The role of ATG16L1 in chronic inflammatory bowel disease

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1 INTRODUCTION

1.1 The role of autophagy in cell homeostasis and inflammation

Autophagy is a dynamic process for capturing cytosolic components like defective proteins or organelles and delivering them to lysosomes for hydrolytic degradation. This multistep process is evolutionarily conserved and documented by the high conservation of the involved proteins which are encoded by Autophagy-related (ATG) genes, throughout the entire animal kingdom. The regulatory mechanisms are highly similar from yeast to mammals. The term “autophagy” is derived from the Latin words for “self” and “eating” and the primary role of this mechanism is to protect cells under stress conditions, such as starvation. Degrading cytoplasmic material during periods of starvation provides the background to produce amino acids and fatty acids that can be used to synthesize new proteins or are oxidized by mitochondria to produce Adenosinetriphosphate (ATP) for cell survival. Defects in autophagy are associated with many diseases, including neurodegenerative diseases, tumorigenesis, diabetes and Crohn disease. There are three different types of autophagy, including macroautophagy, chaperone-mediated autophagy and microautophagy. Macroautophagy (here referred to as ‘autophagy’) is essential for cell homeostasis and subject of this work. It uses specialized cytosolic vesicles that fuse with the lysosomes. By contrast, microautophagy is the direct uptake of cytoplasm at the lysosome surface by invagination of the lysosomal membrane. At last, chaperone-mediated autophagy takes place at the lysosomal membrane, as well. It is a mechanism to translocate unfolded proteins across the membrane.

During autophagy, an expanding double-membrane structure termed the ‘phagophore’ or ‘isolation membrane’ engulfs portions of the cytoplasm. After completion to a closed autophagosome it fuses directly or indirectly with lysosomal vesicles for hydrolytic degradation of the enclosed material. The way of autophagosome formation differs from vesicle formation in other membrane trafficking processes, such as endocytosis and the secretory pathway. In these pathways transport vesicles are generated by sprouting and scission from the membranes of donor organelles. In contrast, autophagosomes are described to be formed in the cytosol in a de novo process. This formation depends on two protein conjugation systems, the ATG12-ATG5 and the ATG8-phosphatidyl ethanolamine...
systems which both act as ubiquitin-like ligation systems\textsuperscript{12}. The following steps are involved\textsuperscript{2} (see Figure 1-1):

**I)** The formation of the phagophore (isolation membrane) is instituted by Beclin/VPS34 complex at the endoplasmic reticulum (ER) or other membrane systems and controlled by mTOR (target of Rapamycin) and class I PI3K (phosphatidylinositol-3-kinase 1) signaling pathway.

**II)** The formation of the ATG12-ATG5 conjugate depends on two enzymes, ATG7 (E1-like) and ATG10 (E2 like). Afterwards, in addition to the covalent interaction with ATG12, ATG5 interacts non-covalently with ATG16L1. Because ATG16L1 has a self-assembling character, the ATG12-ATG5 conjugate forms a multimeric complex with ATG16L1. The complex adheres to the phagophore, mediated by ATG5.

**III)** After processing the C terminus of LC3 by ATG4 to expose the glycine residue, the C-terminal glycine is attached to phosphatidyl ethanolamine (PE) by the E1-like activating enzyme ATG7 and the E2-like conjugation enzyme ATG3. The lipidated form of LC3 is associated with the autophagosomal membrane.

(During formation of the autophagosome, the ATG12-ATG5-ATG16L1 complex (named ATG16L1-complex) and the LC3-PE (LC3-II) cover the expanding phagophore. The ATG16L1-complex is situated mostly on the outer side, whereas LC3-II is found on both sides.)

**IV)** Selective or non-selective cargo is engulfed. When the autophagosome formation is completed, the ATG16L1-complex is released into the cytosol. Concurrently, a significant amount of LC3-II stays in the completed autophagosome.

**V)** The autophagosome fuses indirectly via fusion with late endosomes and formation of amphisomes or directly with the lysosomal compartment. The contents get hydrolyzed and monomers are recycled.
Figure 1-1: Steps of autophagosome formation.
I) Phagophore (isolation membrane) formation by Beclin/VPS34 complex at the endoplasmic reticulum (ER) or other membrane systems under the control of the inhibiting TOR (target of Rapamycin) and class I PI3K (phosphatidylinositol-3-kinase 1) signaling pathway; II) the ATG5-ATG12 complex assembles, supported by ATG16L1 and binds as a multimer to the phagophore; III) processing of LC3 (yeast Atg8) and insertion into the expanding phagophore membrane; IV) engulfment of random or selective cargo, closure of the autophagosome, partial recycling of LC3; V) fusion of the autophagosome with the lysosomal compartment (directly or indirectly via fusion with late endosomes and formation of amphisomes), hydrolysis of the cargo and recycling of monomers. Source: S.Billmann-Born et al. / European Journal of Cell Biology (2011)

Autophagy was originally described to be a non-selective mechanism, being characterized by autophagosomes primarily containing bulk cytoplasmic material. But it is now known that autophagosomes can engulf and degrade substrates in a selective manner\(^\text{13}\). During selective autophagy, only relevant cargoes are isolated into autophagosomes, which accordingly have contours that resemble those of the cargoes. The autophagosomes generated under these conditions contain little bulk cytosol between the cargo and the inner membrane. Examples of selective autophagy are Cvt vesicles (cytoplasm-to-vacuole transport vesicle in yeast), mitophagy (degradation of damaged mitochondria), pexophagy (degradation of peroxisomes), xenophagy (degradation of intracellular viruses and bacteria) and
reticulophagy (degradation of endoplasmic reticulum). Cellular cargo is commonly targeted to autophagosomes by interactions between a molecular tag (such as polyubiquitin), adaptor proteins such as p62/SQSTM1 or NBR1 and LC3. The adaptor proteins recognize the molecular tags and contain an LC3-interacting region (LIR). They enable autophagy to target cargo selectively to growing LC3-positive phagophores. If autophagy-protein-dependent mechanisms fail in immunity, this may afford not only an increased susceptibility to infection, but also to chronic inflammatory diseases and autoimmune diseases like for example Crohn disease (CD). Beside these functions autophagy has important roles in protecting against cell death induced by ER stress. Blocking autophagy by chemical inhibition or deletion of an ATG gene (ATG5, ATG6/Beclin1, ATG8/LC3) in vitro, leads to an increased susceptibility to ER stress-mediated apoptotic cell death. Conversely, induction of autophagy by treatment with the mTOR inhibitor rapamycin, leads to a higher resistance to endoplasmic reticulum (ER) stress in cells.

To sum up, in addition to stress management autophagy is involved in immunity, defense against microbial invasion, normal development, senescence and lifespan extension. Also in many human pathophysologies it plays a role: for example cancer, myopathies, neurodegeneration, heart and liver disease, and gastrointestinal disorders.

1.2 The role of autophagy in endoplasmic reticulum stress

Protein synthesis and maturation (including correct folding) is not the only function of the endoplasmic reticulum (ER). It may also be a major source for building the autophagic isolation membrane by forming omegasomes. ER stress is triggered when unfolded proteins accumulate in the ER due to advanced input of proteins (e.g. when protein synthesis is increased or the protein secretion from the ER is defective) or decreased capacity of the ER to fold proteins (e.g. upon changes in the Ca\(^{2+}\), oxygen and glucose homeostasis). Notably, ER stress is also induced when unfolded proteins accumulate in the cytosol. The unfolded protein response (UPR), the major ER stress pathway, is directly linked to autophagy (see Figure 1-2). There are three branches of the UPR, mediated by three ER membrane-associated proteins, PERK (protein kinase RNA-like endoplasmic reticulum kinase), ATF6 (activating transcription factor) and IRE1 (inositol requiring enzyme 1).
**PERK** arranges the transcriptional activation of the autophagic proteins LC3 and ATG5 in hypoxic responses via the transcription factors ATF4 and CHOP\(^34\). Furthermore, autophagosome formation is induced by the eukaryotic initiation factor 2α (eIF2α) downstream of PERK under conditions of ER stress by inducing LC3 conversion and ATG12 upregulation\(^20\). An additional report of Kouroku et al. confirms this correlation by demonstrating that ER stress (PERK/eIF2α phosphorylation) enhances the ATG16L1 complex-dependent LC3 conversion from LC3I to LC3II. The group proposed that autophagy is a cellular defense mechanism against polyQ72-induced ER-stress-mediated cell death by degrading polyQ72 aggregates\(^20\).

**IRE1**, a kinase and endoribonuclease directly upstream of XBP1 can induce activation of JNK signaling under conditions of ER stress, via its interaction with TRAF2\(^35\). JNK is directly able to induce autophagosome formation via LC3\(^36,37\). XBP1 deletion results in spontaneous enteritis and increased susceptibility to induced Colitis in addition to Paneth cell dysfunction. The hypomorphic XBP1 function is described to cause an overactivation of IRE1 along with increased JNK phosphorylation in small intestinal epithelium of Xbp1-deficient mice, leading to autophagosome formation under conditions of ER stress\(^38\) (see Figure 1-2).

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**Figure 1-2: The link of UPR and autophagy**

ER stress activates unfolded protein response (UPR) signaling mediated by three ER membrane-associated proteins, PERK (protein kinase RNA-like endoplasmic reticulum kinase), ATF6 (activating transcription factor) and IRE1 (inositol requiring enzyme 1). The responses are linked to the induction of autophagy via increasing the expression level of ATG5, ATG12 and LC3, further via leading to LC3 conversion or inhibition of the autophagy induction.
Another example demonstrating the linkage between ER stress and autophagy is given by studies of genetic Atg7 deficiency in mice. Atg7 deficiency correlates with decreased autophagy and increased ER stress in liver. As well, the Atg7 deficiency results in impaired insulin signaling, which was previously reported as a consequence of pathological ER stress in obesity. Further, the deletion of ATG5, Beclin1 or LC3 leads to an advanced susceptibility to apoptotic cell death during ER stress, as mentioned before.

At last, in a recent study Kimura et al. demonstrated that no protein delivery to vacuoles could be reported in the absence of autophagic activity in Aspergillus oryzae. Hence, they conclude that autophagy is responsible for the delivery of misfolded secretory proteins accumulated in the ER to vacuoles for degradation.

1.3 The autophagy protein ATG16L1

The Atg16l1 gene (human: 3411base pairs (bp), RefSeq.NM_030803; mouse: 3149bp, RefSeq.NM_029846) is located on chromosome 2 on position 37.1 and encodes a protein involved in the formation of autophagosomes during autophagy. The autophagy protein ATG16L1 was identified in 2003 by Mizushima et al. and exhibits three isoforms: ATG16Lα, ATG16Lβ and ATG16Lγ. The human/mouse homology is defined by a protein identity of 94% and a DNA identity of 89.1% (generated with “HomoloGene”; human: gi|124256478|ref|NP_060444.3; mouse: gi|27777650|ref|NP_084122.2). It is named after the weak but significant homology in its N-terminal region (amino acid (aa) 106-208) with yeast APG16 (amino acid (aa) 28-145). ATG16L1 is an indispensable protein, whose deficiency, similar to the lethal phenotype of ATG5- and ATG7-deficient mice, leads to death within the 1st day of delivery. This indicates that ATG16L1 is required for neonatal starvation. It is ubiquitously expressed and consists of an N-terminal domain involved in ATG5 binding, a central coiled coil domain required for self oligomerization, and a C-terminal WD40 repeat domain whose role is entirely unknown in this protein (see Figure 1-3). In general, the WD-repeat domains are thought to have the common feature of folding into a beta propeller and forming a platform without any catalytic activity. Hence, it provides the opportunity for multiple protein complexes to assemble reversibly.
ATG16L1 forms a complex with the ATG12-ATG5 conjugate resulting in the ATG16L-ATG5-ATG12 complex, commonly named ATG16L complex. This complex has the capacity of a new type of E3-like enzyme and functions as a scaffold for LC3 lipidation by dynamically localizing to the putative source membranes for autophagosome formation. These findings were supported by results of Saitoh et al. who illustrate that an ATG16L1-deficiency in mouse embryonic fibroblasts (MEFs) hinders the recruitment of the ATG12-ATG5 conjugate to the isolation membrane thus resulting in the loss of LC3 conjugation to phosphatidyl ethanolamine (PE) and the complete inhibition of the basal autophagy-machinery. But it is shown that the truncation of the whole C-terminal WD40 domain had no effect on dimerization with ATG5. Further, the autophagic machinery was implicated in the NLR system by Travassos et al. via demonstrating that in case of pathogen invasion the intracellular sensors NOD1 and NOD2 recruit ATG16L1 to the plasma membrane at the bacterial entry site.

1.3.1 The role of ATG16L1 in Paneth cell vesicle export

1.3.1.1 Paneth cells

Paneth cells are part of the intestinal barrier, which is critical for health and disease. A layer of mucus, produced by specialized goblet cells, is the first line of defense of this barrier. Various bactericidal and bacteriostatic proteins that act together as a primary barrier are included in the mucus. The epithelial monolayer of the gastrointestinal tract provides a physical barrier between the external environment and the circulation. Resident microflora colonizes the lumen, hence being in contact with the apical brush borders, where nutrient absorption occurs. In this setting, epithelial transport must be balanced by keeping most microbial cell populations in check. It is suggested that innate immune mechanisms exist to counter microbial
colonization because of the low bacterial numbers in the small intestine relative to the
distal ileum, cecum, and colon. Mediating this activity is the major role of Paneth
cells, which secrete gene-encoded antimicrobial peptides and additional host
defense proteins into the lumen\textsuperscript{50,51}. These cells are 1 of 4 lineages that are
produced by stem cells localized in the crypts of Lieberkühn. Paneth cells are
unusual in comparison with the other intestinal epithelial lineages (absorptive
enterocytes, mucus secreting goblet cells and hormone secreting enteroendocrine
cells). At the base of the crypts, Paneth cells live >30 days. Their main function is to
produce, package, and export a wide variety of antimicrobial proteins including an
array of $\alpha$-defensins (cryptdins), lysozyme, angiogenin-4, and secretory
phospholipase A2\textsuperscript{50,52}. To produce these proteins, mature Paneth cells contain an
abundant endocytoplasmic reticulum, golgi apparatus, as well as distinct cytoplasmic
granules, which are the hallmark of Paneth cells. The content of these cells is
secreted into the gut lumen whereby the membrane of an individual granule fuses
with the apical cell surface membrane. Lysozyme for example degrades the bacterial
cell wall by its muramidase activity, but it is also capable of lysing bacterial
membranes in a non-enzymatic manner\textsuperscript{53}. Cryptdins have been demonstrated also
to play a role in the clearance of potential pathogens. In 1 example, mice
overexpressing 1 member of the cryptdin family of proteins were much less
susceptible to oral infection by \textit{Salmonella typhimurium}\textsuperscript{54}. Further, Paneth cells have
also been described to produce inflammatory cytokines in both a constitutive (TNF-
$\alpha$\textsuperscript{55}) and inducible manner during injury (interleukin-17\textsuperscript{56}).

\subsection{1.3.1.2 ATG16L1 and Paneth cell vesicle export}
Paneth cells came in the center of attention of Crohn disease, as CD patients with
the \textit{ATG16L1} risk variant T300A show abnormal secretion of their granula\textsuperscript{57}. Consequently, Cadwell et al. generated and characterized mice that were
hypomorphic for \textit{ATG16L1} to analyze the Paneth cell morphology. The hypomorphic
mice, \textit{ATG16L1}\textsuperscript{HM}, expressed \textit{ATG16L1} at 30\% of the wildtype level. They exhibited
notable abnormalities in the granule exocytosis pathway, with disorganized
lysozyme-positive granules, decreased granule number as well as reduced
extracellular lysozyme-mucus co-localization. \textit{ATG16L1}- and \textit{ATG5}-deficient Paneth
cells showed similar aberrations and Paneth cells of patients homozygous for the
\textit{ATG16L1} risk allele bore resemblance to those abnormalities\textsuperscript{58} (see Figure 1-5).
Finally a recent issue points out that ATG16L1 might have an even more extensive function contrary to the expectations. The virus-plus-susceptibility thesis demonstrates a new aspect of the interplay between pathogenesis, gene interaction and a virus factor. Cadwell et al. suggest that interaction between a specific virus infection and a mutation in the Crohn disease susceptibility gene ATG16L1 induces intestinal pathologies in mice. They state that virus-plus-susceptibility gene interaction generates abnormalities in granule packaging and in gene expression patterns of Paneth cells, but yet the molecular mechanism remains unexplained.\textsuperscript{59}

1.4 Inflammatory bowel disease
Crohn disease (CD) and ulcerative colitis (UC) are chronic inflammatory disorders of the intestine that are known collectively as inflammatory bowel disease (IBD). IBD is characterized by chronic inflammation of the intestinal tissue and by subsequent progressive destruction of the mucosal integrity\textsuperscript{60}. Clinical symptoms include frequent and chronically relapsing diarrhea, abdominal pain, rectal bleeding and malnutrition\textsuperscript{61}. UC is restricted to the colon, which is affected by severe mucosal inflammation that is accompanied by extensive superficial mucosal and submucosal ulcerations\textsuperscript{62}. CD in contrast is characterized by discontinuous, transmural inflammation that affects all layers of the intestine in the whole gastrointestinal tract with cardinal incidence in the terminal ileum and colon\textsuperscript{63} (see Figure 1-4).
Figure 1-4: Characterization of CD and UC.
Crohn disease is characterized by a completely inflamed intestine whereas Ulcerative colitis affects only the intestinal mucosa. Source: http://www.inflammation-at-interfaces.de; http://www.internisten-im-netz.de/de.

