid Easy Pure (Invitrogen™, Thermo Fisher Scientific) was used according to manufactures protocol. For the Mini preparation QUIAGEN QIA Prep® Spin Miniprep KIT 250 was used according to manufactures instructions. To evaluate Plasmid DNA concentrations Nano Drop was used according to manufactures instructions. For transfection following reaction mixture was used (table 7).

Table 7: Mix for transfection of HeLa cells with RUSH plasmid

200 µl	Opti-MEM
2 µg	RUSH-Plasmid
12 µl	Polyethylenimine

The mixture was vortexed for 5 sec. and incubated for 15 min. at RT.
On day three DMEM and 10 % FCS was pre-warmed in 37 °C water bath. 4 % paraformaldehyde (PFA) in PBS and place it under the hood was prepared as well as 40 mM biotin (SUPELCO) in pre-warmed DMEM and 10 % FCS. Cells were washed once with PBS. 2 ml DMEM and biotin were added to the 60 min. well, the timer was started for 20 min. and DMEM with or without biotin was added to remaining cells. Cells were incubated 20 min. at 37°C. Medium was removed from next well and 2 ml DMEM and biotin was added to the 40 min. well. Cells were incubated 20 min. at 37 °C. Medium was removed from the next wells and 2 ml DMEM and biotin to the 20 min. well was added. Cells were incubated for 18 min. at 37 °C. Two min. prior the final incubation time elapses, wells were washed one time with PBS. 4 % of PFA in PBS was added to fix the cells for 10 min. at room temperature in the dark. Wells were washed 3 times in PBS and cover slips were mounted on microscope slides.
For additional immunofluorescence staining cells were permeabilized by adding permeabilisation buffer for 5 min. at room temperature. Cells were washed 3 times with PBS and blocked in blocking buffer for 1h at room temperature. The glass cover slips were transferred into a 15 cm dish covered with moist whatman paper and Parafilm. Primary antibodies (1:300 mouse a-P230, 1:1000 sheep a-TGN46, 1:500 mouse a-Calnexin, 1:300 rabbit a-JAGN1, 1:300 rat a-HA) and/or 1:6000 a-DAPI diluted in blocking buffer were added and incubated for 1 h at room temperature. Cells were washed 3 times for 5 min. with PBS. Secondary fluorescently conjugated antibodies (1:1000 a-mouse Alexa-594. 1:1000 a-sheep Alexa-633, 1:1000 a-rat Alexa-647) were added in blocking buffer and incubated for 30 min. at room temperature. Cells were washed 3 times for 5 min. with PBS. Cover slips were mounted with ProLong Gold (Invitrogen™, Thermo Fisher Scientific) on microscope slides.

Acquisition

Images were acquired using the laser scanning confocal fluorescence microscope ZEISS LSM 780 (Carl Zeiss) with a 100x/1.46 oil a-Plan-Apochromat oil objective lens. To quantify secretory vesicle numbers, z-stacks were acquired using the same the objective. For detection of Alexa Fluor, the 488 nm laser line was used. Pictures were acquired using Leica Software (ZEN, 2010) and adjusted with the ImageJ image software. Only cells were analysed that showed appropriate transport of the reporter to the Golgi apparatus after addition of biotin. Cells with ER signal were excluded from the analysis. For the

representation of the whole volume of the cell, typically 9-15 z-stacks with a step-size of 0.35 mm were acquired of each field of view.

Quantification of vesicles

Vesicles were quantified using FIJI software and a programmed makro plugin including the threshold algorithm "Yen". (Deng, Pakdel et al. 2018).

4.4. Statistics

For the secretome studies Perseus software platform was used. Benjamini Hochberg test was performed with a false discovery rate of 0.05. Significant abbundance was calculated using students t-test for the volcano plots.

For statistical evaluation of the quantification of vesicles in the RUSH-system GraphPad Prism for Mac OS X (GraphPad Software) was used (one-way ANOVA). P-value of 0.05 was considered as significant.

5. Results

The aim of the thesis was to analyse the participation of JAGN1 in the secretory pathway. First, a comprehensive mass-spectrometry (MS) analysis was undertaken to define the constitutents of the secretome of iPSC-derived bona fide neutrophil granulocytes.
Second, intracellular protein trafficking has been studies in a newly generated JAGN1 HeLa knockout system by means of immunofluorescence. Third, using the granule protein lysozyme C as a model, the kinetics of protein trafficking has been studied in JAGN1-deficient and wildtype cells by means of the RUSH-system.

5.1. Impaired secretion in JAGN1-deficient iPSc-derived bona fide neutrophil granulocytes

We first asked whether JAGN1 regulates secretion of proteins in neutrophils. To answer this question, *JAGN1* KO clones of iPSc-derived neutrophil-like cells were generated by Dr. Yoko Mizoguchi. The cells were either stimulated with 1,5 µM PMA for 25 min. or left unstimulated. Supernatants of two wildtype control clones and two *JAGN1* KO clones were collected in triplicates, respectively. The supernatants were analysed by mass spectrometry (MS).

To get an overview about the reliability of the experiments, principal components analysis was performed. There was no overlap between the triplicates of each group. All four groups (Control PMA-, control PMA+, *JAGN1* KO PMA- and *JAGN1* KO PMA+) could be clearly discriminated in both independent experiments (figure 7). Even though, in the second experiment, the triplicates of PMA stimulated *JAGN1* KO cells were more heterogeneous than in the first experiment, the principal components analysis indicated that the differences in the secretory profiles were either genotype-dependent or dependent upon the stimulation with PMA.

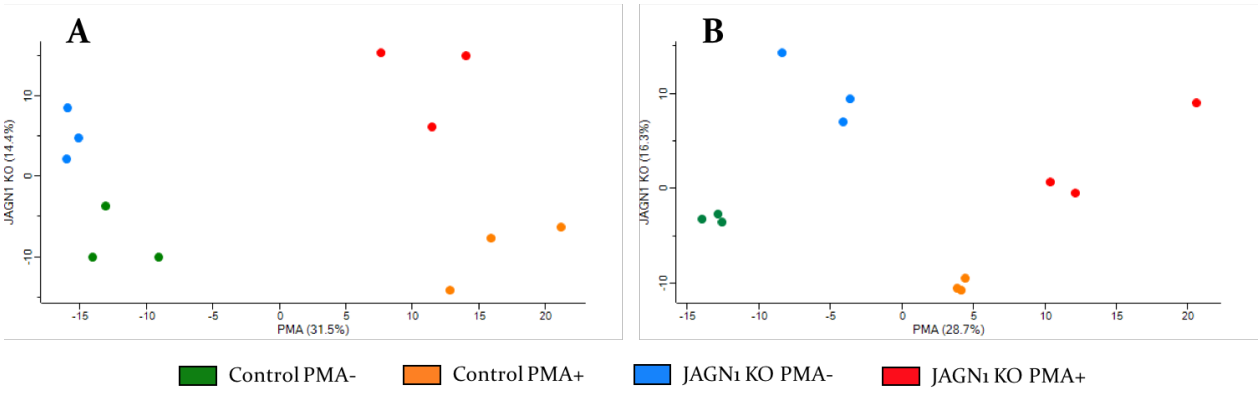


Figure 7 Principal component analysis of supernatant triplicates of iPSc-derived neutrophil-like cells. *Component one was JAGN1 KO compared to control (y-axis). Component two was PMA stimulation compared to unstimulated condition (x-axis). Secretory profiles were genotype-dependent or dependent upon PMA-stimulation.* ***(A)*** *Experiment 1: consistent discrimination of all four groups.* ***(B)*** *Experiment 2: consistent discrimination of all groups. Stimulated JAGN1 KO clones are more heterogeneous in the second experiment than in the first one.*

MS-based quantitation of secreted proteins identified 1726 proteins in the first experiment and 1773 proteins in the second experiment. Proteins that were marked as potential contamination (32 and 30 proteins in the first and second experiment, respectively) or proteins only identified by site of post translational modifications (50 proteins in the first and 42 proteins in the second experiment) were excluded. The remaining proteins were analysed using Perseus software platform.

To determine the effect of PMA stimulation on iPSc-derived neutrophil-like cells, proteins that differed significantly in their secreted abundances, we compared the secreted proteins of control cells without PMA stimulation and control cells after the stimulation with PMA as described in material and methods. Figure 8 shows a Volcano plot of two independent experiments. 35 proteins were consistently found to be secreted in higher levels upon PMA stimulation in both independent experiments, whereas 78 proteins were found to be secreted at lower levels in the stimulated controls.
There were no contradictory results of proteins that were less abundant in one experiment and more abundant in the other one.

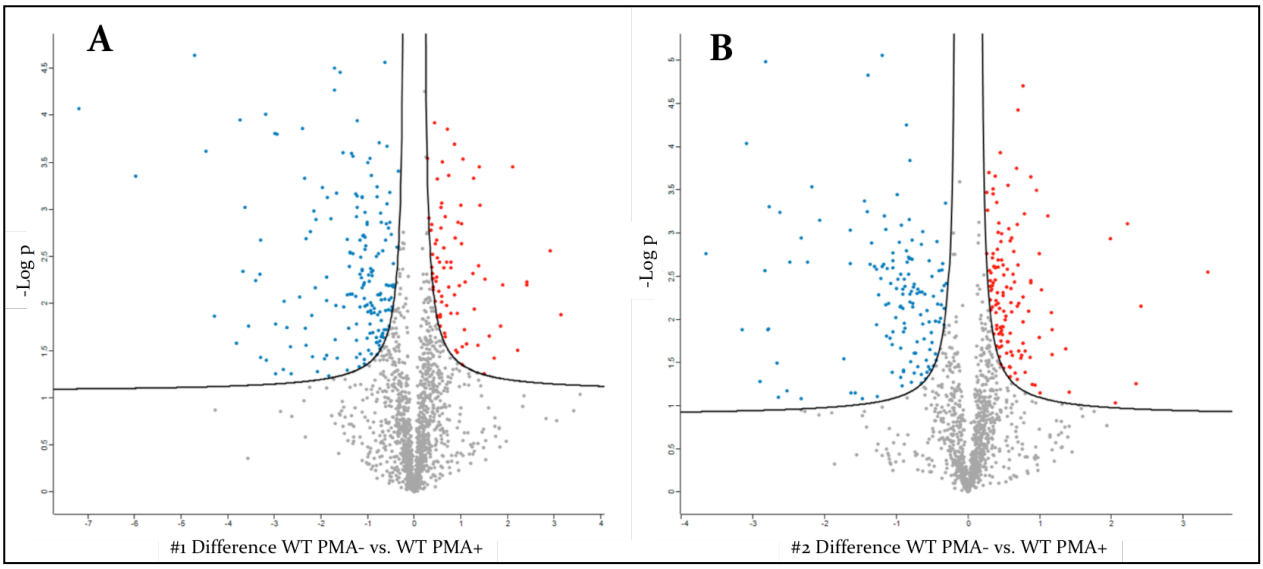


Figure 8: Volcano plots of secreted proteins of iPSc-derived neutrophil granulocytes upon stimulation with PMA. *Supernatants were collected and analysed with mass spectrometry as described in material and methods. Blue dots represent individual proteins that are significantly less abundant and with red dots represent individual proteins that are significantly more abundant upon PMA stimulation in comparison to unstimulated control cells (PMA-)* ***(A)*** *Experiment 1.* ***(B)*** *Experiment 2.*

In view of these consistent cellular responses towards PMA, we conclude that PMA stimulation induces an increase and decrease of specific protein secretion in iPSc-derived neutrophil granulocytes. Next, we used this model system to analyse the secretome in JAGN1-deficient iPS-derived neutrophils, in the presence and absence of PMA, respectively. Table 8 summarizes the experimental findings and lists the number of detected proteins in the indicated experimental condition.

To analyse dependency on PMA stimulation in *JAGN1* KO cells we compared PMA stimulated *JAGN1* KO clones to unstimulated *JAGN1* KO clones. 19 proteins were significantly less secreted and 57 proteins were significantly more secreted upon stimulation with PMA. Granule proteins, such as MPO and BPI, as well as numerous lysosomal hydrolases, such as cathepsin D and beta-galactosidase, were more secreted after the addition of PMA in the control clones. This underlines that the reaction to PMA was not impaired in the absence of JAGN1.

To discover proteins that were dependent on PMA stimulus in the absence of JAGN1, *JAGN1* KO clones were compared to control clones with no addition of PMA. Four proteins were significantly less secreted and 9 proteins were significantly more secreted. In the group of less abundant proteins there was no evidence for extracellular function of

the secreted proteins. In the group of more abundant proteins mostly lysosomal hydrolases were identified, such as cathepsin D and beta-galactosidase.

Table 8: Number of proteins with significant differences of abundance in supernatants of iPSc-derived *JAGN1* KO neutrophil granulocytes

	Number of proteins with decreased level of abundance	Number of proteins with increased level of abundance
Control + / control -	78	35
***JAGN1* KO + / *JAGN1* KO -**	19	57
***JAGN1* KO - / control -**	4	9
***JAGN1* KO + / control +**	4	18

Number of proteins that were identified in both experiments with significant differences of abundance in supernatants of iPSc-derived neutrophil-like cells. Wildtype (n = x clones) and JAGN1-deficient cells (n= z clones) were stimulated with (+) PMA for 25 min. or were unstimulated (-). JAGN1 KO was generated using CRISPR/Cas9 as described in material and methods. Proteins were identified with mass spectrometry and analysis was performed with Perseus software platform. Potentially contaminated proteins were excluded as well as proteins only identified by site.