1.4.1 Genetics in inflammatory bowel disease

Genome-wide association studies (GWAS) have identified 71 risk loci in CD and 47 risk loci in UC. An interesting and unexpected finding from the GWA studies is the important role of autophagy in CD pathogenesis, as the two genes ATG16L1 and IRGM, were found to be significantly associated in multiple GWAS\textsuperscript{64-68}, as well as a third autophagy-associated gene, \textit{LRRK2}\textsuperscript{66,69}. Genetic variations in these genes are supposed to have a major impact on the response of the innate immune system to intestinal microbiota and susceptibility to IBD. A link to innate immunity has been made by molecular studies demonstrating a physical interaction between ATG16L1 and NOD2, a pattern recognition receptor that recognizes bacterial products of the enteric flora\textsuperscript{70-72}. Interestingly, several risk loci identified through GWAS are common for ulcerative colitis and CD, whereas \textit{ATG16L1}, \textit{IRGM} and \textit{NOD2}, seem to be more specific for CD\textsuperscript{73}. Hence, the discovery of the association of NOD2 polymorphism with susceptibility to Crohn disease led to the hypothesis that Crohn disease results from a genetic-based failure in host response to luminal bacteria\textsuperscript{61}. In addition, NOD2 is described to be involved in Muramyl dipeptide (MDP) induced autophagy\textsuperscript{49,74,75}, whereas the Crohn disease associated variant NOD2-L1007fsinsC leads to diminished xenophagy (selective autophagy for degradation of intracellular bacteria)\textsuperscript{49,74}. Equally, a loss-of-function of the above mentioned ATG16L1-T300A Crohn disease variant was described, as leading to reduced xenophagy, as
well\textsuperscript{48,49,74-76}. This led to the received opinion that the increased risk of Crohn disease is caused by impaired bacterial handling and lowered rates of bacterial capture by the autophagy machinery. As NOD2 is described to recruit ATG16L1 to the plasma membrane at the bacterial entry side\textsuperscript{49}, this might be a hind for a crosstalk of NOD2 and ATG16L1 in xenophagy, further underscoring the observed cooperative effect of the CD-associated variants.

\subsection*{1.4.2 The role of ATG16L1 in Crohn disease}

The allelic variant in \textit{ATG16L1} as risk variant for CD was first discovered by our group in 2006. A genome-wide association study using 735 Crohn disease cases and 368 healthy controls was performed resulting in the identification of the coding SNP \textit{rs2241880}. The variant, typed T300A, results in an amino acid exchange from threonine to alanine at position 300 of the amino acid sequence (see Figure 1-3). A three-dimensional model of the WD-repeat domain structure of human ATG16L1 was designed via in silico protein analysis. The WD-repeat is assumed to consist of 32 $\beta$-strands forming an eight-bladed $\beta$-propeller with the location of the variant amino acid T300A in strand $\beta3$.\textsuperscript{64} A following meta-analysis expanded the correlation of the ATG16L1 T300A polymorphism to susceptibility to Crohn disease to a further predisposition to Ulcerative Colitis. Hence, confirming the role for autophagy in the pathogenesis of inflammatory bowel diseases (IBD)\textsuperscript{77}.

Functional information regarding the role of autophagy in humans in the context of CD is almost lacking. But relevant information regarding ATG16L1 has been obtained in \textit{in vitro} studies and \textit{in vivo} in mice. Kuballa et al. showed a diminished capacity of the CD risk variant ATG16L1\textsuperscript{T300A} in the capture of internalized \textit{Salmonella typhimurium} within autophagosomes by using human epithelial cell lines. They concluded that the association of the ATG16L1 T300A variant with increased risk of Crohn disease is due to impaired bacterial handling and lowered rates of bacterial capture by the autophagy machinery\textsuperscript{48}. This observation fits with the current focus on adherent-invasive \textit{E.coli} (AIEC) as one of the microbial malefactors in CD\textsuperscript{78} and has been replicated by several groups\textsuperscript{48,74-76}. Except in one publication the authors did not see significant effects of the T300A variant in an over-expression experiment\textsuperscript{79}. Interestingly, in contrast to bacterial internalizing autophagy (xenophagy), Kuballa et al. asserted that the T300A variant has no impact on basal autophagy in HeLa cells\textsuperscript{48}. Additionally, Saitoh et al. described higher IL-1$\beta$ and IL-18 signaling in ATG16L1-deficient macrophages after stimulation with the TLR-4 ligand LPS. It is
known, that IL-1β increases the intestinal epithelial tight junction permeability\textsuperscript{80}. In a mouse model with ATG16L1 lacking in hematopoietic cells, they showed that the animals were highly susceptible to chemically induced colitis\textsuperscript{44} (see Figure 1-5).

Recent data of Plantinga et al. shows that the \textit{ATG16L1} polymorphism is associated with an increased production of proinflammatory cytokines upon stimulation with NOD2-ligands, but not with TLR2 or TLR4 agonists. This study is the first to link the \textit{ATG16L1} polymorphism with increased production of IL-1β and IL-6 in humans\textsuperscript{81}.

\subsection*{1.4.3 Immunity in inflammatory bowel disease}

In IBD the inflammatory response is characterized by an imbalance between pro- and anti-inflammatory cytokines, which leads to a defective activation of the intestinal immune system. This imbalance is characterized by a domination of pro-inflammatory (TNF\textsubscript{α}, IL-1β, IL-6, IL-8)\textsuperscript{82-84} and a lack of anti-inflammatory cytokines (IL-10, IL-13)\textsuperscript{85}. In CD a shift to advanced levels of Th1-type pro-inflammatory cytokines such as \textit{interferon gamma} (IFN-γ), TNF\textsubscript{α} and IL-6 is described, whereas UC seems to exhibit a Th2-type cytokine profile such as IL-13\textsuperscript{62,86-89}. Confirmative, Plantinga et al. recently showed that the \textit{ATG16L1} risk variant is associated with increased IL-1β and IL-6 production in humans\textsuperscript{81} (see Figure 1-5). Finally, autophagy serves as a guardian of immunological tolerance by appropriate selection of naive T cells before they exit to the periphery. This seems to be important, as genetic disruption of \textit{Atg5} in thymic epithelial cells leads to an altered selection of certain MHC class II restricted T-cell specificities and autoimmunity. The animals develop multiorgan inflammation, including colitis\textsuperscript{90}.
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Figure 1-5: ATG16L1 in inflammation
Grey boxes schematically illustrate the autophagy factor ATG16L1 identified as Crohn disease susceptibility locus. ATG16L1 alleles encode either a protective ATG16L1 300T (top) or a risk form ATG16L1 300A (bottom). Top panel shows intestinal epithelium with different cell types along with the proposed functions of autophagy in the intestinal epithelium. Bottom panel lists six findings regarding the effect of ATG16L1 mutations in different cell lines or patients carrying the risk variant. (modified from Deretic and Levine, Cell Host & Microbe 5, June 2009)

1.4.4 Microbial flora in inflammatory bowel diseases and the link to autophagy
IBD has often been characterized by the failure of tolerance to the normal gut flora and antigens, which finally results in inadequate immunologic responses. Recent
studies in rodents confirm that colonic inflammation is accompanied with changes in bacterial composition\textsuperscript{92}. The bacterial concentrations are increased, whereas microbial diversity is diminished, as an increase of \textit{Enterobacteriaceae}, including \textit{E.coli}, is described for IBD along with a decrease in \textit{Firmicutes phyla} members compared to controls\textsuperscript{93}.

A common hypothesis is that a subset of CD is caused by defective clearance of commensal, opportunistic or pathogenic bacteria with subsequent initiation of compensatory antibacterial effector T cells that cause tissue damage\textsuperscript{94,95}. A link to autophagy is given by polymorphisms of genes, regulating clearance of intracellular pathogens and observed defects in innate antimicrobial function. For example, secretion of both $\alpha$- and $\beta$-defensins is defective in CD\textsuperscript{96,97}, whereas NOD2 deficient mice exhibit decreased expression of $\alpha$-defensin defcr-4\textsuperscript{98}. Deletion of the autophagy gene \textit{ATG16L1} or the endoplasmic reticulum stress protein XBP-1 results in abnormal Paneth cell morphology and reduced expression of antimicrobial peptides\textsuperscript{38,58}. Furthermore, the risk variant \textit{ATG16L1} T300A causes a decline in bacteria degrading autophagy (xenophagy)\textsuperscript{48,49,74-76}.

Decreased antimicrobial peptide secretion, as well as reduced xenophagy could lead to overgrowth, increased mucosal adherence and translocation of commensal bacteria, while defective clearance of invasive or phagocytosed bacteria promotes persistence of viable intracellular bacteria\textsuperscript{99}.

\section*{1.4.5 Mouse models for intestinal inflammation}

Animal models of intestinal inflammation progressed the understanding of inflammatory bowel disease. The common used chemically induced models of intestinal inflammation are trinitro benzene sulfonic acid (TNBS), oxazolone or dextran sodium sulfate (DSS) colitis\textsuperscript{100}. TNBS as well as oxazolone are haptenating substances and are given intrarectal (in ethanol) with the following effect: ethanol has the effect of breaking the mucosal barrier integrity, whereas TNBS and oxazolone induce a T cell mediated response against hapten-modified autologous proteins or luminal antigens. Contrary, DSS affects the integrity of the mucosal barrier by acting directly toxic to gut epithelial cells of the basal crypts\textsuperscript{100}. It is applied to mice in their drinking water and induces an acute colitis, which is characterized by bloody diarrhea, ulcerations and infiltrations with granulocytes\textsuperscript{101}. Importantly, it is shown, that the adaptive immune system is only secondary involved in this model as T and B cell deficient mice also develop severe intestinal inflammation after DSS.
administration\textsuperscript{102}. Therefore, the DSS-induced colitis is a useful model to study the involvement of innate immune mechanisms to colitis.

1.5 Aim of the work

In this work, the specific function of ATG16L1 was studied in mice \textit{in vivo} in a DSS induced colitis model and \textit{in vitro} in intestinal epithelial cells and bone marrow derived macrophages. Therefore three mouse models have been compared, the wildtype mice (WT), which express the complete protein; the \(\Delta\)WD40 mice, which express a truncated form of the ATG16L1 protein lacking the WD40 repeat domain and furthermore, the \(\Delta\)IEC mice as conditional Villin-Cre knockout mice with ATG16L1 deletion in the intestinal epithelium (see Figure 1-6).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1-6}
\caption{Mouse models with genetically altered \textit{Atg16l1}}
\end{figure}

\textbf{WT}: The wildtype mice which express the complete protein; \textbf{ATG16}\(\Delta\)\textbf{WD40}: the \(\Delta\)WD40 mice, which express a truncated form of the ATG16L1 protein lacking the WD40 repeat domain; \textbf{ATG16}\(\Delta\)\textbf{IEC}: the \(\Delta\)IEC mice as conditional Villin Cre knockout mice with ATG16L1 deletion in the intestinal epithelium.

ATG16L1 is assumed to have unique protective functions, including Paneth cell antimicrobial peptide release and the negative regulation of pro-inflammatory cytokine production. A deletion of this protein leads to high susceptibility to Dextran sodium sulfite (DSS) induced colitis. Especially the ATG16L1 WD repeat domain is suggested to fulfill a distinct function in higher eukaryotes, because it is absent in the dimeric coiled coil structure of the yeast APG16 protein in contrast to the structure of the mammalian protein\textsuperscript{103}. Until now, no interaction partner for the WD40 domain is...
described. It is shown that the truncation of the whole WD40 domain has no impact on dimerization with ATG5. Hence, Fujita et al. concluded that the WD40 domain in ATG16L1 is dispensable for canonical autophagy\(^79\). But the risk associated variant T300A is located within this domain. The exchange of threonine to alanine at that position has an impact on Paneth cells of patients carrying the risk variant, as they show abnormal packaging and secretion of their antimicrobial granules. The same phenotype is described for hypomorphic mice, expressing only 30% of ATG16L1. Further it is demonstrated that the risk variant T300A leads to diminished xenophagy which in turn might be a link to impaired gut homeostasis. However, it is unclear how ATG16L1 independently or as part of the classical autophagy pathway interacts with the ER connected vesicle export pathway and how it arranges his influence on the gut homeostasis. Further it is unclear, which specific role does the WD40 repeat domain play within these events.

Therefore, the specific goals of this thesis were:

- to investigate the consequence of an ATG16L1 truncation or deletion in intestinal inflammation in a mouse model

- to monitor the role of ATG16L1 in the autophagic machinery in intestinal epithelial cells and myeloid immune cells (bone marrow derived macrophages)

- to study the contribution of ATG16L1 on vesicle export in Paneth cells

- to investigate a presumed link of ATG16L1 and ER stress in intestinal epithelial cells and myeloid immune cells (bone marrow derived macrophages).
2 MATERIAL AND METHODS

2.1 Material

2.1.1 Organisms
C57BL6/J mice (WT mice) Jackson Laboratory
ATG16L1 conditional knockout mice (∆IEC mice) genOway
ATG16L1 knock-in mice (∆WD40 mice) genOway

2.1.2 Chemical
Acryl amide/bisacryl amide (30:0.8) BioRad
Agarose SeaKem LE Biozym
Amphotericin Sigma
Bovine serum albumin (BSA, Fraction V) ICN Biomedicals Inc.
BrdU CalBiochem
DEPC Sigma
DNA Smart Ladder Eurogentec
dNTPs Fermentas
DTT Fermentas
Dimethyl sulfoxide (DMSO) Roth
Dextran sulfate sodium (DSS, MW 40.000 Da) TdB Cosultancy
EDTA Gibco
Ethanol Merck
Fetal calf serum (FCS) PAA
Formaldehyde Roth
FITC-Dextran 4000 Sigma-Aldrich
Glycine Roth
Glycerol Roth
HCl Roth
Hepes Biochrom KG
Isopropanol Roth
LD-MDP Bachem
2-Mercaptoethanol Merck
Methanol Roth
MATERIAL AND METHODS

2.1.3 Media
DMEM Dulbecco’s minimal essential medium Sigma
MMM Mouse Macrophage medium Sigma
HBSS calcium-magnesium-free medium PAA
LB medium 10g NaCl, 10g tryptone, 5g yeast extract, ad 1l Milli-Q H2O, autoclaved
MEM minimal essential medium PAA, supplemented with 10% (v/v) FCS, 1% (v/v) P/S

2.1.4 Buffers and solutions

2.1.4.1 SDS-polyacrylamide gelelectrophoresis and Western blot
Anode buffer 1 30 mM Tris base, 20% (v/v) methanol
Anode buffer 2 300 mM Tris base, 20% (v/v) methanol
Cathode buffer 25 mM Tris base, 20% (v/v) methanol, 40
MATERIAL AND METHODS

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5x Protein loading buffer (Fermentas) 0.313 M Tris HCl (pH 6.8 at 25°C), 10% (w/v) SDS, 0.05% (w/v) bromophenol blue, 50% (v/v) glycerol, 2M DTT

4x Separation buffer 1.5 M Tris/HCl (pH 8.8), 0.4% (w/v) SDS

4x Stacking buffer 0.5 M Tris/HCl (pH 8.8), 0.4% (w/v) SDS

Stripping buffer 62.5 mM Tris/HCl (pH 6.8), 2% (w/v) SDS, 0.7% (v/v) b-mercaptoethanol

TTBS 20 mM Tris, 137 mM NaCl, 0.1% (v/v) Tween20 milk powder in TTBS

Blocking solution 3% (w/v)

2.1.4.2 Immunohistochemistry

Shadon Gill III Hematoxylin Thermo Scientific
Giemsa’s azure eosin methylene blue Merck
AEC substrate Dako
Blocking reagent Dako
Antibody Diluent Dako
DAPI (20mg/ml) Sigma-Aldrich

2.1.4.3 Cell stimulation

Bafilomycin Sigma
LD-MDP Bachem
Rapamycin Sigma
Tunicamycin (Streptomyces lysosuperficus) Calbiochem
2.1.5 Antibodies

Table 1: List of Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species, Type</th>
<th>Dilution</th>
<th>Manufacturer</th>
<th>Cat. number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-ATG16L1</td>
<td>Mouse, monoclonal</td>
<td>1:500</td>
<td>MBL</td>
<td>M150-3</td>
</tr>
<tr>
<td>Anti-CD3</td>
<td>Rabbit, polyclonal</td>
<td>1:50</td>
<td>Abcam</td>
<td>Ab5690</td>
</tr>
<tr>
<td>Anti-ATF4</td>
<td>Rabbit, polyclonal</td>
<td>1:100</td>
<td>Santa Cruz</td>
<td>Sc-200</td>
</tr>
<tr>
<td>Anti-goat-FITC</td>
<td></td>
<td>1:300</td>
<td>Jackson Immunoresearch</td>
<td></td>
</tr>
<tr>
<td>Anti-goat-HRP</td>
<td></td>
<td>1:1000</td>
<td>Amersham Biosciences</td>
<td>1025</td>
</tr>
<tr>
<td>Anti-grp78</td>
<td>Rabbit, polyclonal</td>
<td>1:500</td>
<td>ENZO</td>
<td>ADI-SPA-826-D</td>
</tr>
<tr>
<td>Anti-LC3</td>
<td>Rabbit, polyclonal</td>
<td>1:300 (IHC) 1:500 (WB)</td>
<td>Novus</td>
<td>ND100-2331</td>
</tr>
<tr>
<td>Anti-Lysozym (α)</td>
<td>Goat, polyclonal</td>
<td>1:500</td>
<td>Santa Cruz</td>
<td>Sc-27958</td>
</tr>
<tr>
<td>Anti-mouse-Alexa fluor</td>
<td></td>
<td>1:300</td>
<td>Invitrogen</td>
<td>A21425</td>
</tr>
<tr>
<td>Anti-mouse-HRP</td>
<td></td>
<td>1:1000</td>
<td>GE healthcare</td>
<td>NA931V</td>
</tr>
<tr>
<td>Anti-PDI</td>
<td>Rabbit, polyclonal</td>
<td>1:500</td>
<td>Abcam</td>
<td>Ab3672</td>
</tr>
<tr>
<td>Anti-rabbit-Alexa fluor</td>
<td></td>
<td>1:300</td>
<td>Invitrogen</td>
<td>A21430</td>
</tr>
<tr>
<td>Anti-rabbit-HRP</td>
<td></td>
<td>1:1000</td>
<td>GE healthcare</td>
<td>NA934V</td>
</tr>
<tr>
<td>Anti-β-actin</td>
<td>Mouse, monoclonal</td>
<td>1:60000</td>
<td>Sigma Aldrich</td>
<td>A-5441</td>
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</tbody>
</table>

2.1.6 Oligonucleotides

All oligonucleotides used were synthesized from Metabion. Sequences are shown in Table 2.

Table 2: Used Primers and their sequences

<table>
<thead>
<tr>
<th>Primer</th>
<th>Orientation</th>
<th>Sequence (5’→3’)</th>
<th>Tanneal (°C)</th>
<th>Amplicon (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATG16l1_A</td>
<td>sense</td>
<td>GGAAGCGTCACATCGCGGAGG CTCCCCACGCTTTGCGAGT</td>
<td>65</td>
<td>278</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATG16l1_B</td>
<td>sense</td>
<td>CCAAGGAGGCGTCACATCGCGGAGG CTCCCCACGCTTTGCGAGT</td>
<td>65</td>
<td>381</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4</td>
<td>sense</td>
<td>CGTGCCTGGGGCAGCTCTTCTGGAGG GCAGAGGCCAGGGCAGGG</td>
<td>66</td>
<td>282</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHOP</td>
<td>sense</td>
<td>CGTCCCTTCACCTTGAGACG CTGGCTAGGCTCTCTCC</td>
<td>60</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Defa3</td>
<td>sense</td>
<td>TGGCCCTTCAGGTCCAGG GACCTTTCTGTGCAAGTG</td>
<td>62</td>
<td>216</td>
</tr>
<tr>
<td></td>
<td>antisense</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**MATERIAL AND METHODS**

<table>
<thead>
<tr>
<th>Gene</th>
<th>sense antisense</th>
<th>Primer sequence</th>
<th>M1</th>
<th>M2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Defa5</td>
<td></td>
<td>AGGCTGATCCTATCCACAAACAG AGCATCAGTGCCCTCAGACC</td>
<td>62</td>
<td>282</td>
</tr>
<tr>
<td>DES</td>
<td></td>
<td>GCGCCAAAGCCAAGCAGGAGA GAGGACGGGGGCCAGGAACCT</td>
<td>66</td>
<td>503</td>
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<tr>
<td>G3PDH</td>
<td></td>
<td>TGAAGGTCGGAGTCAACGGATTTGCT CAGTGCGGCATGAGGTCCACCAC</td>
<td>55</td>
<td>720</td>
</tr>
<tr>
<td>Hspa5</td>
<td></td>
<td>CCCCTGTCGCCCTAGACCA TCGTCCGATGAGGGGCTTG</td>
<td>65</td>
<td>416</td>
</tr>
<tr>
<td>Krt18</td>
<td></td>
<td>GCCGCAGTCTGGAGGAGAGC AGGGTGACTCTCAGGCTCGT</td>
<td>65</td>
<td>416</td>
</tr>
<tr>
<td>Lyz</td>
<td></td>
<td>ACTGCCCAGGCAAGGTCTACA TCGCCATGCAACCATTGCTC</td>
<td>60</td>
<td>381</td>
</tr>
</tbody>
</table>

**2.1.7 Marker**

**DNA-Ladder**

![Figure 2-1: 1kb DNA smart Ladder from Fermentas.](image)

**Protein-Ladder**

![Figure 2-2: Prestained Protein Molecular Marker (10-250 kDa) from Fermentas.](image)
2.1.8 Kits
Advantage RT-for-PCR BD Bioscience/Clontech
BrdU In-Situ Detection Kit BD Pharmingen
ECL-PLUS Amersham
GoTaq DNA polymerase Promega
QiaShredder Qiagen
RNeasy Kit Qiagen

2.1.9 Electric devices and other materials

2.1.9.1 Centrifuges
Centrifuge 5417R Eppendorf
Varifuge 3.0R Haraeus

2.1.9.2 Incubators
HeraCell CO2-Incubator Heraeus Instruments
Orbital Incubator SI50 Stuart Scientific

2.1.9.3 Electrophoresis devices and power supplies
Geldok 2000 Biorad
PP 4000 Programmable High Voltage Power Pack Biometra

2.1.9.4 Microscopes
Fluorescence microscope with integrated Apotome Carl Zeiss Micro Imaging GmbH

2.1.9.5 Other devices
Gene Amp PCCP System 9700 AppliedBiosystems
NanoDrop ND-1000 spectrophotometer Thermo Scientific
Sterile working bench HeraSafe Heraeus
Heating block Grant Instruments

2.1.9.6 Consumables
0.5ml, 1.5ml and 2ml micro tube Sarstedt
Pipette tips Sarstedt
Tube 15ml and 50ml Sarstedt
Hyperfilm Amersham Biosciences
PVDF-membrane Highbond P Amersham Biosciences
Tissue culture dish 6-well \hspace{1cm} Sarstedt
100 Sterican size 18 \hspace{1cm} Braun
Injekt-F (1ml) \hspace{1cm} Braun
Surgical Disposable Scalpels \hspace{1cm} Braun

2.2 Methods

2.2.1 Isolation of RNA
Extraction of RNA from mouse intestinal epithelial cells and bone marrow derived macrophages was performed using the RNeasy Kit from Qiagen according to manufacturer's protocol.

2.2.2 cDNA synthesis
cDNA synthesis was performed using Advantage® RT-for-PCR kit from BD Bioscience/Clontech according to manufacturer's protocol.

2.2.3 Polymerase chain reaction (PCR)
Nucleic acid sequences can be amplified by using polymerase chain reaction. This method can be divided into three different steps: (i) denaturation, (ii) annealing and (iii) elongation. After DNA denaturation specific primers bind during the annealing phase to the template and the defined region of the DNA is replicated during the elongation phase. By repeated denaturation, annealing and elongation for several cycles the specific DNA sequence is amplified exponentially. Standard PCR was performed using the components shown in Table 3. The PCR was carried out with the following PCR program: 95°C 5min, up to 35x (95°C 1min, T\textsubscript{anneal} 1min, 72°C 1min), 72°C 5min. T\textsubscript{anneal} equals the primer annealing temperature as estimated by the program UCSC In-Silico PCR. PCR products were analyzed by agarose gel electrophoresis and finally detected under UV light.

Table 3: Components of a standard PCR

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume [µl]</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA Template</td>
<td>2</td>
</tr>
<tr>
<td>GoTaq Buffer (5x)</td>
<td>4</td>
</tr>
<tr>
<td>dNTP mix (each 10mM)</td>
<td>0,5</td>
</tr>
<tr>
<td>Primer mix (each 10µM)</td>
<td>0,5</td>
</tr>
<tr>
<td>GoTaq DNA Polymerase</td>
<td>0,25</td>
</tr>
<tr>
<td>Milli Q H\textsubscript{2}O</td>
<td>12,75</td>
</tr>
</tbody>
</table>
2.2.4 Reverse-transcriptase (RT) PCR
RT-PCR is a PCR amplification of a product from the reverse transcription (RT) reaction, whereby a template mRNA is translated into a single-stranded cDNA. In this study, a RNA-dependent DNA polymerase (=reverse transcriptase) derived from the retrovirus moloney-murine leukemia virus (MMLV) was used in combination with oligo-(dT)18 primer to ensure its binding at the 3' poly(A) tail of the mRNA. All used components originate from the Advantage® RT-for-PCR kit (BD Bioscience/Clontech). The following reaction mixture was used:

- RNA: 1 µg
- Oligo (dT): 1 µl
- DEPC treated H2O: add 13.5 µl

The mixture was heated at 70°C for 5 min and then cooled on ice. The following components were subsequently added:

- Reaction buffer (5x): 4 µl
- dNTPs 10 mM each: 1 µl
- RNase-Inhibitor: 0.5 µl
- Reverse Transcriptase (200 U/ml): 1 µl

The reaction mix was incubated for 1 hour at 42°C. Afterwards the Reverse Transcriptase was heat inactivated at 70°C for 10 min. The mixture was filled add 100 µl with DEPC treated H2O. 2 µl of the resulting cDNA was used directly for the subsequent PCR.

2.2.5 Agarose gel electrophoresis
Nucleic acids were separated in 1% (w/v) agarose gels supplemented with 0.3% (v/v) SYBR safe. The electrophoresis was performed in horizontal gel chambers at 90 to 120 V. Fragment sizes were estimated using DNA ladders.

2.2.6 Protein extraction
Protein extracts were prepared as described by Laemmli et al.\textsuperscript{104} by resuspending cell pellets in denaturing lysis buffer containing 2% (w/v) SDS, 0.06M Tris (pH 6.8), 0.05% (w/v) bromophenol blue, 50% (v/v) glycerol and 0.1M Dithiothreitol (DTT), shearing them up to ten times through syringe needle (sterican size 18, braun) and boiling them for five minutes at 100°C in a heating block. Purified supernatant was applied directly to a SDS-polyacrylamide gel or transferred into a fresh microcentrifuge tube and stored at +4°C for one week.
2.2.7 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)
SDS-PAGE is a method to separate proteins according to their electrophoretic mobility and allows the analysis of proteins via staining or Western blotting. Furthermore, molecular weight of analyzed proteins can be determined using molecular weight marker. Proteins were separated by discontinuous gel electrophoresis. Compositions of the different gels are shown in Table 4. Electrophoresis was performed in 1x SDS running buffer. Protein samples were supplemented with 5x protein loading buffer and loaded onto the gel. Electrophoresis was carried out at 120-180V for 90 to 120min.

Table 4: Gel components for SDS-PAGE

<table>
<thead>
<tr>
<th>Component</th>
<th>Stacking gel [µl]</th>
<th>Separating gel [µl]</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. bidest</td>
<td>1950</td>
<td>2500</td>
</tr>
<tr>
<td>4x Stacking buffer</td>
<td>750</td>
<td>-</td>
</tr>
<tr>
<td>4x Separation buffer</td>
<td>-</td>
<td>2500</td>
</tr>
<tr>
<td>(Bis) Acrylamide</td>
<td>300</td>
<td>5000</td>
</tr>
<tr>
<td>10% APS</td>
<td>30</td>
<td>100</td>
</tr>
<tr>
<td>TEMED</td>
<td>3</td>
<td>10</td>
</tr>
</tbody>
</table>

2.2.8 Western Blot
After separation of protein samples by SDS-PAGE, proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Schwalbach) at 0.8mA/cm2 and 1 to 1.5 hours. After blocking for one hour with 5% (w/v) low-fat milk powder in TTBS, membranes were probed with specific primary antibodies overnight at 4°C, washed and incubated with horseradish peroxidase (HRP)-conjugated IgG as secondary antibody for one hour at room temperature. After another washing step, proteins were visualized by chemiluminescence (ECL, Amersham Biosciences). To determine even transfer and equal loading, membranes were stripped and reprobed with antibody specific for β-actin. The bands were quantified by densitometry.

2.2.9 Animal treatment
All experiments were performed according to German guidelines for animal care and protection (V742-72 241.121-3 (20-2/04) and (76-7/00) as well as V312-72241.121-3). Mice were maintained in a 12h light-dark cycle under standard conditions and were provided with food and water ad libitum. All animals were pathogen free as assessed by regular microbiological screening and kept under barrier conditions at 21°C ± 2°C and 60% ± 5% humidity in individually ventilated cages.
2.2.10 Induction of DSS-colitis and determination of clinical scores
Chronic colitis was induced by administration of 2 % dextran sulfate sodium (DSS) in the drinking water for 5 d followed by 5 d of regular drinking water over a period of 30 d. Acute colitis was induced by 4 %DSS in the drinking water for 5 d. Groups of four to 11 mice were used to monitor the disease activity index (DAI) or after sacrifice via Immunhistochemistry of sections of the Colon the histological scoring (see Table 5). Clinical parameters were recorded daily. DAI displaying the combined score of weight loss, stool consistency, and rectal bleeding, was performed as previously described by Siegmund et al.\textsuperscript{105}. A high resolution mouse video endoscopic system was used (HOPKINS® Optik 64019BA; KARL STOLZ AIDATM VET, Berlin, Germany). Histological scoring was obtained by combination of the score of inflammatory cell proliferation and tissue damaging as described by Siegmund et al.\textsuperscript{106} and Atreya et al.\textsuperscript{107}.

<table>
<thead>
<tr>
<th>Component</th>
<th>DAI</th>
<th>Histological scoring</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight loss</td>
<td></td>
<td>Inflammatory cell infiltration</td>
</tr>
<tr>
<td>Stool consistency</td>
<td></td>
<td>Tissue damaging</td>
</tr>
<tr>
<td>Rectal bleeding</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.2.11 FITC dextran and BrdU administration
Intestinal permeability was assessed by administration of the non-metabolizable macromolecule FITC-dextran 4000 (Sigma-Aldrich, Deisenhofen, Germany). FITC-dextran was administered by gavage (0.6 g/kg body weight) 4 h before sacrifice. Whole blood and serum were obtained by cardiac puncture and centrifugation at 5,000 rpm for 15 min respectively. Dilutions of FITC-dextran 4000 in PBS were used as a standard curve and absorption of 100 μl serum or standard was measured in a fluorometer at 488 nm.

BrdU (5-bromo-2-deoxyuridine) is a synthetic nucleoside that is an analogue of thymidine and provides a simple technique for labeling and identifying proliferating cells. BrdU can be incorporated into the newly synthesized DNA of replicating cells (during the S-phase of the cell cycle), substituting for thymidine during DNA replication. To study proliferation of epithelial cells 50mg BrdU/kg body weight (CalBiochem, Schwalbach, Germany) was injected intraperitoneally 2h before sacrifice.
2.2.12 Statistical analysis
All parametric data are presented as the means ± standard deviation. Data from two groups were analyzed for significance using a student’s unpaired t-test (http://www.physics.csbsju.edu/stats/t-test.html). A p value of p<0.05 was considered as statistically significant (*) and p<0.01 was considered strong significant (**) and p<0.001 was considered as highly significant (***) . Experiments and measurements were replicated at least three times.

2.2.13 Densitometrical analysis with ImageJ
The densitometrical analysis of pictures or X-ray films received from agarose gel electrophoresis combined with UV light visualization and detection of proteins by chemiluminescence was performed with the non-commercial software ImageJ (http://rsb.info.nih.gov/ij/).

2.2.14 Immunohistochemistry

2.2.14.1 Processing of tissues
Jejunum tissue and colon tissue, especially the distal part, was removed and fixed in 4 % paraformaldehyde in 1xPBS for 24 h. Tissues were dehydrated in an ascending ethanol and xylol series. Samples were embedded in paraffin and dissected with 5 μm using a microtome (Leica RM2165).

2.2.14.2 Hematoxylin-Eosin (HE) staining
Processed tissue sections were incubated in Shadon Gill III Hematoxylin, differentiated in 0.5% acetic acid, washed and stained with Giemsa’s azur eosin methylen blue solution. The stained sections were analyzed by microscopy.

2.2.14.3 BrdU staining
To visualize BrdU incorporation sections were stained with 1:500 dilution of mouse anti-BrdU mAb in Antibody diluent overnight. Sections were then incubated for 1h with biotinylated anti-mouse secondary antibody (1:50 in Antibody Diluent) and developed with AEC substrate. Counterstaining was performed with Shadon Gill III Hemytoxylin. Stained sections were analyzed by microscopy.

2.2.14.4 Immunofluorescence staining
Sections were boiled in citrate buffer for 3 or 20 min (depending on the primary antibody) followed by sequential incubation with blocking reagent. Slides were then
incubated for 1 h at room temperature with primary antibody. After washing with PBS, slides were incubated for 45 min at room temperature with Alexa fluoro555- or FITC-labeled secondary antibodies (see Table 1). Nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole; 20 mg/ml).

2.2.14.5 Electronmicroscopy and Toluidine blue staining
Electronmicroscopy and Toluidine blue staining of semi thin sections was performed at Institute of Anatomy (AG Lucius) according to standard protocols\textsuperscript{108,109}.

2.2.15 Isolation of primary cells

2.2.15.1 Intestinal epithelial cells (IECs)
Briefly, mouse Jejunum was everted and incubated in calcium-magnesium-free HBSS with 1 mM EDTA for 30 min at 37°C with gentle shaking to liberate intestinal epithelial cells (IECs). The isolated cells were seeded in 6-well plates (Nunc) at 1x10^6 cells/well in minimal essential medium (MEM), supplemented with 20 % fetal calf serum (FCS) and 1 % Penicillin/Streptomycin (P/S) and were left resting for 3 h before stimulation.

2.2.15.2 Bone marrow derived macrophages (BMDMs)
Primary bone marrow-derived macrophages (BMDM) were obtained from femoral bone marrow. In brief, cells from the femur were isolated and cultured at 10^6 cells/ml for 7 days in 1:1 Dulbecco's minimal essential medium (DMEM, Sigma) and Mouse Macrophage Medium (MMM, Sigma), supplemented with 10 % fetal calf serum (FCS), Penicillin/Streptomycin (1x), Amphotericin (2.5 µg/µl) and rm M-CSF (20 ng/ml). At 3 days after washing and reculturing in fresh medium, the cell preparation contained a homogenous population of >95 % macrophages. The BMDMs were plated in 6-well culture plates (8x10^5 cells/well).