To determine the secretory output of *JAGN1* KO cells after stimulation with PMA we compared PMA stimulated *JAGN1* KO clones to PMA stimulated control clones. 4 proteins showed a significant decrease in abundance and 18 proteins were secreted at significantly higher levels. 13 of these proteins were annotated with extracellular functions. 14 proteins showed signal sequence in Uniprot Database, which is a necessary part of the amino acids for the translocation to the ER and subsequently for the secretory pathway. The other four proteins (Phosphoserine aminotransferase, D-3-phosphoglycerate dehydrogenase, Coagulation factor XIII A chain and Tripeptidyl-peptidase 1) showed no signal sequence for the destination towards the secretory pathway.
Interestingly, two primary granule proteins, MPO and BPI, were secreted at significantly higher levels in PMA stimulated *JAGN1* KO clones compared to control clones. A third granule protein, lysozyme C, which can be found in all three granule subtypes was also found to be secreted at higher levels in stimulated *JAGN1* KO clones, albeit only in one experiment. Cathepsin D was also more secreted in *JAGN1* KO clones compared to control clones in the stimulated and unstimulated conditions. It was also significantly more secreted in stimulated control clones compared to unstimulated control clones.

Furthermore, lysosomal hydrolases (such as lysosomal alpha-mannosidase, beta-galactosidase, N-acetylglucosamine-6-sulfatase, arylsulfatase B, beta-mannosidase, acid ceramidase, gamma-interferon-inducible lysosomal thiol reductase, tripeptidyl-peptidase 1, ganglioside GM2 activator) were significantly more secreted in *JAGN1* KO clones compared to control clones.
The histone H2A was significantly less secreted in stimulated *JAGN1* KO clones compared to stimulated control clones. Other histones (such as histone H2B type-1, core histone macro-H2A.1, histone H3, histone H2 type-1, histone H4, and histone H1.2) showed also a reduction of secretion in the *JAGN1* KO clones but only in one experiment (figure 9).

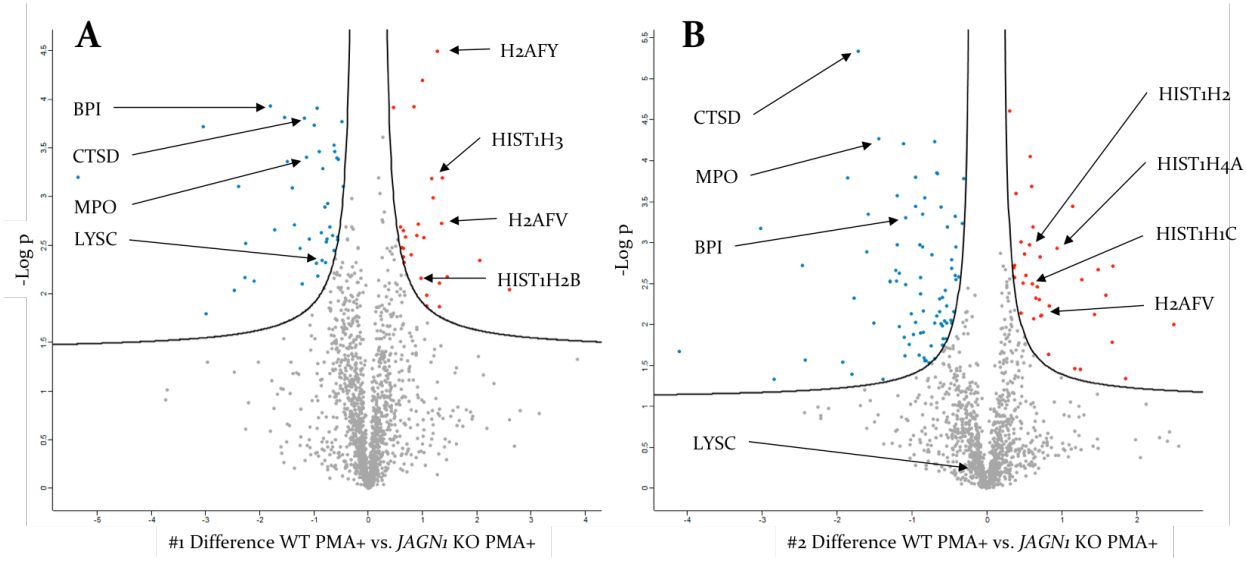


Figure 9: Volcano plots of secreted proteins of iPSc-derived JAGN1 KO neutrophil granulocytes in comparison to WT controls. *Supernatants were collected and analysed with mass spectrometry as described in material and methods. Blue dots indicate proteins that are secreted at significantly reduced levels, red dots indicate proteins that are secreted at higher levels. Control clone after incubation with 1,5µM PMA for 25min. at 37°C vs. JAGN1 KO clone in the same conditions. Highlighted are BPI (bactericidal permeability-increasing protein), CTSD (cathepsin D), MPO (myeloperoxidase), LYSC (lysozyme C), HIST1H2B (histone H2B type-1), H2AFY (core histone macro-H2A.1), HIST1H3 (histone H3), H2AFV (histone H2A), HIST1H2 (histone H2 type-1), HIST1H4A (histone H4), HIST1H1C (histone H1.2); **(A)** Experiment 1. **(B)** Experiment 2.*

Additionally, almost all proteins that were more abundant in the MS-analysis in the absence of JAGN1 were N-glycosylated proteins. There was no distinct pattern with respect to post-translational modification in less secreted proteins.

In principle, these data can be reconciled with with two possible and non mutually exclusive interpretations. The differences in protein secretion could possibly be due to a higher degree of apoptosis in the absence of JAGN1. Alternatively, JAGN1 might specifically control secretion of defined proteins such as granule proteins and lysosomal hydrolases. Since histone proteins were found at lower levels in supernatants of JAGN1-deficient cells, it is possible that NETosis is impaired in JAGN1-deficiency.

In an attempt to distinguish between these interpretations and to directly visualize secretion, we switched to another model system and analysed the kinetics of secretion in adherent HeLa cells.

5.2. CRISPR/Cas9 mediated *JAGN1* knock-out in HeLa cells

In a first step, JAGN1-deficient HeLa cells were created using CRISPR/Cas9 mediated gene editing. *JAGN1-deficient* clones were tested by Western blot. Nine out of 68 clones showed no JAGN1-specific band after incubation with the anti-JAGN1 polyclonal rabbit antibody. Actin was used as a loading control for all samples (figure 10).