2.2.16 Stimulation of BMDMs or IECs
For stimulation experiments, seeded cells were stimulated with the following stimuli or were left untreated:

<table>
<thead>
<tr>
<th>Reagent</th>
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</tr>
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<tr>
<td>Rapamycin</td>
<td>50µg/ml (20min)</td>
</tr>
<tr>
<td>Bafilomycin</td>
<td>100nM (20min)</td>
</tr>
<tr>
<td>LD-MDP</td>
<td>100µg/ml (2h)</td>
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<td>Salmonella typhimurium</td>
<td>MOI 100 (1h)</td>
</tr>
<tr>
<td>Tunicamycin</td>
<td>5µg/ml (4h)</td>
</tr>
</tbody>
</table>

Table 6: Reagents used in cell culture experiments.
2.2.17 Transgenic animals

2.2.17.1 Generation of Atg16l1 conditional knockout mice (∆IEC)
The Atg16l1 knockout mice were generated in collaboration with GenOway. The Atg16l1 targeting strategy was designed to introduce a single distal loxP site upstream Atg16l1 gene exon 1, within the promoter region, and a loxP site together with an FRT flanked neomycin selection cassette within the intron 1, and leading to conditional disruption of the translation of all Atg16l1 isoforms by partial gene deletion. The resulted mouse line was bred with deleter-mice constitutively expressing the Flp-recombinase to remove the neomycin selection cassette and thus creating a line of floxed mice in which Atg16l1 gene exon 1 is flanked by loxP sites. This floxed mouse line then was mated with Cre-recombinase expressing mice (under the direction of the mouse villin 1 promoter) to obtain a tissue specific Atg16l1 knock-out model (recombination occurs in villi and crypt cells of the small and large intestines).

2.2.17.2 Generation of Atg16l1 knock-in mice (∆WD40)
The Atg16l1 knock-in mice were generated in collaboration with GenOway. The targeting strategy was designed for insertion of a Stop-polyadenylation signal cassette in 3′ of Atg16l1 exon 10, and leading to the constitutive expression of truncated Atg16l1 isoforms missing the C terminal region encoding for the WD40 domains. The resulting lines of mice were bred with deleter-mice constitutively expressing the Flp-recombinase, in order to remove the neomycin selection cassette and thus create the Atg16l1 Knock-in mouse line devoid of selection cassette.
3 RESULTS

3.1 The role of ATG16L1 in DSS induced colitis.

Autophagy-related protein 16-1 (ATG16L1) is a component of a large protein complex essential for autophagy. As autophagy is described to be genetically linked to Crohn’s disease with a polymorphism in ATG16L1 as revealed by our group in a genome wide association study in 2007\textsuperscript{110}, it was interesting to understand the specific role of the ATG16L1 WD40 repeat domain, which comprises the Crohn associated coding variant T300A (threonine-to-alanine substitution), in inflammatory bowel disease. Three mouse models have been compared: the wildtype mice (WT), which express the complete protein; the \(\Delta\)WD40 mice, which expressed a truncated form of the ATG16L1 protein lacking the WD40 repeat domain and furthermore, the \(\Delta\)IEC mice as conditional Villin-Cre knockout mice (see Figure 3-1 A). Before testing the gene expression of \textit{Atg16l1} in intestinal epithelial cells (IECs) a purity control was performed to estimate the extent of contamination by further cell lineages in the isolated IECs. Therefore, the expression of the transcripts \textit{Cd4}, \textit{Des} and \textit{Krt18} was investigated via RT-PCR. \textit{Cd4} encodes for the protein CD4, expressed on the surface of CD4\textsuperscript{+}T cells, additionally \textit{Des} encodes for the Protein desmin, a subunit of intermediate filaments in skeletal muscle tissue. \textit{Krt18} encodes for the protein keratin 18, a type I cytokeratin which is expressed in single layer epithelial tissues. As depicted in Figure 3-1 B, the RT-PCR of the intestinal epithelial cells shows that in fact IECs were isolated (387bp), only low expression of \textit{Cd4} (282bp) and no expression of \textit{Des} was detected which indicates that due to the method of primary IEC isolation, there is a negligible low grade of contamination with immune cells and no contamination with muscle cells.
Figure 3-1: Domain structure and expression profile of murine ATG16L1 displaying the three variants of genotypes.

A) The N-terminal domain (ATG16) of the ATG16L1 protein is involved in complexation of essential autophagy proteins ATG5 and ATG12 and self-dimerisation. The C-terminal eight WD repeats are of unknown function and comprise the Crohn disease associated coding SNP T300A. The wildtype mice express the whole protein, the ΔWD40 ubiquitously express the truncated form, missing the WD40 repeat domain, and the ΔIEC mice do not express ATG16L1 in the IECs. Marked with bars are the DNA binding sites for the primer pairs A(167-423) and B(7667-1128).

B) Intestinal epithelial cells were isolated from WT mice and RT-PCR experiments were performed with cDNA and sequence-specific primers for CD4, DES and Krt18.

C) RT-PCR experiments were performed with cDNA and sequence-specific primers for Atg16l1 in the area of the ATG16 domain coding sequence (A167-423) and of the beginning of the WD40 repeat domain coding sequence B(7667-1128) and G3PDH.

D) Protein extracts of intestinal epithelial cells of WT, ΔWD40 and ΔIEC mice (each n=3) were separated by SDS-PAGE and immunoblotted against ATG16L1 and β-actin; representative image.

E) Jejunum tissue section of WT, ΔWD40 and ΔIEC mice stained with anti-ATG16L1 (red). Arrows show ATG16L1 positive, non-epithelial cells; dotted line denotes crypt unit; representative images.

Furthermore, for genotype validation the expression of the Atg16l1 gene was tested in intestinal epithelial cells (IECs) by RT-PCR via two different pairs of oligonucleotides. One pair (A(167-423)) amplified a DNA fragment from nucleotide 167 to 423 in the DNA sequence coding for the coiled coil domain, the other one (B(767-1128)) a fragment from nucleotide 767 to 1128 within the DNA sequence coding for the WD40 domain (see Figure 3-1 A,C). In wildtype mice with both primer pairs the desired DNA sequences were amplified, whereas in ΔWD40 mice only primer pair A bound to the DNA sequence coding for the coiled coil domain. In ΔIEC
mice no bands were detected apart from very weak amplifications, which can be explained by contamination of the intestinal epithelial cells by CD4⁺ T cells. G3PDH was used as a loading control (see Figure 3-1 C). Additional genotype validation was performed by investigating the protein expression of ATG16L1. After preparation of whole protein extracts from intestinal epithelial cells, SDS PAGE and immunoblotting were performed with a specific antibody raised against the N-terminal region of ATG16L1 and β-actin as equal loading control of proteins. Figure 3-1 D shows that ATG16L1 could be detected in WT mice with a protein size of ~70 kDa. Again, a very weak signal was observed in ∆IEC mice, which can be explained by contamination with non-intestinal epithelial cells. For the truncated version of ATG16L1 in ∆WD40 mice no specific signal was detected via immunoblotting, which is assumed to be a complicacy with the antibody especially in this analysis method. At last, ATG16L1 protein expression was validated via immunohistochemistry in Jejunum sections of all three genotypes using the same ATG16L1 antibody as before. As depicted in Figure 3-1E ATG16L1 positive signals were detected in intestinal epithelial cells of WT and ∆WD40 mice. ∆IEC Jejunum sections exhibit no ATG16L1 positive epithelial cells. All three genotypes show ATG16L1-stained non-epithelial cells (see Figure 3-1, E).

These results indicate on the one hand that the isolation method for the intestinal epithelial cells is sufficient for further experiments and on the other hand that the mouse models generated in collaboration with genOway are validated.

ATG16L1 is described to be linked with Crohn disease⁶⁴, but two different opinions exist about its role during DSS induced colitis. Cadwell et al. detected no differences in responses to DSS-induced intestinal injury in mice expressing ATG16L1 at 30 % of the wildtype level⁵⁹. Contrariwise Saitoh et al. reported higher responsibility to DSS treatment for chimeric mice deficient for Atg16l1⁴⁴. Hence, it was interesting to compare Cadwells and Saitohs findings with our mouse models. Wildtype mice as well as ∆WD40 and ∆IEC mice were either treated with 2 % DSS in the drinking water for five days followed by five days of water over a period of 30 days (chronic colitis) or with 4 % DSS in the drinking water for 5 days (acute colitis). During this DSS induced colitis almost all mice developed loose stools or diarrhea. DSS is toxic to gut epithelial cells of the basal crypts and affects the integrity of the mucosal barrier¹¹¹ via extensive crypt and epithelial cell damage. Significant
infiltration of granulocytes and mononuclear immune cells and decreased epithelial cell proliferation are observed consequences. The wall appears thickened, and stricture appears early in the disease. Coloscopical examination indicated that in both experiments all strains developed colitis as exemplarily depicted in Figure 3-2 for acute colitis.

![Figure 3-2: Colonoscopy of DSS treated WT, ΔWD40 and ΔIEC mice.](image)

Acute Colitis was induced by giving animals 4% DSS in the drinking water for 5 days. On the last day of the experiment colonoscopy was performed.

Disease severity was investigated daily by weight progression, hemoccult test and stool consistency, thus termed the disease activity index (DAI) (see Figure 3-3 A). The combined score ranged from 0 (no changes) to 12 (strong disease severity with weight loss >20 %, liquid stools and gross rectal bleeding). A group of six to ten wildtype, eight to eleven ΔWD40 and five to seven ΔIEC animals were used. The DAI validation sustained the above described coloscopical findings in mice of all three strains. Interestingly, compared to WT as well as to ΔWD40 mice the ΔIEC mice exhibited a significantly higher responsibility to DSS in both colitis experiments, as already shown likewise by Saitoh et al. No clear differences appeared by comparison of ΔWD40 mice with WT mice. All WT and ΔWD40 mice survived, but one ΔIEC mouse died.

As displayed in Figure 3-3 B and C, during chronic and acute DSS colitis the ΔIEC mice exhibited significantly increased intestinal injury response, whereas no clear difference could be observed between ΔWD40 and WT mice.
RESULTS

<table>
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<td>6-10%</td>
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<td>&gt;20%</td>
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<td>well-formed pellets</td>
<td>-</td>
<td>-</td>
<td>pasty and semiformal pellets that did not adhere to the anus</td>
<td>-</td>
<td>liquid stools that did not adhere to the anus</td>
</tr>
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<td>no blood by using hemocult</td>
<td>-</td>
<td>-</td>
<td>positive hemocult</td>
<td>-</td>
<td>gross bleeding</td>
</tr>
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</table>

Figure 3-3: DAI score during DSS-treatment.
A) Components of the disease activity index (DAI). B) DSS was applied in drinking water for 5d followed by 5d of drinking water over a period of 30d. The disease activity index (DAI) displays the results of daily assessment of change in body weight, diarrhea score, and bleeding score during chronic colitis or acute colitis (index with values are shown as means ± SEM; *, p ≤ 0.05).

HE staining of the colon of mice treated with DSS affirmed fewer signs of inflammation in WT and ΔWD40 mice but strong signs in ΔIEC mice. In all three genotypes the epithelium was afflicted by DSS toxicity to gut epithelial cells. A clear difference was detected in the architecture of the crypts. In mildly inflamed mice like WT and ΔWD40 animals the crypts are almost intact. In higher inflamed ΔIEC mice the crypt structure was occasionally lost after day five (see Figure 3-4 A).

Histological scoring is the combined score of inflammatory cell infiltration (score 0-3) and tissue damage (score 0-3)\textsuperscript{106,107} (Figure 3-4 B). The combined score ranged from 0 (no changes) to 6 (extensive cell infiltration and tissue damage). As shown on Figure 3-4 C, the histological score is significantly increased in all three genotypes from untreated mice to DSS challenged mice (p ≤ 0.001), and it is significantly elevated in DSS treated ΔIEC mice compared to WT mice (p ≤ 0.05).
RESULTS

Figure 3-4: Histological scoring for unchallenged and DSS treated WT, ΔWD40 and ΔIEC mice. A) HE staining of colons from WT, ΔWD40 and ΔIEC mice challenged for 5d with 4%DSS or left untreated; at a magnification of 100. B) Components of histological scoring. C) The histological displays the inflammatory cell migration, as well as tissue the tissue damage of WT (n=10), ΔWD40 (n=11) and ΔIEC (n=4) mice. Values are shown as means ± S.D; * p≤0.05; *** p≤0.001.

To confirm the above findings, infiltration of immune cells was visualized by immune fluorescent staining with anti-CD3 as marker for infiltrating T-Lymphocytes. An optical evaluation was performed with Colon sections of three DSS treated animals per genotype, depicted representatively on Figure 3-5. It shows a high increase of CD3 positive cells in the Lamina propria of ΔIEC mice, whereas the rate of infiltration was less severe in WT and ΔWD40 mice.
A possibility to explain higher susceptibility to DSS induced colitis in ΔIEC mice might be the impairment of regenerative proliferation of epithelial cells. To analyze the regenerative response of the gut epithelium during DSS-induced colitis, cell proliferation was measured via Bromdesoxyuridine (BrdU) staining. Cells were labeled by intraperitoneal injection of BrdU two hours before sacrifice. As shown on Figure 3-6 A, the cell proliferation during DSS-induced colitis decreases from WT mice to ΔWD40 and from ΔWD40 mice to ΔIEC mice. This observation was confirmed by counting ~3 BrdU positive cells per crypt in 154 crypts of 5 WT animals, ~2 positive cells in 327 crypts of 5 ΔWD40 and ~1 positive cell in 312 crypts of 5 ΔIEC mice. The decrease of proliferation from WT mice to ΔWD40 and to ΔIEC mice is highly significant (p≤0.001), as well as the decrease of proliferating cells from ΔWD40 to ΔIEC mice (p≤0.01).
RESULTS

For maintaining the intestinal barrier function an efficient regenerative response is required. A fast and severe progression of the disease is the result of impaired barrier integrity. Therefore, general membrane condition in the gut was measured by permeability for FITC-Dextran in unchallenged mice. Four hours before sacrifice FITC-Dextran was administered by gastric gavage. Afterwards, blood samples were collected and serum was analyzed for presence of FITC-Dextran. As a positive control, Agr2 KO mice were used, which are characterized by impaired intestinal homeostasis with developing spontaneous colitis. In unchallenged WT, ΔWD40 and ΔIEC mice, no difference in permeability could be detected (Figure 3-7). These results indicate that no general barrier impairment exists in the more disease susceptible ΔIEC mice.

Figure 3-6: BrdU Immunohistochemistry in the colon of DSS treated WT, ΔWD40 and ΔIEC mice.
A) BrdU staining of colons from WT, ΔWD40 and ΔIEC mice challenged for 5d with 4%DSS. Magnification of 100. B) BrdU positive cells per crypt (154 crypts of 5 WT, 327 crypts of 5 ΔWD40 and 312 crypts of 5 ΔIEC mice). Values are shown as mean ± S.D; ** p≤0.01, *** p≤0.001.
3.2 Influence of ATG16L1 truncation or deletion on autophagy

Afterwards, it was investigated whether primary bone marrow-derived macrophages (BMDMs) or intestinal epithelial cells (IECs) of the three genotypes show different autophagic activity. As a reliable marker of autophagosomes, LC3, a mammalian homolog of yeast Atg8, was used to determine the autophagic activity. The PE-conjugated form localizes both on the isolation membrane and the autophagosome membrane\(^{116,117}\). The unconjugated (LC3-I) and conjugated forms (LC3-II) of LC3 can be separated by SDS-PAGE. Because the amount of LC3-II correlates with the number of autophagosomes, the conversion of LC3 can be used to monitor autophagic activity\(^{116}\). Untreated cells were applied to display the basal level of autophagy, whereas the induced turnover could be analyzed by treating cells with either Rapamycin (50 µg/ml; 20 min.) or Rapamycin in combination with Bafilomycin (100 nM; 20 min.). Rapamycin, the inhibitor of mTOR, induces autophagy\(^{118}\). In this experimental setup it was used to increase the amount of LC3-II to measure the induced autophagic activity. But as half-life of LC3-II is short because autophagosomes are transient structures, LC3-II represents the autophagic activity at the moment in time\(^{119}\). Hence, Bafilomycin, a specific inhibitor of the vacuolar type H\((+)-ATPase\) (V-ATPase) in cells\(^{120}\) and therefore an inhibitor of the lysosomal degradation, was used. It blocks the autophagosome degradation and allows an assessment of the induced turnover. Bone marrow derived macrophages of \(\Delta\)IEC mice were used as control, as they are not affected by the conditional knockout.
In 2009 Fujita et al. described that the deletion of the ATG16L1 WD repeat domain deletion has little impact on canonical autophagy\(^7\). In contrast, this work indicates that the truncation of the ATG16L1 WD repeat domain leads to lower LC3 expression levels and reduced LC3-II conversion in basal autophagy (exemplarily shown on Figure 3-8 A and B). As expected, BMDMs of \(\Delta\)IEC mice did not show any difference compared to the WT. The fold induction of the amount of LC3-II to LC3-I depicts more detailed the autophagic activity (see Figure 3-8 C and D). The results show no significance, but clear tendency. Thus it becomes apparent that in comparison of the WT (11.68 fold induction) to the \(\Delta\)WD40 mice (7.44 fold induction) the basal autophagic activity was reduced about 46 %. Stimulation with Rapamycin led to an induced autophagic activity of about 32.29 fold induction in WT and 11.38 in \(\Delta\)WD40 mice. This is a reduction of about 65 % in \(\Delta\)WD40 mice. As lower LC3-II level might follow from high lysosomal activity, the stimulation of Bafilomycin in combination with Rapamycin gives information about this possibility. Monitoring the induced turnover via inhibiting the lysosomal degradation caused autophagic activity of 41.38 fold induction in WT and 19.41 in \(\Delta\)WD40 mice with a reduction of about 53 %. Again, no difference was observed by comparing autophagic activity of WT and \(\Delta\)IEC mice. These results indicate that the \(\Delta\)WD40 mice have a lower autophagic activity compared to the WT mice.
RESULTS

Figure 3-8: LC3 expression in bone marrow derived macrophages. 
A) Bone marrow derived macrophages (BMDMs) were stimulated with Rapamycin (50µg/ml for 20 minutes) or with Rapamycin and Bafilomycin (100nM for 20 minutes) or were left untreated. Protein extracts were separated by SDS-PAGE and immunoblotted against LC3 and β-actin; representative image. B) Detected bands for LC3-I and LC3-II were densitometrically analyzed by ImageJ and related to β-actin; representative result of 3 independent experiments. C) Fold induction was assessed by determining the ratio between LC3-II and LC3-I; WT and ∆WD40 or D) ∆IEC mice (values are shown as means ± S.D; n=3).