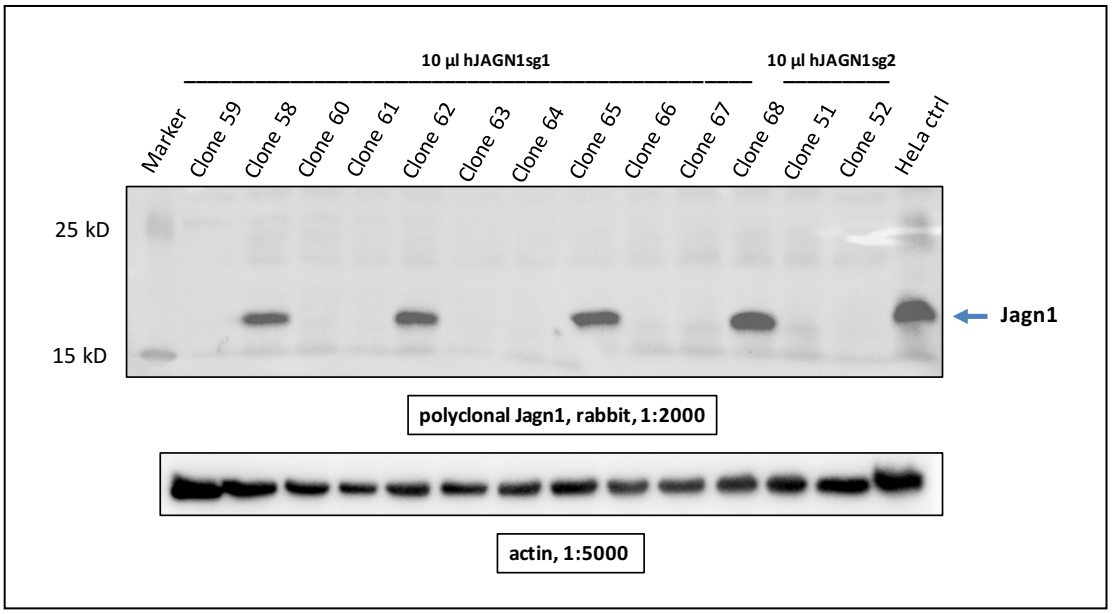


Figure 10: Absence of Jagn1 in cellular lysates of HeLa JAGN1 KO clones. *Nine positive CRISPR/Cas9 mediated JAGN1 KO clones in HeLa cells were detected in Western blot. 15%*

SDS gel, 1:2000 JAGN1 polyclonal rabbit antibody, 1:5000 secondary anti-rabbit HRP. As loading control actin antibody was used 1:5000.

To confirm the KO of *JAGN1* in HeLa cells the DNA was sequenced using the *JAGN1* primer hJAGN1_fwd and hJAGN1_rev as described in material and methods. In all nine clones *JAGN1* was mutated. For further experiments, two clones with frameshift mutations were chosen. Clone one showed a homozygous two base pair deletion, and clone two had a homozygous one base pair insertion at position 23 of the *JAGN1* coding sequence (figure 11).

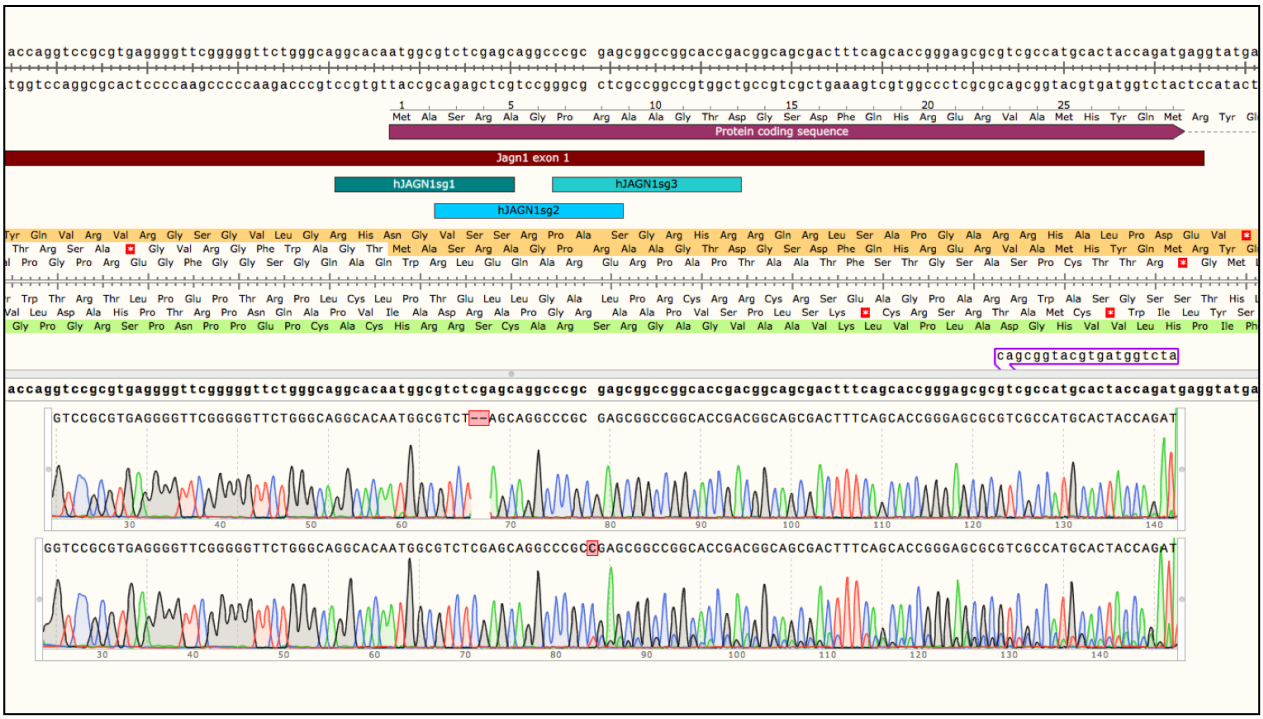


Figure 11: Sequencing analysis of CRISPR/Cas9 mediated JAGN1 KO clones. *Two clones showed frame shift mutations in JAGN1 coding sequence by sequencing analysis. The first clone exhibited a two base pair deletion at position 10 and 11. The second clone had a one base pair insertion at position 23 of the JAGN1 coding sequence.*

Thus, two JAGN1-deficient HeLa clones were successfully generated. These cells were used for further analysis of impaired secretion in the absence of *JAGN1*.