To further monitor the autophagic activity of cells with either truncated or deleted ATG16L1, the above described experiments were repeated in intestinal epithelial cells (IEC). The results reflect the above findings for ∆WD40 cells regarding reduced autophagic activity. As depicted exemplarily in Figure 3-9 A and B there is a distinct reduction of LC3-II amount and autophagic activity in ∆WD40 intestinal epithelial cells. The assessment of the fold induction of LC3-II to LC3-I could strengthen this result by displaying strong significant decrease of LC3 conversion in all three parameters: the basal autophagy was reduced about of 44 % from WT to ∆WD40 mice (unst. p≤0.001), the Rapamycin induced autophagic activity was reduced about 52 % (Rapa p≤0.01) and the accumulation of LC3-II by stimulation with both Rapamycin and Bafilomycin was reduced about 48 % (Rapa+Baf p≤0.01). As illustrated in Figure 3-9 A and B the basal autophagy was completely inhibited in
RESULTS

ATG16L1 deficient IECs, as no LC3-II conversion was observed. Even stimulation with the mTor inhibitor Rapamycin did not return this dysfunction. Again, the absence of the WD repeat domain led to reduced autophagic activity in IECs as already shown for BMDMs.

Figure 3-9: LC3 expression in intestinal epithelial cells.
A) Intestinal epithelial cells (IECs) were stimulated with Rapamycin (50 µg/ml for 20 minutes) or with Rapamycin and Bafilomycin (100 nM for 20 minutes) or were left untreated. Protein extracts were separated by SDS-PAGE and immunoblotted against LC3 and β-actin; representative image. B) Detected bands for LC3-I and LC3-II were densitometrically analyzed by ImageJ and related to β-actin; representative experiment. C) Fold induction was assessed by determining the ratio between LC3-II and LC3-I; WT and ΔWD40 (values are shown as means ± S.D; n=3; **p≤0.01; ***p≤0.001).

3.3 Evident morphological alterations in Paneth cells in ΔWD40 and ΔIEC mice
In intestinal epithelial cells the truncation of the WD40 repeat domain and the knockout of Atg16l1 led to either less LC3 expression or even suppression of LC3 conversion. Furthermore the colitis experiments developed that ΔIEC mice are highly susceptible to DSS-induced colitis. As Cadwell et al. hypothesized that Paneth cells might have unique sensitivity to autophagy gene disruption58, this led us focus on the Paneth cells. They are described as critically important intestinal innate immune cells.
More precisely Paneth cells are secretory epithelial cells of the small intestine. They are thought to play a role in the control of intestinal microbiota via secretion of granule contents including antimicrobial peptides and lysozyme\textsuperscript{121}. Cadwell et al.\textsuperscript{58} discovered that mice, expressing ATG16L1 at 30 % of the wildtype level (hypomorphic mice), exhibited striking abnormalities in the granule exocytosis pathway of Paneth cells. The following investigations are based on their experimental setup but in contrast, for the present study mice were used with a total deletion of \textit{Atg16l1} in the intestinal epithelial cells (\textit{\textDelta}IEC) and mice with a general deletion of the ATG16L1 WD40 domain (\textit{\textDelta}WD40). For quantifying abnormalities in secretory granules lysozyme staining was performed in sections of the Jejunum. Lysozyme is normally packed in the secretory granules of Paneth cells \textsuperscript{121}. Afterwards, based on Cadwells work Paneth cells were divided into four patterns of lysozyme positive granules (see Figure 3-10 A): normal (D\textsubscript{0}), disordered (D\textsubscript{1}), depleted (D\textsubscript{2}) and diffuse (D\textsubscript{3})\textsuperscript{58}. As illustrated in Figure 3-10 B and C the WT Paneth cells show normal patterns of lysozyme expression (70 % of total Paneth cells), whereas the \textit{\textDelta}IEC Paneth cells show a significant large population (p≤0.01 compared to WT; p≤0.05 compared to \textit{\textDelta}WD40) of cells with diffuse lysozyme staining (64 % of total Paneth cells). Interestingly, the \textit{\textDelta}WD40 Paneth cells displayed different pattern. They exhibited a striking population of cells with significantly disordered lysozyme positive granules (52 % of total Paneth cells; p≤0.01 compared to WT; p≤0.05 compared to \textit{\textDelta}IEC).

These results imply that due to the deletion of the WD40 domain a regular formation of granules might not be possible in Paneth cells leading to a disordered granula allocation. Furthermore, the total lack of ATG16L1 leads to a totally disabled lysozyme packaging to the granules. A diffuse lysozyme allocation is the result.
RESULTS

Figure 3-10: Lysozyme allocation in Paneth cells.
A) Lysozyme expression and allocation patterns are sorted to the following four classes (lysozyme represented in green, white represents areas that exclude lysozyme): normal (D0), disordered (D1), depleted (D2), and diffuse (D3) as described by Cadwell et al. 58 B) Representative images of indirect immunofluorescence of jejunum sections stained for lysozyme (green) in WT, ΔWD40 and ΔIEC mouse crypts (dotted line denotes crypt unit), magnification of 600. C) Number of Paneth cells of WT, ΔWD40 and ΔIEC mice displaying each pattern of lysozyme expression (n=318 cells from 3 WT mice, 300 cells from 3 ΔWD40 mice and 221 cells from 3 ΔIEC mice; values are shown as means ± S.D. * p≤0.05; ** p≤0.01; *** p≤0.001.

Afterwards the characteristics of lysozyme (Lyz1) mRNA expression in IECs and BMDMs were investigated by RT-PCR analysis as shown exemplarily on Figure 3-11 A. GAP3H was used as a loading control. Figure 3-11 B illustrates quite clearly the significant down regulation of Lyz1 in ΔWD40 and ΔIEC cells on basal level in unstimulated IECs (p≤0.05 in ΔWD40 and p≤0.001 in ΔIEC compared to WT).
contrast, the basal Lyz1 mRNA expression level in the BMDMs of ∆WD40 mice was significantly higher than in BMDMs of WT or (as control) ∆IEC mice (p≤0.01 or p≤0.05) (Figure 3-11 C).

To investigate the influence of different stimuli on the expression of Lyz1, the IECs were treated with Rapamycin (50 µg/ml; 20 min.), MDP-LD (100 µg/ml; 2 h), Salmonella typh. (MOI 100; 1 h) and Tunicamycin (5 µg/ml; 4 h). Afterwards the mRNA expression was evaluated semi quantitatively via RT-PCR and following densitometry of the gel-bands. It appeared that in contrast to the WT IECs the Lyz1 expression was not affected in ∆WD40 and ∆IEC cells by one of the stimuli.

To further monitor the connection between ATG16L1 truncation or deletion and vesicle export, the number of vesicles was counted in Paneth cells of Jejunum sections stained with toluidine blue (see Figure 3-12 A for exemplary images). As diagrammed in Figure 3-12 B, two classifications of vesicle numbers have been
compared (less than 10 and more than 10 vesicle per Paneth cell). There is a significant higher amount of ‘less than 10’ vesicles per Paneth cell in ∆WD40 (p≤0.001) or ∆IEC mice (p≤0.01) compared to the WT. Furthermore a significant difference between ∆WD40 and ∆IEC mice was detected as well, as the ∆WD40 genotype represents more cells in the ≤10 class (p≤0.05). The distribution of vesicle numbers in the classification of ‘more than 10’ showed that WT mice were most frequently represented here, whereas the Paneth cells of the ∆WD40 (p≤0.001) and the ∆IEC mice (p≤0.05) displayed significant lower ratio in this class. Again, a significant difference between ∆WD40 and ∆IEC mice was monitored with higher amount of ∆IEC cells in this category (p≤0.05).

Figure 3-12: Number of vesicles in Paneth cells.
A) Representative images of jejunum sections stained with Toluidine blue in WT, ∆WD40 and ∆IEC mouse crypts, magnification of 600. B) Vesicles were counted and divided into two groups: ≤10 or >10 vesicles per Paneth cell. (n=401 cells from 4 WT mice, 725 cells from 5 ∆WD40 mice and 927 cells from 5 ∆IEC mice); values are shown as means ± S.D; * p≤0.05; ** p≤0.01; *** p≤0.001.
To determine if the above investigated abnormalities were limited to Paneth cells as already described by Cadwell et al.\textsuperscript{58}, the numbers of goblet cells per crypt on the one hand and on the other hand the numbers of Paneth cells per crypt were counted. The number of Goblet cells was determined via Periodic acid-Schiff (PAS) staining. As depicted in Figure 3-13 A and B, the number of goblet cells differed in neither of the three mouse genotypes. In contrast the difference in numbers of Paneth cells, which was determined via Lysozyme staining, was significantly distinct in all three mouse strains (see Figure 3-13 C). The WT mice exhibited the highest number of Paneth cells with about 6 per crypt. Whereas the \(\Delta WD40\) mice showed 38\% less Paneth cells with about 4 per crypt (\(p \leq 0.001\)) and the \(\Delta IEC\) mice 71\% less with about 2 per crypt (\(p \leq 0.001\)) in comparison to WT mice. The decrease in number of cells from \(\Delta WD40\) to \(\Delta IEC\) is highly significant as well (\(p \leq 0.001\)).

Electron microscopy of Jejunum sections of three animals per genotype was performed to clarify more detailed the phenotypically differences within the Paneth cells of the genetically altered mouse strains. As illustrated exemplarily in Figure 3-14 \(\Delta IEC\) mice exhibited a comparable phenotype to the hypomorphic mice shown by
RESULTS

Cadwell et al.\(^{58}\). Highlighted in red, large areas of structurally altered cellular components were detected in \(\Delta WD40\) mice and more evident in \(\Delta IEC\) mice, compared to the WT. Cadwell et al. discussed these areas as accumulated cytoplasmic vesicle\(^{58}\), but another explanation might be a dilated endoplasmatic reticulum.

![Figure 3-14: Electron microscopy of Jejunum sections. EM of Jejunum sections of WT, \(\Delta WD40\) and \(\Delta IEC\) crypts (red lines accent the assumed area of dilated endoplasmatic reticulum).](image)

Paneth cell secretory granules are loaded with diverse host defense proteins and peptides, including the above described lysozyme, but also \(\alpha\)-defensins\(^{121,122}\). Since defensins were first identified as antimicrobial peptides, it is suggested that defensins interact with host immune cells, thereby playing important roles in both innate and adaptive immune responses against bacterial infection\(^{123,124}\). Thus it was interesting to investigate the expression of \(\alpha\)-defensins/cryptdins in intestinal epithelial cells. mRNA expression of \(Defa3\) and \(Defa5\) was analyzed by RT-PCR and densitometrically evaluated. Figure 3-15 A and B illustrate quite clearly the significant down regulation of basal \(Defa3\) and \(Defa5\) expression in \(\Delta WD40\) and \(\Delta IEC\) mice (\(p\leq0.01\) or \(p\leq0.05\)) compared to WT mice. \(GAP3H\) was used as loading control. Consequently a deficiency of ATG16L1 not only influences the Lysozyme expression in intestinal epithelial cells, it also influences the expression of \(\alpha\)-defensins on mRNA level.
Figure 3-15: Defa3 and Defa5 mRNA expression in IECs.
A) After RT-PCR and agarose gel electrophoresis the detected bands for Defa3 or B) Defa5 were densitometrically analyzed by ImageJ and related to G3PDH; values are shown as means ± S.D; (Defa3 n=5; Defa5 n=4); * p≤0.05; ** p≤0.01.

Further, the relation between lysozyme expression and LC3 expression in Jejunum sections of all three genotypes was investigated via LC3 and lysozyme co-staining of 3 animals per genotype. As illustrated exemplarily in Figure 3-16, the LC3 expression decreased from WT to ΔIEC mice. The WT sections showed clear LC3 dots, whereas in the ΔIEC sections diffuse LC3 staining could be detected. Interestingly, the ΔWD40 sections showed both, clear LC3 dots and diffuse LC3 expression, as well (i-iii).
Figure 3-16: LC3 and lysozyme staining in Jejunum tissue sections of WT, ΔWD40 and ΔIEC mice.

Jejunum tissue sections of WT, ΔWD40 and ΔIEC mice stained with anti-LC3 (red) and anti-lysozyme (green) antibodies; magnification of 600 x; representative images of n=3 animals per genotype. i, ii and iii show sections of the LC3 staining in magnification of 1300 x to visualize LC3-dots. Counterstaining of nuclei with DAPI appears in blue. Colocalization appears in yellow. Representative images of n=3 per genotype. Optical investigation was performed in n=150 cells from 3 WT mice, n=170 cells from 3 ΔWD40 mice and n=88 cells from 3 ΔIEC mice.
Interestingly, the LC3 signals in Paneth cells of WT and ∆WD40 mice overlaid with some of the lysozyme filled vesicles (see Figure 3-17). High magnification (of 3000 x) showed that the colocalization appeared congruent within the lysozyme filled vesicles for both genotypes. In contrast, no comparable co-staining of LC3 and lysozyme could be observed in the ∆IEC cells.

Figure 3-17: Colocalization of LC3 and lysozyme expression in Jejunum tissue sections of WT, ∆WD40 and ∆IEC mice. Partial excerpt of Figure 3-16: magnification of 1300 x LC3 + lysozyme merged and LC3 single stained images of WT, ∆WD40 and ∆IEC mice. Arrows indicate colocalization of LC3 and lysozyme expression within a cell (yellow). Small boxes indicate details of LC3 images in magnification of 3000 x. Representative images of n=3 per genotype. Optical investigation was performed in n=150 cells from 3 WT mice, n=170 cells from 3 ∆WD40 mice and n=88 cells from 3 ∆IEC mice.
Afterwards, the correlation between lysozyme expression and ATG16L1 expression was analyzed, as well, in Jejunum sections of 3 animals per genotype via ATG16L1 and lysozyme co-staining. As depicted in Figure 3-18 the ATG16L1 staining showed remarkable colocalization with lysozyme positive granules of WT and ΔWD40 mice. Expectedly, no ATG16L1 protein expression was observed in ΔIEC mice apart from unspecific signals.
Figure 3-18: ATG16L1 and lysozyme staining in Jejunum tissue sections of WT, ∆WD40 and ∆IEC mice. Jejunum tissue sections of WT, ∆WD40 and ∆IEC mice stained with anti-ATG16L1 (red) and anti-lysozyme (green) antibodies to visualize potential colocalization. Counterstaining of nuclei with DAPI appears in blue. Colocalization appears in yellow; magnification of 600 x. Representative images of n=3 per genotype. Optical investigation was performed in n=117 cells from 3 WT mice, n=79 cells from 3 ∆WD40 mice and n=41 cells from 3 ∆IEC mice.

More detailed, the colocalization could be illustrated in higher magnification of 1300 x (investigated in 3 animals per genotype). In WT mice nearly all lysozyme positive
vesicles exhibited ATG16L1 co-staining, whereas this seems to decrease in $\Delta$WD40 mice (see Figure 3-19). High magnification of 3000 x showed that the ATG16L1 staining is congruent with the lysozyme filled vesicles. Again, $\Delta$IEC cells showed no ATG16L1 staining apart from unspecific signals.

Figure 3-19: Colocalization of ATG16L1 and lysozyme expression in Jejunum tissue sections of WT, $\Delta$WD40 and $\Delta$IEC mice
Partial excerpt of Figure 3-18: magnification of 1300 x of ATG16L1 + lysozyme merged and ATG16L1 single stained images of WT, $\Delta$WD40 and $\Delta$IEC mice. Arrows that indicate colocalization (yellow) of ATG16L1 and lysozyme within a cell. Small boxes indicate details of ATG16L1 images in magnification of 2800 x. Representative images of n=3 per genotype. Optical investigation was performed in n=117 cells from 3 WT mice, n=79 cells from 3 $\Delta$WD40 mice and n=41 cells from 3 $\Delta$IEC mice.
In this study it could be shown by abnormal lysozyme expression patterns that the vesicle export might be constrained in case of ∆WD40 and especially ∆IEC mice. Hence, it was interesting to visualize a putative connection between the endoplasmic reticulum (ER) and the lysozyme allocation via anti-PDI antibody (Protein Disulphide Isomerase). The investigation was performed optical, using three animals per genotype. As the ER is involved in vesicle packaging and export, Paneth cells exhibit a unique morphology that is characterized by an extensive endoplasmic reticulum and Golgi network\textsuperscript{125} as seen in Figure 3-20. It becomes apparent that in WT and ∆WD40 mice the lysozyme filled granules were separated from the ER. Whereas in Paneth cells of ∆IEC mice the total sources of lysozyme was inside the ER. Additionally, these pictures demonstrate clearly the decline of number of Paneth cells from WT, to ∆WD40 and to ∆IEC mice, that was already described in Figure 3-13, C.
Figure 3-20: PDI and lysozyme staining in Jejunum tissue sections of WT, ∆WD40 and ∆IEC mice.

Jejunum tissue sections of WT, ∆WD40 and ∆IEC mice stained with anti-PDI (red) and anti-lysozyme (green) antibodies to visualize potential colocalization. Counterstaining of nuclei with DAPI appears in blue. Colocalization appears in yellow; magnification of 600x. Representative images of n=3 per genotype. Optical investigation was performed in n=166 cells from 3 WT mice, n=119 cells from 3 ∆WD40 mice and n=65 cells from 3 ∆IEC mice.
3.4 ER-stress in intestinal epithelial cells of ∆IEC and ∆WD40 mice

The results suggested that the observed abnormalities in Paneth cell morphology might be linked with the endoplasmic reticulum. It is known that the unfolded protein response (UPR) is connected closely to autophagy \(^{30,126}\). Thus I analyzed if the modification of ATG16L1 leads to higher susceptibility to ER stress in the ∆WD40 or ∆IEC mice.

∆IEC as well as ∆WD40 and WT mice were treated with Tunicamycin (5 µg/ml for 4h), a commonly used ER stress inducer, by inhibiting the GlcNAc phosphotransferase (GPT), which catalyzes the transfer of N-acetylglucosamine-1-phosphate from UDP-N-acetylg glucosamine to dolichol phosphate in the first step of glycoprotein synthesis \(^{127,128}\), or were left untreated. Afterwards the transcripts of two specific ER-stress markers were examined by expression analysis: Heat Shock 70 kDa Protein 5 (Hspa5), also referred to as 78 kDa glucose-regulated protein (GRP78) and CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP). Figure 3-21 A illustrates exemplarily the significant increase of mRNA expression of Hspa5 and CHOP in intestinal epithelial cells of both ∆WD40 and ∆IEC mice. Densitometric analysis showed that on basal level the expression of Hspa5 and CHOP was significantly increased in ∆WD40 (Hspa5: \(p \leq 0.05\); CHOP: \(p \leq 0.01\)) and ∆IEC mice (Hspa5: \(p \leq 0.01\); CHOP: \(p \leq 0.01\)) compared to the WT mice. Stimulation with Tunicamycin induced a significant upregulation of Hspa5 in WT mice (\(p \leq 0.05\)), but not in ∆WD40 or ∆IEC mice, whereas CHOP was significantly upregulated in both, WT (\(p \leq 0.05\)) and ∆WD40 mice (\(p \leq 0.01\)), but again not in ∆IEC mice after stimulation. In contrast, in BMDMs no difference between ∆WD40 and WT mice occurred. ∆IEC mice were used as a control and showed no difference to the WT mice. All genotypes show an upregulation of Hspa5 and more evident of CHOP on basal level or after stimulation with Tunicamycin. The stimulation with other stimuli like MDP, Salmonella typh. or Rapamycin did not affect the expression of Hspa5 or CHOP in both IECs or BMDMs (data of relative mRNA expression not shown).
RESULTS

Figure 3-21: Hspa5 and CHOP mRNA expression in IECs and BMDMs.
A) IECs of WT, ΔWD40 and ΔIEC mice were stimulated with Rapamycin (50µg/ml, 20Min), MDP-LD (100µg/ml, 2h), Salmonella typh. (MOI100, 1h), Tunicamycin (5µg/ml 4h) or left untreated. mRNA expression of Hspa5, CHOP and GAPDH was performed by RT-PCR and analyzed by agarose gel electrophoresis (representative image).
B) Detected bands for Hspa5 and (C) CHOP of unstimulated and Tunicamycin stimulated IECs were densitometrically analyzed by ImageJ, first related to G3PDH and afterwards to WT unstimulated.
D) Detected bands for Hspa5 and (E) CHOP of unstimulated and Tunicamycin stimulated BMDMs were densitometrically analyzed by ImageJ, first related to G3PDH and afterwards to WT unstimulated; values are shown as means ± S.D; n=4; * p≤0.05; ** p≤0.01.

The above findings were demonstrated exemplarily by antibody staining against activation transcription factor 4 (ATF4) of Jejunum sections of all three genotypes (see Figure 3-22). ATF4 is a transcription factor downstream of CHOP and a marker for ER stress. Comparable to the results shown in Figure 3-21: Hspa5 and CHOP mRNA expression in IECs and BMDMs. Figure 3-21 C, an increase of ATF4 expression is illustrated in untreated ΔWD40 and ΔIEC mice, versus untreated WT mice.
Figure 3-22: ATF4 and lysozyme staining in Jejunum tissue sections of WT, ∆WD40 and ∆IEC mice.

Jejunum tissue sections of WT, ∆WD40 and ∆IEC mice stained with anti-ATF4 (red) and anti-lysozyme (green) antibodies to visualize ER stress; magnification of 600 x. Counterstaining of nuclei with DAPI appears in blue.
4 DISCUSSION

The autophagy protein ATG16L1, as crucial part of the phagophore assembly mechanism, is considered to play a role in gut homeostasis by holding protective functions. Atg16l1 deficient mice are not viable. As shown by other groups, mutations of this protein lead to reduced xenophagy, increased IL-1β levels, high susceptibility to Crohn disease and abnormal granula secretion in Paneth cells. The special function of ATG16L1 in these events is still unknown, especially the role of the WD40 repeat domain. The aim of the present study was to clarify the specific role of ATG16L1 in DSS-induced colitis, Paneth cell vesicle export with antimicrobial peptide expression, in the autophagic machinery and ER stress. I hypothesized, that by using the two genetically modified mouse lines (∆IEC and ∆WD40) I could explain the functions of ATG16L1 and the special purpose of the WD40 repeat domain in the intestinal epithelium.

4.1 Influence of ATG16L1 deletion on DSS-induced colitis

A deregulation of the immune response to commensal microorganisms in the gut may evoke dysfunctional immune activation resulting in inflammation of the intestine. Neutrophilic infiltration and the development of ulcerations and bloody diarrhea are the characteristics of acute DSS colitis. Colitis can be induced in mice with severe combined immunodeficiency lacking T and B cells. Therefore the DSS induced model appears to be independent of a T cell response and is an appropriate tool to study the innate mechanisms of colitis. Within this model the mechanism of inflammation is believed to involve the direct toxic effect of DSS on colonic epithelium leading to the direct activation of immunological cells.

In the present study the results of the DSS induced colitis were assessed by disease activity index (DAI) and evaluating colon tissue by histological examinations (histological score and infiltration of CD4+ cells). Possible causes for the different phenotypes as diminished regenerative proliferation of inflamed intestinal epithelial cells or general impaired intestinal barrier function were determined on the one hand via in vivo administration of BrdU and on the other hand by the administration of FITC-Dextran.

In my experiments I could recapitulate results of other studies (Saitoh and Cadwell) suggesting that ATG16L1 deficiency leads to an increased susceptibility to intestinal
inflammation. In contrast toCadwell et al., who used *Atg16l1* hypomorphic mice, the present study employed a complete deletion of ATG16L1 in the intestinal epithelium. 30% of the normal amount of ATG16L1 in the case of Cadwell’s study seemed to be enough to conserve the ability of the cells, affected of DSS treatment, to resist against inflammation. Here we could show that during DSS induced colitis only the ∆IEC mice differ from the wildtype mice, whereas the ∆WD40 mice do not clearly differ from the wildtype mice phenotype, which could be revealed by DAI, colon length and infiltration of CD4+ cells. But histological investigations showed contradictory results. In case of the histological score and the regenerative response of the intestinal epithelial cells, the ∆WD40 mice exhibit a phenotype, which is located between the WT and the ∆IEC mice. This leads either to the assumption that the WD40 repeat domain is not involved in the development of inflammation, as for example being involved in pathways for activating further immune cells like dendritic cells or macrophages, and that the remaining part of the protein can accommodate with the situation. But another possible reason should be kept clearly in mind, as most of the following examinations of this study show clear differences between the WT and the ∆WD40 mice. The analogy of the achieved results during DSS induced colitis of the WT and the ∆WD40 mice might be due to the large margin that was detected between single individuals especially of the ∆WD40 strain during assessing the DAI. All following experiments that will be discussed later, led to the assumption that a higher number of animals might reveal differences between the wildtype and ∆WD40 strains, as well.

An explanation for the higher susceptibility to DSS induced colitis of the ∆IEC mice might be a dysfunctional intestinal barrier of the ∆IEC mice, as it was shown in a mouse model with *Atg16l1* deficient hematopoietic cells and cells of patients carrying the T300A risk variant. The IL-1β levels of these mice were increased, whereas IL-1β itself increases the intestinal epithelial tight junction permeability, as shown by Al-Sadi and Ma. A functional intestinal barrier is important for the gut homeostasis. Thereby the para cellular pathway functions as gate keeper with apically located tight junctions and lateral adherent junctions and desmosomes. The present study could show that neither truncation of the ATG16L1 WD40 domain nor deletion of the whole ATG16L1 protein in the intestinal epithelium had impact on the intestinal barrier function in unchallenged mice, shown by FITC administration. Hence, no general barrier dysfunction exists in ∆IEC mice. The cause for the high susceptibility
to DSS treatment in these mice might have another explanation, as impairment of regenerative proliferation during inflammation could be one possibility. The regenerative proliferation was tested by BrdU staining and was approved to be impaired in the ΔIEC mice. Interestingly, the regenerative response is impaired in the ΔWD40 mice as well, leading to the above mentioned assumption that the ΔWD40 genotype might also cause to increased susceptibility to DSS-induced colitis. As DSS is toxic for the IECs, an impaired regenerative response during inflammation could lead to a directly impaired immunological function of the intestinal epithelium. Since the ΔIEC mice have genetically normal immune cells, this impaired IEC response may be caused by inadequate chemokine and cytokine signals and a secondary overreaction of innate immune cells like dendritic cells or macrophages. Hence, the direct activation of immunological cells might proceed inadequate, leading to higher inflammatory responses and higher severity of the colitis.

4.2 Discussion of the methodology of in vitro studies for autophagy

The autophagic process consists of continuous dynamic membrane formation and fusion. Hence, it is important to estimate autophagic activity accurately and quantitatively when studying autophagy in vitro.

4.2.1 Estimation of autophagy

Many methods for monitoring autophagy have been established, including GFP-LC3, tandem fluorescent LC3, and LC3-II turnover assay. In this study the estimation of autophagy was performed by a LC3-II conversion assay, a measurement of autophagic turnover in which LC3-II is assayed by immunoblotting with anti-LC3 antibody in the presence and absence of a lysosomal inhibitor. Bafilomycin A1 (an inhibitor of V-ATPase) was chosen as lysosomal inhibitor for monitoring the LC3-II turnover. The accumulation of LC3-II reflects the activity of the process delivering LC3-II into lysosomes, that is, autophagic turnover. V-ATPases are multisubunit complexes organized in two domains. These evolutionary highly conserved enzymes acidify a wide array of intracellular organelles and pump protons across the plasma membranes of numerous cell types. At low concentrations bafilomycin A1 (BafA1) inhibits the V-ATPase by binding to the proteolipid subunit, subunit a might also participate in binding. At these low concentrations, BafA1 is highly specific for V-ATPases. Therefore, these
inhibitors have been widely used in studies of V-ATPase mediated acidification\textsuperscript{142,143}. The vacuolar H+-ATPases (V-ATPases) are responsible for establishing and maintaining intracellular pH gradients across specialized organellar membranes, including the trans-Golgi network (TGN), secretory granules, endosomes and lysosomes. Hence it has to be noted, that Bafilomycin as V-ATPase contributes to the acidification of other organelles, including Golgi and endosomes and therefore may show off-target effects\textsuperscript{144,145}.

4.2.2 Primary cell culture of intestinal epithelial cells

Intestinal epithelial cells play a key role in the homeostasis of the organ. The main functions are to absorb and assimilate selected nutrients and to produce secretions with protective (mucous barrier) or signaling (growth factors or cytokines) properties\textsuperscript{146,147}. They also have the ability to protect the organism from various pathogens and toxins, which are found even under normal conditions in the intestine. Hence, they provide an effective barrier to the complex antigenic load of intestinal contents\textsuperscript{148}. The epithelium of the small intestine is one of the most rapidly proliferating tissues in the body. A process, involving cell generation and migration from stem cells located at the bottom of the crypt to the extrusion of the terminally differentiated cells at the tip of the villus, ensures that the epithelium is continuously renewed\textsuperscript{149}. The proliferation, migration and differentiation events are tightly regulated by a variety of growth factors and macromolecules of the extracellular matrix\textsuperscript{150}. So far the study of human intestinal cell regulation has been limited to cell cultures generated from experimental animals\textsuperscript{151} and human cancer tissues\textsuperscript{152} as primary cells can only be obtained in short-term cultures at low amounts. The normal physiology of the intestinal epithelium has been studied on the whole organ and mucosal preparations, which are limited both by rapid necrosis and degeneration of the epithelium. Finally, it is impossible to differentiate between the functions of the different mucosal cytotypes\textsuperscript{149}.

Significant advances have been made in single-cell preparation and maintenance in short-term cultures \textsuperscript{153}. It is assumed that proliferation of normal gut epithelium \textit{ex vivo} is dependent on the maintenance of the structural integrity of this tissue. Thus, for the present study a preparation of IECs was tried to be performed after Evans et al.\textsuperscript{154}. They established an isolation method of the epithelia by preservation the three-dimensional integrity with the result of epithelial cells detaching as intact units, called organoids. But this preparation method failed for the present study, wherefore
single cell preparation and short term culture were chosen. But recently, Sato et al. established other long-term culture conditions for generating crypt-villus organoids\textsuperscript{155}. These organoids are built by single crypts undergoing multiple crypt fission events. Thereby the fissioned crypts simultaneously generate villus-like epithelial domains in which all differentiated cell types are present. They showed that single sorted Lgr5\textsuperscript{+} stem cells can also initiate these crypt–villus organoids. Thus, a future prospect will be to generate such crypt-villus organoids for further experiments with intestinal epithelial cells.

However, it is also evident that an important limitation of these systems is that observations made with experimental animal models cannot always be transferred to humans. A fact possibly due to the difference of brush border enzyme expression and its regulation by hormones and growth factors. Further, the fundamental differences between animal and human tissues in the composition of the epithelial basement membrane along the crypt-villus axis are important\textsuperscript{156}.

According to anatomical data obtained with mouse small intestine\textsuperscript{157,158} it is important to take care that contamination by stromal cells (fibroblasts) or immune cells (macrophages, lymphocytes) is difficult to control. In this study a purity control was performed to estimate the extent of contamination of the isolated epithelial cells by further cell lineages. But only low expression of CD4\textsuperscript{+}T cells was obtained.

\subsection*{4.2.3 Primary cell culture of bone marrow derived macrophages}

Macrophages are central players in both the innate and the adaptive immune system. They have a crucial phagocytic function and are involved in important cytotoxic activity and in the destruction of microorganisms. Macrophages present antigens to primed T-Lymphocytes and secrete a number of important cytokines and chemokines, which regulate a wide range of immune responses\textsuperscript{147}. Both mature Macrophages and macrophage precursors have their source in the bone marrow. By harvesting immature cells from the femurs of mice and culturing them with specific growth factors, large numbers of macrophages can be derived from bone marrow precursors. This results in a quite homogenous population of cells\textsuperscript{159}.

The cells undergo differentiation and polarization into macrophages with variable functional properties, in response to the in vivo microenvironment. Cytokines and microbial products function alone or in combination to induce differences in functional phenotypes, affecting capacity for phagocytosis and inflammatory responses\textsuperscript{160}. This should be kept in mind for ex vivo cellular interaction studies. They rely on
macrophage differentiation of harvested cellular populations and are performed under largely artificial condition. Further, differences in media components could have an influence on macrophage phenotypes. Currently, ex vivo studies variably rely on culture in recombinant MCSF, or culture in conditioned medium from one of several cell lines that are known to produce MCSF\textsuperscript{161}.

In this study BMDMs were used as a contrast to epithelial cells. Differences in the results would be a hint for cell specific characteristics. Further this cell line provided in contrast to IECs a negative control for the ∆IEC mice with the $\text{Atg16l1}$ knockout only in the intestinal tissue.

4.3 Influence of ATG16L1 truncation or deletion on autophagy

For the CD associated risk variant ATG16L1 T300A it was shown by several groups that bacterial handling is impaired\textsuperscript{48,49,74-76} which might be a reason for reduced autophagic activity. In this study, the autophagic activity was examined by LC3-II turnover. In contrast to the work of Fujita et al., who described that an ATG16L1 WD repeat domain deletion has little impact on canonical autophagy\textsuperscript{44}, it could be shown in the present study that the truncation of the WD40 repeat domain leads to lower LC3 expression levels and reduced LC3-II conversion in basal autophagy in both intestinal epithelial cells and in bone marrow derived macrophages. Even stimulation with the mTor inhibitor Rapamycin or lysosomal inhibition by Bafilomycin did not lead to an enhancement. The truncation of the WD40 repeat domain leads to a decreased autophagic activity of about 50 %, indicating a specific role for the WD40 repeat domain during basal autophagy, especially during LC3-II turnover. The fact, that in ∆WD40 mice the conversion of LC3 is still functional, but in a minor extent, indicates that on the one hand, due to the binding of ATG5 on the N-terminal site of ATG16L1, the complex accumulation of ATG5, ATG12 and ATG16L1 is still running, but on the other hand especially the WD40 repeat domain might be involved in ensuring an accurate and compact complex formation and therefore by missing this domain the complex formation is reduced or loosely. The findings of this study suggest that the function of the ATG16L1 WD40 repeat domain might be the intensifying of the protein interactions during complex formation. The WD40 domain therefore might not be essential for the complex formation and following LC3 conversion, but it strengthens it. But how does this phenotype fit to the results of the DSS induced colitis? There, no striking difference to the WT mice could be shown via DAI. But the distinct reduction
of the autophagic activity enforces the assumption that with more animals a difference in the DAI of the colitis experiment could appear as well. The $\Delta$IEC mice showed high sensitivity to DSS treatment which might be due to an impaired intracellular bacterial killing by autophagic mechanisms. Komatsu et al.\cite{43} and Saitoh et al.\cite{44} described that $\text{Atg16l1}$-deficiency hinders the recruitment of the ATG12-ATG5 conjugate to the isolation membrane, resulting in the loss of LC3 conjugation to phosphatidyl ethanolamine (PE) and the complete inhibition of the basal autophagy machinery. This study confirms these findings. ATG16L1 deletion in the intestinal epithelium leads to the loss of LC3-II turnover and to higher susceptibility to DSS induced colitis. Interestingly, the untreated animals of this genotype do not differ from the wildtype mice in their outward appearance or in histology of the gut. This leads to the assumption, that missing autophagic activity in the intestinal epithelial cells must be absorbed by other mechanisms, whereas during DSS treatment, this failure cannot be compensated anymore and leads to the distinct phenotype.