5.3. Impaired vesicular trafficking of lysozyme C in JAGN1-deficient HeLa cells

The *JAGN1* KO HeLa clones were generated to study the influence of JAGN1 on intracellular protein trafficking and secretion of proteins.
Lysozyme c was chosen as reporter protein for several reasons. Lysozyme c was identified in the secretome of JAGN1-deficient iPSC derived-neutrophil like cells in higher levels when compared to control cells. And lysozyme c is a granule protein, which is present in all three subtypes of neutrophil granules.
As the secretion of proteins is a highly dynamic process it is crucial to monitor the kinetics of this process. The retention using selective hooks (RUSH)-system was established by Boncompain and Perez (Boncompain and Perez 2012) to study secretory cargo sorting. The system allows synchronizing and visualizing the release of candidate secretory reporter proteins tagged with EGFP. The reporter is retained in the ER by interacting with its streptavidin binding protein (SBP) sequence to a streptavidin-KDEL hook that is co-translationally inserted into the ER compartment. Upon biotin addition to the cell culture medium, the reporter is released from the hook and its transport kinetics is followed by fluorescent microscopy (figure 12).

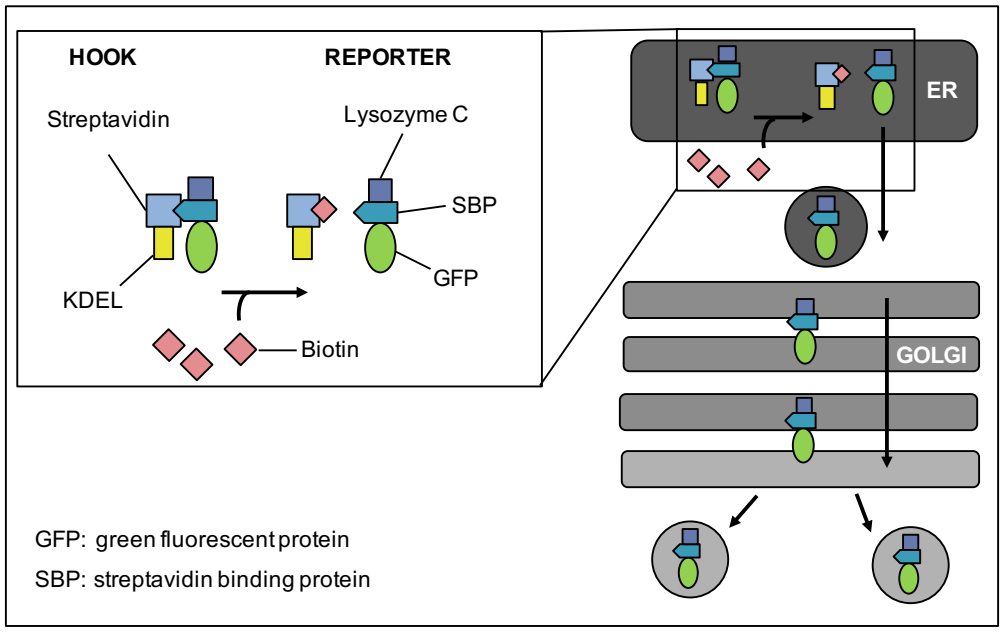
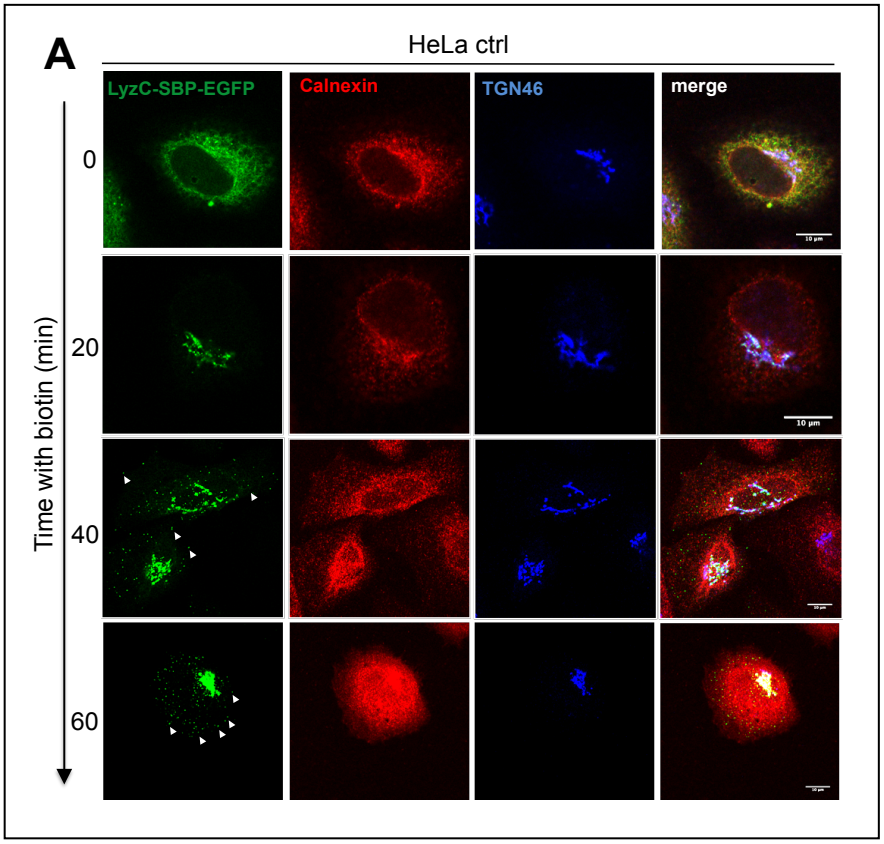


Figure 12: Molecular basis of the retention using selective hooks- system. *The KDEL sequence that is fused to streptavidin retains the hook in the endoplasmic reticulum (ER). Under steady-state conditions the hook binds the reporter of interest (in this case lysozyme C) by its affinity to streptavidin binding peptide. After the addition of excess of biotin the fusion between the hook and the reporter is outcompeted and the reporter can travel to the*

next compartment, such as the Golgi apparatus (GOLGI) and its final destination. The reporter is visualized by the fusion to a fluorescent protein, such as GFP. Adapted from (Boncompain and Perez 2012).

HeLa control cells and HeLa *JAGN1* KO cells were transfected with the Lysozyme C-RUSH construct pIRESneo3-Str-KDEL_LyzC-SBP-EGFP .
The RUSH system was used to analyse defective protein trafficking in HeLa *JAGN1* KO cells. In absence of biotin, by using SBP-EGFP tagged lysozyme C (LyzC-SBP-EGFP) as a reporter, we observed its localization and ER retention by co-localizing with ER marker calnexin. After 20 min. of biotin addition, the reporter co-localized with TGN marker TGN46 to the Golgi apparatus. After 40 and 60 min. of biotin addition, LyzC-SBP-EGFP localized to the Golgi apparatus and to secretory vesicles. The experiment was done three times and representative images are shown in figure 13.