4.4 Influence of ATG16L1 truncation or deletion on the secretory pathway

Paneth cells are part of the intestinal barrier with the main function of secreting antimicrobial peptides including $\alpha$-defensins and lysozyme\cite{50,52}. These cells increasingly got into focus in the context of their abnormal behavior in inflammatory bowel disease associated with autophagy. For example, mice with reduced expression of the ATG16L1 protein (Atg16l1$^{HM}$) show that Paneth cell packaging of antimicrobial proteins into granules and thus their proper export into the lumen of the gut is defective\cite{58}. Further $\text{Atg5}$ Villin-Cre and $\text{Atg7}$ Villin-Cre mice display morphological abnormalities in their Paneth cells that are indistinguishable from the Atg16l1$^{HM}$ mice. Moreover these mice do not display obvious morphological abnormalities outside the Paneth cells within the ileal epithelium\cite{58,162}.

In this study histological analyses were performed to investigate the effect of the $\text{Atg16l1}$ Villin-Cre deletion or the truncation of the WD40 repeat domain on the morphology of Paneth cells. First, the lysozyme allocation was compared. As this antimicrobial peptide is normally packaged in the secretory granules of Paneth cells\cite{121}, this study confirms the findings of Cadwell et al. Comparable to the morphology of their ATG16L1$^{HM}$ mice\cite{58}, in this study the Paneth cells of the $\Delta$IEC
mice display diffuse lysozyme staining (64 % of total Paneth cells), which implies that
the total lack of the autophagy protein ATG16L1 causes a totally disabled lysozyme
packaging, resulting in diffuse allocation of this antimicrobial peptide within the
Paneth cell.
Interestingly, yet the truncation of the WD40 repeat domain leads to an altered
morphology. The ΔWD40 mice exhibit a striking population of Paneth cells with
disordered allocation of lysozyme positive granules (52 % of total Paneth cells). This
indicates that already the deletion of the WD40 repeat domain causes abnormal
granula formation, leading to the assumption that the WD40 repeat domain is
involved in the procedure of the secretory pathway.
Already the mRNA expression of lysozyme (Lyz1) is modified due to the deletion or
truncation of ATG16L1. Both genotypes ΔWD40 and ΔIEC show significantly
decreased Lyz1 mRNA levels in intestinal epithelial cells. But in contrast, BMDMs of
ΔWD40 mice exhibit significantly increased mRNA levels of lysozyme. This might be
one explanation why the truncation of the WD40 repeat domain does not lead to
higher susceptibility to DSS induced colitis. These animals exhibit macrophages with
higher mRNA expression of lysozyme. In case of higher expression on protein levels,
the cells might be highly effective against intracellular bacteria because of the
enzymatic activity of lysozyme. It hydrolyses glycosidic bonds and is thus able to
hydrolyse the cell wall peptidoglycans of some micro-organisms and thereby kill the
harmful organism\textsuperscript{163}. The high antimicrobial potency of the macrophages might
compensate the reduced lysozyme level of the intestinal epithelial cells in ΔWD40
mice, whereas the ΔIEC lack such compensation due to normal lysozyme expression
in their non-epithelial cells, which in turn leads to higher susceptibility to DSS induced
colitis. But the question why the genetically altered \textit{Atg16l1} leads to such different
expression levels of lysozyme in different cell lines still remains unsolved.
Further, the association between the autophagy proteins ATG16L1 and LC3 and
lysozyme positive granules was monitored via fluorescence staining. LC3 dots,
representing autophagosomes, decreased from WT mice to ΔWD40 mice and were
not detectable in ΔIEC mice. This is concordant with the results of the reduced or
inhibited LC3-II conversion, obtained from the LC3 turn-over experiments.
Interestingly, both proteins ATG16L1 and LC3 show colocalization with lysozyme in
the WT mice. This is still existent in the ΔWD40 mice, but seems to be decreased
due to the truncation of the WD40 repeat domain. This leads to the assumption that
there is an interaction between Paneth cell lysozyme filled secretory vesicles, and LC3, as well as ATG16L1. This interaction might be implemented by ATG16L1, and strengthened by the WD40 repeat domain. But if this interaction is a function of the autophagy protein, autonomous from the autophagy machinery, still remains to be answered. Further, a direct connection between autophagy, Paneth cell vesicle export and the ER could be demonstrated by co-fluorescent staining of PDI (Protein Disulphide Isomerase) as ER marker and lysozyme. This study could demonstrate that in WT mice and ΔWD40 mice the lysozyme filled granules are separated from the ER, whereas the total sources of lysozyme in ΔIEC mice are within the ER. Another evidence for the association of ATG16L1 truncation or deletion on Paneth cell vesicle export is given by subsequent morphological changes, shown by examination of granule numbers in Paneth cells as well as detected by electron microscopy of Jejunum sections of the different mouse genotypes. Cadwell et al. described reduced vesicle numbers in the ATG16L1HM mice which could be confirmed by this study. The numbers of granules differ between WT, ΔWD40 and ΔIEC mice. Both, the truncation of the WD40 repeat domain and the deletion of the ATG16L1 protein in the intestinal epithelial cells lead to decreased granule number within the Paneth cell. Via electron microscopy Cadwell et al. detected areas within the Paneth cells of hypomorphic mice, which they construed as areas of increased cytoplasmic vesicles. In this study, comparable morphological changes were shown for ΔWD40 mice and more evident for ΔIEC mice. One reason might be a hindered retrieval vesicle pathway from the endosome to the ER. But in relation to the findings of the PDI staining, where it could be shown, that the lysosome of ΔIEC mice is located within the ER, this supposes that the large area of stained ER might coincide with the altered cellular structures detected by electron microscopy. This might be a hint for dilated ER structures within the genetically altered mouse strains. Hence, this work delineates an association between the autophagy protein ATG16L1 (with special function for the WD40 repeat domain), Paneth cell granula, the endoplasmic reticulum and supposable cytoplasmic vesicular trafficking.

A study of Zhao et al. affirms the association between autophagy proteins and cytoplasmic vesicular traffic. They described that Atg5 expression in phagocytic cells is essential for cellular immunity to intracellular pathogens in vivo. They showed that Atg5 was required for the recruitment of the IFNγ-inducible p47 GTPase IIGP1 (Irga6) to the vacuole membrane, while, via electron microscopy, they did not detect
double membrane shaped autophagosomes enveloping *T. gondii*. Therefore they concluded that an autophagy protein can participate in immunity and intracellular killing of pathogens via autophagosome-independent processes such as GTPase trafficking\textsuperscript{164}. The present study shows for the first time that both autophagy proteins LC3 and ATG16L1 are located at Paneth cell lysozyme filled granules. Referred to Zhao et al., our findings may indicate an autophagy-independent role for LC3 and ATG16L1 in vesicular trafficking.

Still the question remains why the above named morphological changes are located on Paneth cells within the intestinal epithelium after ATG16L1 deletion or truncation. This study demonstrates that histologically all three genotypes do not seem to differ in the investigated tissue and organ system apart from the one cell lineage in focus, the Paneth cells. Even the number of Paneth cells is significantly decreased by truncation of the WD40 repeat domain and more by ATG16L1 deletion. By contrast, goblet cells as one of the other cell lineages found in the intestinal epithelium are not affected in their number.

4.4.1 Decreased defensin expression by ATG16L1 truncation or deletion

Defensins are antimicrobial peptides produced at a diversity of epithelial surfaces. They assist in maintaining the balance between protection from pathogens and tolerance to normal flora in the intestinal tract and therefore contribute to host immunity. Based on structural differences relating to the bonds between cysteine pairs, defensins are differentiated into two groups, the α- and β-defensins\textsuperscript{165}. Although certain Paneth cell α-defensins (cryptdins) also have been detected in different tissues, α-defensins are specific to Paneth cells in the small intestine\textsuperscript{166,167}. They are processed from inactive proforms by specific proteolytic cleavage steps, as mediated by matrix metalloproteinase-7 (MMP-7; matrilysin) in Paneth cells. It was shown that, MMP-7-null mice, lacking functional cryptdin peptides, have a defect in clearance of intestinal infections\textsuperscript{168}. Thus, altered expression of defensins compromises host immunity and is suggested to be an essential factor in the development of inflammatory bowel disease (IBD)\textsuperscript{169}. The group of Shi found out that α-defensins, including mouse Paneth cell defensins cryptdin-3 and cryptdin-4, human neutrophil defensin HNP-1, and human Paneth cell defensin HD-5, blocked the release of IL-1β from LPS-activated monocytes. They concluded that α-defensins may play an important role in intestinal homeostasis by controlling the production of IL-1β\textsuperscript{170}.  

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In the present study, quantitative analysis of α-defensin mRNA expression was performed. It could be shown that the truncation or deficiency of ATG16L1 influences the expression of α-defensins in intestinal epithelial cells. A significant downregulation of Defa3 and Defa5 mRNA was discovered in both genotypes, which indicates a special function for the WD40 repeat domain in this context. The extreme down regulation of defensin mRNA is likely not explainable by the decreased number of Paneth cells, alone. In that case, a decreasing trend in mRNA levels would have been expected, comparable to the decline of the Paneth cell number from WT to ΔWD40 to ΔIEC mice. An explanation might be the involvement of ATG16L1 in expression pathways that regulate the amount of defensins. It is described that reduced Paneth cell defensin expression has been linked with susceptibility to Crohn disease\textsuperscript{96,97}. Hence, the downregulation of Defa3 and Defa5 mRNA might be a reason for the high susceptibility to DSS induced colitis in ΔIEC mice.

ΔWD40 mice have the same mRNA level of α-defensins as the ΔIEC mice, whereas during DSS-induced colitis, they instead differ from ΔIEC mice by resembling the WT mice. This leads to the conclusion that always a combination of different factors leads to the development of a disease. But as noted above, a higher number of ΔWD40 animals in the DSS-induced colitis may further strengthen the notion that their phenotype might be located between the WT and the ΔIEC mice.

As NOD2 and ATG16L1 are described to interact during autophagy\textsuperscript{49,74,75}, the received opinion, that a variety of different factors play a role in the development of Crohn disease, might be supported by the findings of Wehkamp and colleagues\textsuperscript{171}. They suggest an association between the intracellular receptor NOD2 (which mediates the intracellular recognition of the bacterial cell wall component muramyl dipeptide (MDP) and is also described to recruit ATG16L1 to the plasma membrane at the bacterial entry site), and the mechanisms for regulating the expression of α-defensin\textsuperscript{172}. The NOD2 receptor is most highly expressed in circulating blood monocytes and in intestinal epithelial cells, with particularly high expression in Paneth cells\textsuperscript{173,174}. In their study, Wehkamp and colleagues observed that patients with a NOD2 mutation exhibited a significantly reduced production of Paneth cell HD5 mRNA compared to CD patients with wild type NOD2\textsuperscript{171}. If NOD2 and ATG16L1, which interact during xenophagy, are also connected during the control of defensin production, still remains unsolved.
4.5 ER stress induced by truncation or deletion of ATG16L1

How does a defect of an autophagy protein cause such specific effects on Paneth cells, while other intestinal epithelial cell lineages do not exhibit obvious morphological abnormalities? In contrast to other intestinal epithelial cells, Paneth cells contain an outsized endoplasmic reticulum (ER). The loss or the mutation of key autophagy proteins may disrupt autophagy-mediated organelle turnover, hence disturbing the ER homeostasis. Kaser et al. demonstrated that Paneth cells are particularly sensitive to ER stress by examining the intestinal role of Xbp1, a transcription factor important in ER homeostasis. Further, Cadwell et al. have shown that ATG16L1<sup>HM</sup> Paneth cells exhibit degeneration of mitochondria and replacement of ER with abundant vesicular structures. As it is known that the unfolded protein response (UPR) is linked to autophagy by affecting the expression of ATG5, ATG12 and LC3 as well as the LC3 conversion, this study shows that also ATG16L1 is linked to unfolded protein response in intestinal epithelial cells. This study demonstrates that the truncation of ATG16L1 in ∆WD40 mice and even more the deletion in ∆IEC mice exhibit significant higher mRNA levels of Hspa5 and CHOP in comparison to WT mice, approved by histological staining of ATF4 in intestinal epithelial cells. In contrast, in bone marrow derived macrophages, no ER stress, measured by Hspa5 or CHOP expression levels could be observed. This indicates a specific link between autophagy proteins, intestinal epithelial cells and ER stress.

4.6 Involvement of the autophagy protein ATG16L1 in intestinal inflammation

Despite intensive study of IBD pathogenesis, the mechanisms which contribute to disease initiation and progression still largely remain unknown. The results obtained in this work indicate that many factors are involved in the pathogenesis of DSS-induced colitis. First the function of the autophagy protein ATG16L1 and its WD40 repeat domain during DSS-induced colitis were analyzed. Interestingly, by investigating the DAI, the WT and the ∆WD40 mice did not differ in their phenotype, whereas the ∆IEC mice were significantly higher susceptible to DSS treatment. But as the ∆IEC mice exhibit unaltered and functional immune cells, this works indicates that an inadequate communication between intestinal and immunological cells must be one basis for the high susceptibility of ∆IEC mice. Recent reports suggest that
autophagy takes part in innate and adaptive immunity processes. It was demonstrated that IFN-gamma induces autophagy in macrophages, whereby this immunological induction was achieved through a process that included the GTPase IRGM. Further, the role of autophagy in MHC II presentation of endogenously synthesized viral proteins or non-viral peptides from cytoplasmic sources was displayed. On the one hand ATG16L1 might be directly involved in immunological pathways and its deficiency in intestinal epithelial cells leads to dysfunctions in immunological answers. On the other hand the deficiency of ATG16L1 leads to a blockade of LC3 conversion and therefore to a loss of autophagic activity with reduced intracellular bacterial killing as a result. But also the truncation of the WD40 domain leads to reduced autophagic activity, which would imply that the WD40 mice should differ in their disease severity from WT mice. The WD40 mice ubiquitously exhibit the truncation of the ATG16L1 protein, hence exhibiting the mutation in their immune cells, as well. As demonstrated in this study, the bone marrow derived macrophages of the WD40 mice express a higher level of Lyz1 mRNA in comparison to the WT mice. Therefore their macrophages might be highly effective against intracellular pathogens, which might be one explanation for no higher susceptibility of the WD40 mice.

Paneth cells play an important role in the maintenance of the intestinal barrier by producing, packaging and exporting antimicrobial proteins. Especially the autophagic protein ATG16L1 was described to be involved in morphological and vesicular changes in Paneth cells of Atg16l1 hypomorphic mice comparable to the cellular morphological changes of patients carrying the Crohn associated risk variant T300A. This study could affirm these findings by demonstrating that the truncation of the WD40 repeat domain leads to diminished lysozyme granula packaging, whereas the deletion of ATG16L1 in intestinal epithelial cells leads to complete inhibition of forming lysozyme filled granula. This was confirmed by the findings of this study that the lysozyme is completely located to the endoplasmic reticulum (ER) in ATG16L1 deficient Paneth cells, while the lysozyme filled granules of WT and WD40 mice are separated from the ER. Interestingly, electron microscopic analysis gives a hint that the ER might be dilated due to genetically alteration or deletion of Atg16l1.

Further, this work demonstrates that the autophagy proteins ATG16L1 and LC3 are located to lysozyme filled granules in WT and WD40 mice, indicating that the
granules might interact with these proteins. These findings, and the observation of reduced granula numbers with accompanied supposable increased cytoplasmic vesicles (a possible result of hindered retrieval pathway from the endosome to the ER) due to ATG16L1 truncation or deletion, denote that the autophagy protein ATG16L1 might play an autophagy-independent role in vesicle export. This was already shown for example for ATG5 as participating in immunity and intracellular killing of pathogens via autophagosome-independent-processes such as GTPase trafficking\textsuperscript{164}. Further, Huang et al. gave a link from the ER to autophagosomes. They suggested that antibacterial autophagy occurs at omegasomes (cup-shaped projections from the ER that serve as a platform for autophagosome biogenesis in mammalian cells) and revealed that the Rab1 GTPase plays a crucial role in mammalian autophagy\textsuperscript{178}.

The failure of ∆IEC mice in packaging lysozyme into Paneth cell granules might be another reason for the high susceptibility to DSS-induced colitis, beside the inhibited autophagic machinery in the intestinal epithelial cells. Hence, the intestinal homeostasis is impaired by the lack of exported lysozyme in the mucus\textsuperscript{58}.

This work furthermore demonstrates that the impaired vesicular trafficking of Paneth cells with ATG16L1 truncation or deletion is accompanied by a strong reduction of α-defensin expression on mRNA level. This implies that ATG16L1 is apparently autophagy-independently involved in the regulation of Defa3 and Defa5 production. If directly or indirectly via NOD2 for example (NOD2 is described to be involved in human α-defensin 5 production\textsuperscript{171}) is not clear. While it is supposed that α-defensins may play an important role in intestinal homeostasis by controlling the production of IL-1β\textsuperscript{170}, this leads to the assumption that ATG16L1 might be involved in this regulation. Further it is described that ATG16L1-deficiency causes increased production of IL-1β by Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF)-dependent activation of caspase-1\textsuperscript{44}. This demonstrates that ATG16L1 is an essential component for the control of the inflammatory immune response.