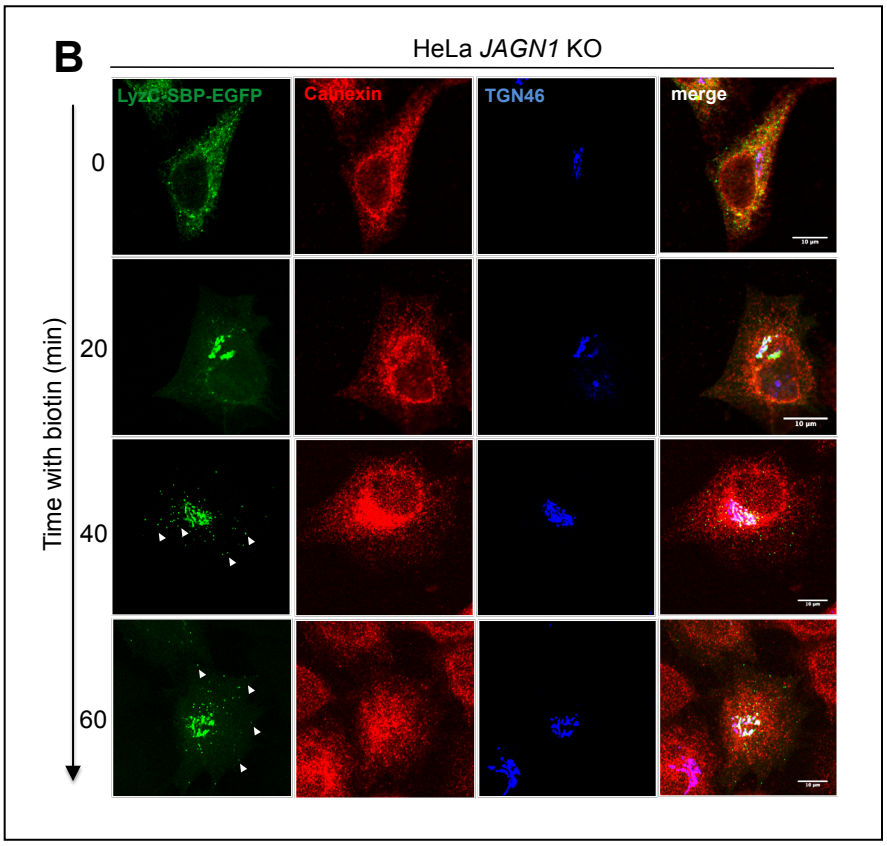


Figure 13: Immunofluorescent images of HeLa control and HeLa JAGN1 KO cells after transfection with RUSH plasmid LyzC-SBP-EGFP. *HeLa cells were transiently transfected with RUSH reporter protein LyzC-SBP-EGFP for 24 h. To study LyzC-SBP-EGFP transport kinetics cells were fixed with PFA at time points 0, 20, 40 and 60 min. after biotin addition. Images were acquired by fluorescence microscopy. Calnexin antibody labelled with Alexa594 was used as an ER marker and TGN46 antibody labelled with Alexa594 as a trans Golgi network marker. Arrows highlight secretory vesicles. Experiment was done three times. Representative images are shown for HeLa control cells (A) and HeLa JAGN1 KO cells (B).*

LyzC-SBP-EGFP secretory vesicles were quantified using FIJI software and a programmed customized ImageJ macro plugin and compared between HeLa control cells and CRISPR/Cas9 mediated *JAGN1* KO HeLa cells. Whereas at time point 20 min. no significant difference was seen, WT and JAGN1 KO cells showed differences after 20 and 40 min, resprectively. The mean number of EGFP tagged lysozyme c positive vesicles in HeLa control cells and HeLa *JAGN1* KO cells after 40 min. were 59.40 (SEM = 27.99) and 36.75 (SEM = 25.24), respectively ($p<0.001$). After 60 min. there were significantly ($p<0.05$) less EGFP tagged lysozyme C positive vesicles in *JAGN1* KO HeLa cells (mean = 62.19; SEM = 29.67) compared to controls (mean = 49.61; SEM = 29.95) (figure 14).

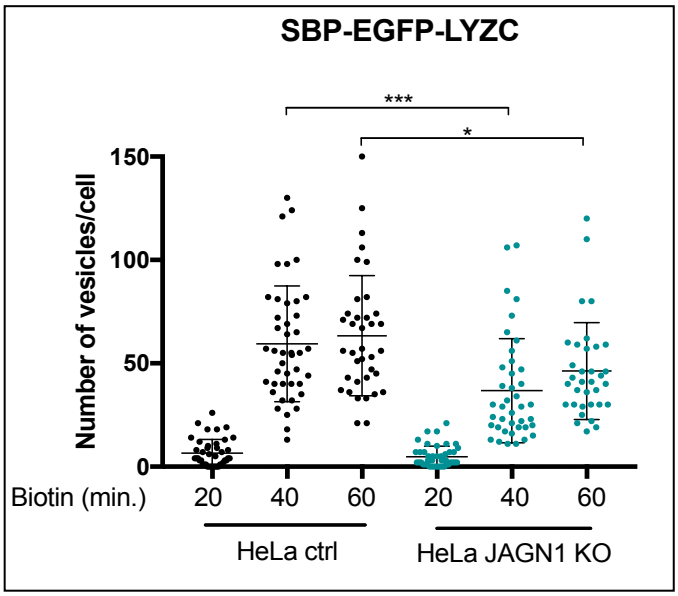


Figure 14: Quantification of LyzC-SBP-EGFP secretory vesicles. *The number of LyzC-SBP-EGFP vesicles per cell were quantified in HeLa control cells or JAGN1 KO after 20, 40, and 60 min. of biotin addition using custom-made ImageJ macro plugin (mean ± SEM, n= 37-42 cells); * p< 0.05, *** p< 0.001, (one-way ANOVA).*

Thus, the vesicular transport of Lysozyme C was delayed in JAGN1-deficient cells, indicating that the vesicular trafficking in *JAGN1* KO cells for lysozyme C is defective. At first sight, this finding is in contrast to our observation of increased lysozyme C secretion in JAGN1-deficient neutrophil granulocytes. However, the experimental conditions cannot easily be compared. iPS derived neutrophils are different cells, furthermore they were stimulated with PMA. Taken together, both experimental systems document that the secretory pathway and vesicular trafficking are affected in JAGN1-deficient cells.

6. Discussion

The reason for studying vesicular trafficking in JAGN1-mutated cells was mainly to elucidate the relation between the ER located protein JAGN1 and the phenotype of JAGN1-deficient patients suffering from SCN. Although there is evidence that JAGN1 is involved in secretion the detailed mechanism is not known so far. Therefore, this thesis aimed to shed light the role of the JAGN1 protein in the secretory pathway using novel cellular model systems.

6.1. Interpretation of the results in view of current scientific knowledge

The release of granule proteins of neutrophil granulocytes has to be regulated in a sensible way to avoid proteostatic stress to neutrophils, to prevent tissue damage and to fulfil their duty to kill pathogens. Therefore, the content of granules has to be controlled during the development of the neutrophils.
The main findings of the secretome data showed the loss of JAGN1 seems to impair this delicate balance. In JAGN1-deficient cells, there were consistent differences in protein abundance. In JAGN1-deficient neutrophil granulocytes, MS analysis of secreted proteins revealed an increased abundance of granule proteins and lysosomal hydrolases. Furthermore, at least one subtype of histones was significantly less abundant in *JAGN1* KO clones. Because the vast majority of secreted proteins was not affected in the absence of JAGN1, the data suggest that there is rather a specific defect. In particular secretion of specific proteins upon stimulation with PMA appears to be dependent on the presence of JAGN1 in iPSc-derived neutrophil-like cells.

To visualize protein trafficking intracellularly, the RUSH-system provides an excellent option. The CRISPR/Cas9 mediated HeLa *JAGN1* KO cells showed a delay of transporting the granule protein lysozyme C into vesicles. Thus, JAGN1 is necessary for proper lysozyme C vesicular trafficking in HeLa cells. Although the studies were not done in neutrophils it is remarkable that lysozyme has been described as one of the most effective enzymes in host defence by destroying the wall of pathogens (Callewaert and Michiels 2010). Furthermore, it is stored in all subtypes of granules. If the intracellular trafficking of such an essential protein of neutrophils is perturbed, one might speculate that neutrophils are not able to fulfil their function properly.

Interestingly, the affected granule proteins in the secretome data were MPO and BPI as well as cathepsin D, that are all granule proteins stored in the azurophilic granules. This observation is in line with earlier findings that JAGN1-deficient neutrophils exhibit a

paucity of primary granules (Boztug, Jarvinen et al. 2014, Wirnsberger, Zwolanek et al. 2014). Of note, our studies confirmed that protein expression of BIP is increased in *JAGN1*-mutated granulocytes, again in line with previously published data from primary human neutrophil granulocyts (Boztug, Jarvinen et al. 2014).
Based on observations in Drosophila mutants, Lee et al. have come up with the hypothesis that JAGN1 may be important to orchestrate nutrient transport through feeder cells during vitellogenesis (Lee and Cooley 2007). Our data in JAGN1-deficient cells, both in iPS-derived neutrophils and in HeLa cells, confirm a specific role of JAGN1 in controlling vesicular transport.
Nevertheless, it is not yet clear how an ER-resident protein directly influences the secretory pathway. The secretion defect in JAGN1-deficient cells could be secondary to a defect of the COPI complex. The sorting machinery of the COPI complex might depend on proper function of JAGN1. The COPI complex orchestrates the first checkpoint in the early secretory pathway between the ER and the ERGIC and is involved in intraGolgi transport (Duden 2003). Through mutations in JAGN1 the interaction between COPI and JAGN1 might be dysregulated and subsequently the maintainance of ER- and Golgi-resident proteins is disturbed. Too many proteins consecutively accumulate in the Golgi apparatus. One might speculatet that an overload of the TGN might lead to dysregulation of sorting machineries in the TGN and finally in secretion defects, including lysosomal hydrolases and granule proteins.

It is possible that JAGN1 is involved in sorting hydrolases into granules through a regulated pathway. As there were mainly lysosomal hydrolases affected in the secretome study, it is thinkable that lysosomal hydrolases that should be routed to granules by a regulated pathway are wrongly sorted into the constitutive pathway in the absence of JAGN1. As mentioned above the constitutive pathway is depending on M6P and MPR. Subsequently, lysosomal hydrolases are rather constitutively secreted than stored into the granules. This missorting in the Golgi apparatus could explain the higher abundance of the mentioned proteins in the extracellular space.
Sorting of lysosomal hydrolases is depending on M6P moieties on their N-linked oligosaccharide chains. JAGN1-deficient cells showed N-glycosylation defects. As most of the significantly more abundant proteins in the MS-analysis in the absence of JAGN1 were N-glycosylated, the dysregulation in sorting might be related to glycosylation. This would also confirm the findings of Boztug et al. that N-glycosylation defects play a role in the pathophysiology of JAGN1 deficiency (Boztug, Jarvinen et al. 2014). It has also been recently described that azurophilic granule proteins (such as MPO and BPI) are dominated by paucimannosidic N-linked carbohydrate structures (Thaysen-Andersen, Venkatakrishnan et al. 2015), which are added in the ER and modified in the Golgi apparatus (Hang, Lin et al. 2015).

As described before, missing N-glycans could result in ER retention (Vagin, Kraut et al. 2009). The knowledge that so called paucimannosidic N-linked carbohydrate structures are only found in azurophilc granules, would agree with the finding that proteins from azurophilic granules are affected. If JAGN1 is participating in glycosylation in the ER the lack of N-glycans on proteins in the absence of JAGN1 would be followed by a higher rate of proteins in the ER and finally culminate in ER-stress. If the so far described glycosylation defects play a specific role for the transport of lysozyme C is not clear. It has been described that knockdown of a transport machinery of the early secretory pathway perturbs glycosylation followed by a slower rate of secretion in the Golgi apparatus (Wakana, van Galen et al. 2012).

Furthermore, many proteins that were detected in the supernatants of JAGN1-deficient cells showed no signal sequence or annotation as being secreted. This raises the question if there are unconventional secretory pathways. Another explanation to the fact, that some proteins showed no signal sequence could be a higher rate of apoptosis in the abesnce of JAGN1. If lysosomal hydrolases are not any more efficiently packed into the regulated secretory pathway and subsequently into storage granules but are transported unregulated, this can lead to an accumulation of lysosomal hydrolases and consequently to toxic effects in the cell. This might explain the higher rate of apoptosis in JAGN1-deficient cells, which has been described before (Boztug, Jarvinen et al. 2014). For example, cathepsin D was more secreted in *JAGN1* KO clones compared to control clones in the stimulated and unstimulated conditions. Cathepsin D was shown to induce neutrophil apoptosis through direct cleavage of caspase-8 in human and mouse neutrophils (Conus, Perozzo et al. 2008, Conus, Pop et al. 2012). Thus, more secretion of cathepsin D in the absence of JAGN1 could be related to the higher susceptibility to apoptosis.