This work describes that intestinal epithelial cells of ∆WD40 and ∆IEC mice show ER stress in an increasing trend from truncation to deletion, whereas the bone marrow derived macrophages are not affected. This implicates that the cause for this phenotype might be found within the physiology of intestinal epithelial cells. Especially the Paneth cells with ATG16L1 truncation or deletion show abnormalities in their morphology, granula export and probably in their cytoplasmic vesicle export.
On the one hand an explanation might be that the hindered vesicle export, shown for Paneth cells, with accompanied accumulation of lysozyme in the ER and further failing autophagosome turnover leads to ER stress. But on the other hand it was recently shown by other groups that ER stress may alter the ER export and the dynamic of post-ER compartments\textsuperscript{179}, indicating that ER stress might be the reason for the phenotypes of the $\Delta$WD40 and $\Delta$IEC mice. This works strengthens this assumption by showing that an increased expression of ATF4 is detected in all intestinal epithelial cells, with no special role for Paneth cells. This might indicate that ATG16L1 truncation or deficiency directly leads to ER stress in all IECs. As Paneth cells have an exceptional position within the intestinal epithelial cells with an outsized ER and specialized secretory vesicle export, especially these cells show the observed morphological abnormalities after ATG16L1 truncation or deletion. The direct connection between ATG16L1 and the ER is not known but might be the omegasome formation. The omegasome is described to be one possible origin of the autophagosome membrane. It is a specialized subdomain of the ER, and autophagosome formation takes place inside the omegasome\textsuperscript{180}. It is not described that ATG16L1 directly interacts with the ER during autophagosome formation, but it might be involved indirectly by interacting with unknown interaction partners. For example, Matsunaga et al. revealed the role of ATG14L in autophagosome formation as it mediates the localization of the autophagy-specific PI3-kinase complex to the ER\textsuperscript{181}.

But nevertheless the data of this work demonstrate that inflammatory bowel disease is controlled by a complex molecular framework. Many factors lead to morphological and functional changes of Paneth cells, which in turn influence the gut homeostasis and might also trigger immunological answers, finally leading to chronically inflammatory responses. A further interesting aspect regarding the connection between ER stress and intestinal inflammation is illustrated by the group of Kaser et al. They showed that induction of ER stress in intestinal epithelium through XBP1 deletion results in spontaneous intestinal inflammation. The XBP1-deficient IECs show diminished antimicrobial activity and response to inflammatory signals in the local milieu\textsuperscript{38}.

At last, an important aspect of this work is the fact that the truncation of the WD40 repeat domain does not lead to a loss-of-function phenotype. As the risk variant T300A is located within this domain which is completely missing in the yeast ATG16
protein, it was argued that the WD40 repeat domain might play an important role in eukaryotes\(^2\). The \(\Delta\)WD40 mice exhibit a mostly different phenotype to the WT mice but they also differ from the \(\Delta\)IEC mice. Their phenotype is a phenotype “in between”. This indicates that on the one hand the WD40 repeat domain is dispensable as no loss-of-function is observed. But on the other hand, it has an important function in possibly strengthening the binding of ATG16L1 with interaction partners which might be necessary for ensuring correct autophagic and non-autophagic activity.

### 4.7 Conclusion

The autophagy protein ATG16L1 seems to play an important role in the maintenance of intestinal homeostasis. A deletion of this protein leads to higher susceptibility to DSS induced colitis with highly inflamed intestinal tissue. This work gives a hint that especially the morphology of Paneth cells is affected by genetically altered \(Atg16l1\), and changes within these cells may be involved in the development of inflammation. The deletion or truncation of the ATG16L1 protein might be the trigger for ER stress which in turn is assumed to be the reason for diminished or defective granular packaging within the ER/trans Golgi network, leading to a reduced number of secretory vesicles. Further the retrieval pathway from endosomes to the ER could be affected, as well. Moreover, this work shows that ATG16L1 is involved in regulating the expression of \(\alpha\)-defensins on mRNA level. Further, as a reason of reduced or impaired autophagic activity due to the truncation or deletion of ATG16L1, rising numbers of intracellular pathogens might damage the intestinal cells. Taken together all these effects might be involved in intestinal inflammation (see Figure 4-1).
Figure 4-1: Supposed effects of ATG16L1 truncation or deletion on intestinal inflammation

Top panel shows the proposed functions of ATG16L1 in the Paneth cell. Bottom panel shows the assumed effect of ATG16L1 truncation or deletion on the Paneth cell leading to intestinal inflammation. Red arrows indicate the secretory pathway, blue arrows the retrieval pathway to the endoplasmic reticulum. (GN: Golgi Network; ER: Endoplasmic Reticulum)
4.8 Future prospect

ATG16L1 deletion in the intestinal epithelial cells leads to higher susceptibility to DSS induced colitis. Furthermore, ATG16L1 truncation and deletion lead to reduced or impaired autophagic activity in BMDMs and IECs and to morphological changes of Paneth cells with concurrent impaired granula packaging and vesicle export. This might be a result of ER stress. Further the α-defensin production is inhibited. Based on these findings and interpretations, some future prospects have been derived for additional experiments.

I. The ∆WD40 mice and the ∆IEC mice show decreased or impaired lysozyme packaging into granula and additionally dilated ER. This indicates that the secretory vesicle pathway and possibly the retrieval pathway are impaired. Further, this work leads to the assumption, that the truncation or deletion of ATG16L1 might be the trigger for ER stress. And in turn, that the ER stress might be the reason for the impaired vesicle pathways. Future experiments will have to show how ATG16L1 is involved in ER stress. Is there a direct connection partner during omegasome formation? Or is the correlation between ER stress and ATG16L1 given by different interaction like for example Clathrin, COPI or COPII?

II. The truncation or deletion of ATG16L1 leads to a complete downregulation of Defa3 and Defa5 on mRNA level. This indicates that the control of the α-defensin production might be an important function of ATG16L1 for regulating the gut homeostasis. Is ATG16L1 involved in the control of other cytokines or chemokines as well, leading to abnormal immunological responses during DSS induced colitis?
5 SUMMARY

The chronic inflammatory disorders of the gut Crohn disease and Ulcerative colitis are counted among inflammatory bowel disease (IBD) and are characterized by bloody diarrhea, ulcerations and infiltrations of inflammatory cells. The molecular mechanisms of the disease still remain unknown. But it has recently been shown, that genome wide association (GWA) studies are the most productive approach for discovering the genetic variations responsible for IBD susceptibility. As the autophagic proteins ATG16LI and IRGM were implicated in CD by GWA, Genetic and functional studies indicate that autophagy is a key pathway in the pathogenesis of CD.

For ATG16L1, a non-synonymous SNP was identified, which causes an amino acid change at position 300 in the amino acid sequence (alanine to threonine). Following experiments in human cell lines demonstrated that ATG16L1 *300A cells show impaired bacterial handling. Additional experiments indicate that chimeric mice with Atg16l1-deficient hematopoietic cells have increased levels of the inflammatory cytokines IL-1β and IL-18 and are high susceptible to dextran sodium sulfate (DSS) induced colitis. Atg16l1-deficient mice die shortly after birth and cells derived from such animals show autophagy defects in vitro, indicating that the protein ATG16L1 is essential for surviving the period of neonatal starvation. While the functions of the N-terminal domain as well as the central coiled coil domain are known, the function of the C-terminal WD40 domain of the ATG16L1 protein still remains entirely unknown.

The aim of this work was to investigate the function of ATG16L1 as well as of the WD40 domain in the pathogenesis of IBD. Since Atg16l1 deficient mice are not viable, conditional Villin Cre knock out mice with a deletion of the ATG16L1 protein in the intestinal tissue (∆IEC) were used for this study, as well as mice with a truncation of the WD40 repeat domain (∆WD40). To elucidate the role of ATG16L1 in IBD, mice were treated with DSS. Interestingly, ∆IEC mice were highly susceptible to DSS-induced colitis. Furthermore, ATG16L1 truncation or deletion leads to reduced or impaired autophagic activity in BMDMs and IECs and to morphological changes in Paneth cells including impaired granula packaging and vesicle export. This work gives a hint, that these morphological changes might be a result of ER stress, which
in turn was shown to be triggered by ATG16L1 deletion or truncation. Further, it could be shown that the α-defensin production is inhibited on RNA level in both genotypes, demonstrating that ATG16L1 plays an essential role in the control of the inflammatory immune response.

Further, this work indicates that the WD40 domain of the ATG16L1 protein is dispensable, as in ΔWD40 mice the autophagic machinery is reduced but not inhibited and as morphological changes of the Paneth cells are not as drastic as in ΔIEC mice. Further, these animals do not show disease severity as strong as the ΔIEC animals. As ΔWD40 mice can be located phenotypically mostly “in between” the WT and the ΔIEC phenotypes, this indicates that the main function of the WD40 domain might be the strengthening of protein bonds for adequate protein interactions. The loss of the domain does not induce a loss of function but a decrease of function.

Taken together, all results demonstrate that ATG16L1 plays an important role in the homeostasis of Paneth cells, indicating that genetically alteration of Atg16l1 may play a crucial role in the pathogenesis of intestinal inflammation.
6 ZUSAMMENFASSUNG


Im Falle von ATG16L1 wurde ein nicht-synonymer SNP (Variation einzelner Basenpaare im DNS-Strang) identifiziert, welcher einen Aminosäureaustausch an Position 300 der Aminosäuresequenz (Alanin zu Threonin) bewirkt. In humanen Zelllinien konnte gezeigt werden, dass die ATG16L1 Variante *300A einen eingeschränkten Wirkungsmechanismus gegen Bakterien hervorruft. Nachfolgende Versuche bei chimären Mäusen mit ATG16L1 defizienten hämatopoetischen Stammzellen bewiesen, dass die Tiere erhöhte Level der inflammatorischen Zytokine IL-1β und IL-18 aufweisen, ebenso wie eine hohe Anfälligkeit für eine durch DSS hervorgerufene Kolitis. Mäuse mit einem komplett ausgeschalteten Gen, welches für ATG16L1 kodiert, sterben kurz nach der Geburt. Mittels in vitro Studien konnte gezeigt werden, dass primäre Zellen dieser Tiere Autophagiedefekte aufweisen, was zu der Annahme führte, dass ATG16L1 eine essentielle Rolle während der Phase des neonatalen Nährstoffmangels spielt. Während die Funktion der N-terminalen Domäne und der zentralen coiled coil Domäne des ATG16L1 Proteins bekannt ist, ist die Funktion der C-terminalen WD40 Domäne gänzlich ungeklärt.

Ziel dieser Arbeit war es, die spezielle Funktion von ATG16L1 und der WD40 Domäne während einer chronisch entzündlichen Darmerkrankung zu klären. Basierend auf dem Wissen, dass Atg16l1 defizierte Tiere nicht lebensfähig sind, wurden für diese Arbeit Tiere mit einem konditionalen Knockout, katalysiert von der Cre-Rekombinase unter der Direktion vom Villin Promotor, verwendet (ΔIEC). Folglich ist in diesen Tieren die ATG16L1 Deletion nur auf das intestinale Epithelium...
ZUSAMMENFASSUNG


Schließlich deuten alle Ergebnisse dieser Arbeit darauf hin, dass das Protein ATG16L1 eine wichtige Rolle in der Paneth Zell Homöostase spielt. Folglich können Mutationen oder Deletionen dieses Proteins an der Pathogenese von chronisch entzündlichen Darmerkrankungen beteiligt sein.
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## 10 APPENDIX

### 10.1 Abbreviations

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<tbody>
<tr>
<td>µg</td>
<td>Microgram</td>
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<tr>
<td>µl</td>
<td>Microliter</td>
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<td>Amino Acid</td>
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<td>Ab</td>
<td>Antibody</td>
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<td>APS</td>
<td>Ammonium Persulfate</td>
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<tr>
<td>ATF</td>
<td>Activating Transcription Factor</td>
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<tr>
<td>ATG</td>
<td>AuTophaGy related</td>
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<td>ATP</td>
<td>Adenosine 5’-Triphosphate</td>
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<tr>
<td>Baf</td>
<td>Bafilomycin</td>
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<tr>
<td>BMDM</td>
<td>Bone Marrow Derived Macrophage</td>
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<tr>
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<td>BrdU</td>
<td>5-Bromo-2-Deoxyuridine</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>C</td>
<td>Carboxy</td>
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<td>Coiled Coil</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn Disease</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>Cvt vesicle</td>
<td>Cytoplasm-To-Vacuole Transport Vesicle</td>
</tr>
<tr>
<td>d</td>
<td>Day</td>
</tr>
<tr>
<td>DAI</td>
<td>Disease Activity Index</td>
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<td>4,6-Diamino-2-Phenylindole Dihydrochloride</td>
</tr>
<tr>
<td>DEPC</td>
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<td>DMEM</td>
<td>Dulbecco’s Minimal Essential Medium</td>
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<td>Dimethyl sulfoxide</td>
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<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>dNTP</td>
<td>2’-Desoxyribonucleotide-5’-Triphosphate</td>
</tr>
<tr>
<td>DSS</td>
<td>Dextran Sodium Sulfate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithithreitol</td>
</tr>
<tr>
<td>E</td>
<td>Enzyme</td>
</tr>
<tr>
<td>ECL</td>
<td>Electrochemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>eIF2α</td>
<td>Eukaryotic Initiation Factor 2α</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>G3PDH</td>
<td>Glyceraldehyde-3-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine Triphosphate</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome Wide Association Studies</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's Balanced Salts</td>
</tr>
<tr>
<td>HE</td>
<td>Hematoxylin-Eosin</td>
</tr>
<tr>
<td>Hepes</td>
<td>(4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory Bowel Disease</td>
</tr>
<tr>
<td>IEC</td>
<td>Intestinal Epithelial Cell</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IRE1</td>
<td>Inositol Requiring Enzyme</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
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<tr>
<td>L</td>
<td>Liter</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny Broth</td>
</tr>
<tr>
<td>LC3</td>
<td>Microtubule-Associated Protein 1 Light Chain 3</td>
</tr>
<tr>
<td>LIR</td>
<td>LC3-Interacting Region</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>Lyz</td>
<td>Lysozyme</td>
</tr>
<tr>
<td>MDP</td>
<td>Muramyldipeptide</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse Embryonic Fibroblast</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal Essential Medium</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>MHC II</td>
<td>Major Histocompatibility Complex (Class II)</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MMM</td>
<td>Mouse Macrophage Medium</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity Of Infection</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian Target Of Rapamycin</td>
</tr>
<tr>
<td>N</td>
<td>Amino/NH₂</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD-Like Receptor</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-Binding Oligomerization Domain</td>
</tr>
<tr>
<td>PAS</td>
<td>Periodic Acid-Schiff</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PDI</td>
<td>Protein Disulphide Isomerase</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidyl ethanolamine</td>
</tr>
<tr>
<td>PERK</td>
<td>Protein Kinase RNA-Like Endoplasmic Reticulum Kinase</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-Kinase 1</td>
</tr>
<tr>
<td>PVDVF</td>
<td>Polyvinylidene Fluoride</td>
</tr>
<tr>
<td>Rm M-CSF</td>
<td>Recombinant Mouse Macrophage Colony-Stimulating Factor</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
</tbody>
</table>
10.2 Publications

**NOD2-C2 - a novel NOD2 isoform activating NF-kappaB in a muramyl dipeptide-independent manner.**

**Molecular signatures of a disturbed nasal barrier function in the primary tissue of Wegener's granulomatosis.**
Mucosal Immunol. 2011 Mar 16

**The complex interplay of NOD-like receptors and the autophagy machinery in the pathophysiology of Crohn disease.**
European Journal of Cell Biology, Volume 90, Issues 6-7, June-July 2011, Pages 593-602, SFB 415: Specificity and Pathophysiology of Signal Transduction Pathways
10.3 Curriculum vitae

Lebenslauf

Persönliche Daten

Name: Dipl. Biol. Janne Böck (geb. Wessel)
Anschrift: Hansastraße 72, 24118 Kiel,
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e-Mail: j-boeck@web.de
Geburtstag: 26.02.1981 in Kiel
Familienstand: verheiratet, 1 Kind (geb. am 22.09.2011)

Schulbildung

1987 – 1991 Claus-Rixen-Grundschule in Altenholz
(Leistungskurse Deutsch und Biologie)

Wissenschaftlicher Werdegang

09/2011 – 09/2012 Elternzeit

04/2008 – 08/2011 Promotion (rer. nat) am Institut für klinische Molekularbiologie Kiel (Direktor: Prof. Dr. Stefan Schreiber)
Arbeitsthema: The role of ATG16L1 in chronic inflammatory bowel disease

09/2007 – 03/2008 Diplomarbeit im Fach Zellbiologie am Forschungszentrum Borstel (Abteilung Biochemische Immunologie), „Aktivierung humaner Leukozyten durch Varianten des CXC-Chemokins Plättchenfaktor 4“, Betreuer: PD Dr. Frank
Petersen, Prof. Dr. Sylvia Bulfone Paus (Note: sehr gut)

07/2007 – 08/2007 Praktikum im Forschungszentrum Borstel
(Abteilung Biochemische Immunologie, PD Dr. Frank Petersen)

10/2002 – 03/2008 Studium der Biologie an der Christian-Albrechts-
Universität zu Kiel mit dem Hauptfach Zellbiologie (Note 1,0), dem biol. Nebenfach Mikrobiologie (Note 1,3) und dem nicht-biol. Nebenfach Rechtsmedizin (Note 1,7), Abschluss: Diplom

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Frau Dr. Olga Gavrilova danke ich für die Unterstützung bei der Durchführung der Maus-Experimente.


Vor allem aber möchte ich mich bei Steffen Böck bedanken. Danke Steffen, für deinen Glauben an mich.
10.5 Eidesstattliche Erklärung

Hiermit versichere ich, Janne Böck, an Eides statt, dass ich die vorliegende Arbeit selbstständig und nur mit Hilfe der angegebenen Hilfsmittel und Quellen unter Anleitung meiner akademischen Lehrer angefertigt habe.

Diese Dissertation wurde bisher an keiner anderen Fakultät vorgelegt. Ich erkläre, kein anderes Promotionsverfahren ohne Erfolg beendet zu haben, und dass keine Aberkennung eines bereits erworbenen Doktorgrades vorliegt.

Ich erkläre, dass die Arbeit unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft entstanden ist.

Kiel, den 10.02.2012
Janne Böck