Another possibility is, that the secretion defect is related to ER stress. As mentioned above, increased synthesis of secretory proteins can lead to ER stress. The presence of JAGN1 might participate in the quality control in the ER. Loss of function of JAGN1 possibly perturbs the export of proteins from the ER into the early secretory pathway. The mechanism behind that needs further biochemical analysis. But it has already been shown that loss of JAGN1 leads to an increasing ER-stress, including enlarged ER structures (Wirnsberger, Zwolanek et al. 2014).

It has been demonstrated that the subtypes of granules vary in their kinetics of mobilization in response to inflammatory signals. The mechanism is not understood but the calcium levels of the cell appear to are involved (Amulic, Cazalet et al. 2012). It is also known that intracellular calcium levels are mainly regulated by the ER (Capiod 2011, Pinto,

Kihara et al. 2015). If JAGN1 is involved in calcium homeostasis this might be an indication why the kinetics of lysozyme C rich vesicles was impaired in the absence of JAGN1. Consequently the kinetics of mobilization of the granules to the cell surface might also be linked to the presence of JAGN1 by its role in the organization of the ER.

As mentioned above PMA stimulates NETosis including the involvement of histones. Less secretion of histone H2A in *JAGN1* KO clones could be related to less efficient formation of NETs in the absence of JAGN1 (Hirsch 1958, Park, Yi et al. 2000). As recently described the release of MPO in histones was reduced in the absence of JAGN1 (Khandagale, Lazzaretto et al. 2018). This finding might be connected with our observation that MPO is more secreted in the absence of JAGN1. If MPO was rather released by the constitutive pathway than stored to granules the percentage of MPO for the formation of NETs would be also impaired.

Taken together, our data, interpreted in light of current knowledge, does not yet allow a definitive conclusion on the exact mechanisms how JAGN1 is involved in the secretory pathway. JAGN1 can either directly or indirectly be involved in the sorting of granule proteins of azurophilic granules and lysosomal hydrolases.

6.2. Limitations of the experiments

Even though our findings document a role for JAGN1 in controlling the secretory pathway, several methodological caveats have to taken into consideration.
First, the secretome analysis and the RUSH-experiment both were not performed in primary neutrophil granulocytes but in cellular model systems. IPSc-derived neutrophils behave in many respects like primary neutrophil granulocytes: they share expression of the genes that are physiologically expressed, they roll, adhere, and transmigrate like primary neutrophils, they phagocytose and kill bacteria like primary neutrophils (Mizoguchi et al, unpublished). However, the secretome of iPS derived neutrophils has not yet been directly compared the secretome of primary neutrophils.

The RUSH-system was performed in HeLa cells, which reflects a model to study general defects in trafficking rather than the pathophysiology of neutrophils. In HeLa cells lysozyme C is not stored into granules but directly released into the extracellular space. It is unknown whether the secretory pathway in HeLa cells is also present in neutrophils. Clearly, neutrophil granulocytes need to assemble a machinery of primary, secondary and tertiary granules. However, sorting of cargo at the TGN might be related to the phenotype of SCN patients with JAGN1-deficient neutrophils. But too little is known about the sorting mechanisms in neutrophils.

Even though primary neutrophil granulocytes from patients and healthy control individuals would, in principle, represent the best cellular model system, there are several limitations. First, JAGN1-deficiency is a very rare disease, currently there are less than 20 families described worldwide. Second, affected patients are children and thus represent a highly vulnerable group. For ethical reasons, one cannot simple perform venipunctures to obtain blood samples for basic science studies. Third, it is technically quite challenging to study secretory mechanisms in primary neutrophils. They are postmitotic and quite short-lived cells and cannot easily be genetically manipulated.

Furthermore, it has to be mentioned that the results of the two experiments are not comparable for two reasons. As mentioned above, the data was collected in different cell systems. And the point of time at which the supernatants for the MS data was collected and the point of time at which the IF images were taken for the RUSH experiment varied. This might explain why lysozyme C was only in the secretome of one PMA stimulated JAGN1 clone significantly more secreted (in the other KO clone it was also more secreted but not significant). Nevertheless, the cause for the results of the two different approaches might be the same.
Lastly, although most of the secreted proteins are glycosylated, the findings do not indicate a clear evidence for the involvement of glycosylation in the secretion defects. It would be necessary to discriminate if the significant different abundant proteins show also abnormal N-glycans.

Regarding the results of the RUSH-system it has to be discussed that the delay of lysozyme C was significant in two time points (40 min. and 60 min. after addition of biotin). This indicates that the lower rate of secretion is not compensated immediately. Even though, there has to be considered, that there might occur compensation of this delay in later time-points. This has to be analysed in further experiments including later time points.

6.3. Outlook and open questions

Several open questions that deserve additional research remain.
MS-analysis as presented in this thesis could be used for more comprehensive data in additional cell lines, to determine if the secretome of SCN subtypes is genotype-dependent in neutrophils from patients as well. To enhance the statistical read-out of secretome studies, it would be essential to use more cells and analyse a third clone in furhter experiments.

Even though this work suggests that the effects of JAGN1 are quite specific and not global, this hypothesis should be tested in greater detail. For example, the kinetics of other granule proteins, such as MPO, BPI or cathepsin D could be monitored using the RUSH-system. This would answer the question, if the delay of lysozyme C rich vesicle formation is a protein specific phenotype of JAGN1 deficiency. Similar methods than the RUSH-system might also be useful to analyse trafficking defects in other diseases, such as VPS45 deficiency, LAMTOR2 deficiency, Chediak-Higashi-syndrome or Hermansky-Pudlak syndrome type 2.

It remains currently unclear whether the observed phenotype in HeLa cells is a cell-type specific delay in vesicular transport or is causative for JAGN1 deficiency. Therefore, the RUSH system might be adapted for neutrophils.

Further biochemical analysis might shed light in the unknown mechanisms of secretion and solve open questions. For example, there exists no data how exactly the protein JAGN1 is participating in the secretory pathway in neutrophils. Another aspect of interest is how the regulated secretory pathway is orchestrated in neutrophils. This mechanism is poorly understood and definitely needs more investigation.
Additionally, many biochemical aspects of NET formation are not completely understood. The observed lower abundance of histones in the absence of JAGN1 could possibly discover JAGN1 as a new component in this complex machinery. But it clearly needs further analysis, which might be challenging.
Analysis of further patients with mutations in *JAGN1* is necessary to fully explained how JAGN1 participates in the differentiation, maintenance and decay of neutrophils.

Taken together, this thesis has made use of novel technologies (iPS-derived neutrophil granulocytes, Crispr/Cas9 gene editing, RUSH system) to study the role of JAGN1 in the secretory pathway of neutrophil granulocytes. We could document that JAGN1 orchestrates the secretion of specific proteins, in particular lysozyme C. The details of the molecular mechanisms deserve further scientific experiments.

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