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Biochemical and cell biological effects of the single A-subunit of the bacterial subtilase cytotoxin

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List of Abbreviations

%	Percentage
°C	Degree Celsius
µ	Micro- (10^{-6})
approx.	Approximately
A-domain	Enzymatically active domain of AB-toxins
Asp	Aspartic acid
a.u.	Arbitrary units
BAF	Bafilomycin A1
B-domain	Binding/translocation domain of AB-toxins
BFA	Brefeldin A1
BiP	Binding immunoglobulin protein
BSA	Bovine serum albumin
CO_2	Carbon dioxide
COG	Conserved Oligomeric Golgi
CRM	Cross-reacting material
CT	Cholera toxin
CTB	Cholera toxin binding domain
C-terminus	Part of a polypeptide/protein with a free carboxy group
DAEC	Diffusely adherent *E. coli*
DMEM	Dulbecco's modified Eagle's medium
DSS-crosslinker	Disuccinimidyl suberate-crosslinker
DTT	Dithiothreitol
E	Glutamic acid
EAEC	Enteroaggregative *E. coli*
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetate
eEF-1	Eukaryotic elongation factor 1
eGFP	Enhanced green fluorescent protein
EHEC	Enterohemorrhagic *E. coli*

EIEC	Enteroinvasive *E.coli*
EPEC	Enteropathogenic *E.coli*
ER	Endoplasmic reticulum
et al.	and other [lat.]
ETEC	Enterotoxigenic *E. coli*
ExPEC	Extraintestinal pathogenic *E. coli*
FACS	Fluorescence-activated cell sorting
FCS	Fetal Calf serum
g	Gram
Gly	Glycine
h	Hour
HCT	Human colon cancer cells
HeLa	Human epithelial cells
His	Histidine
HSA	Human serum albumin
HUS	Hemolytic uremic syndrome
InPEC	Intestinal pathogenic *E. coli*
JTB	Jumping translocation breakpoint
KH_2PO_4	Potassium dihydrogen phosphate
KCL	Potassium chloride
l	Liter
L	Leucine
L1CAM	L1 cell adhesionmolecule
LEE	Locus of enterocyte effacement
m	Milli (10^{-3})
MEM	Minimum Essential Medium
$MgCl_2$	Magnesium dichloride
min	Minute
ml	Milliliter
mM	Milli Molar
MM	Master mix
n	Sample size

NaCl	Disodium chloride
NC	Negative control
NEAA	Non-essential amino acids
Neu5Ac	N-acetylneuraminic acid
Neu5Gc	N-glycolylneuraminic acid
NMEC	Neonatal meningitis *E. coli*
OEP	Outer membrane efflux protein
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBS-T	PBS containing 0.1 % Tween-20
PDI	Protein disulfide isomerase
PFA	Paraformaldehyde
rcf	Relative centrifugal force
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SE-PAI	Subtilase encoding pathogenicity island
Ser	Serine
Sf	Serum-free
SMP	Skimmed milk powder
STEC	Shiga toxin producing *E.coli* strains
Stx	Shiga toxin
T3SS	Type III secretion system
TDE	Thiodiglycol (2,2′-Thiodiethanol)
Tia	Toxigenic invasion locus A
TIR	Translocated intimin receptor
UPEC	Uropathogenic *E. coli*
UPR	Unfolded protein response
V	Volume
VTEC	Vero cytotoxin-producing *E. coli*
WB	Western blot
WT	Wildtype

1 Introduction

1.1 *E.coli* as an intestinal pathogen

Escherichia coli is a Gram-negative bacterium which is naturally occurring in the intestinal microbiota of humans and animals and usually harmless. However, through genome plasticity evolution has led to the development of pathogenic strains with relevance to public health. Pathogenic *E.coli* can be mainly devided into two groups: extraintestinal pathogenic *E. coli* (ExPEC) and intestinal pathogenic *E. coli* (InPEC). The ExPEC pathotype includes uropathogenic *E. coli* (UPEC), neonatal meningitis *E. coli* (NMEC), and septicemic *E. coli*, wheras the InPEC pathotype is able to cause diarrheal diseases and can be further subdivided into at least six well-described categories: enteropathogenic *E.coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E.coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC) and enterotoxigenic *E. coli* (ETEC) (Nataro und Kaper 1998; Kaper et al. 2004; Harrington et al. 2006). These *E.coli* enteropathotypes share virulence traits as well as distinct clinical, epidemiologic and pathogenic profiles (Kaper et al. 2004). *E.coli* strains can also be classified by serotyping based on their O (lipopolysaccharide,LPS) and H (flagellar) antigens that define serogroups (O antigen only) or serotypes (O and H antigens). So far, 117 serotypes have been identified and since some serotypes can belong to more than one pathotype (e.g., O26:H11 can be either EPEC or EHEC) this classification does not allow a pathotype identification (KAUFFMANN 1947; Clements et al. 2012; DebRoy et al. 2018).

1.2 Shiga toxin producing *E. coli* strains

Shiga toxin producing *E.coli* strains (STEC) are zoonotic pathogens which are characterized by the production of at least one member of the cytotoxin family called Shiga toxin (Stx), which was named after the similarity to a cytotoxin produced by *Shigella dysenteriae* serotype 1 (O'Brien et al. 1982). An equivalent term for STEC is the name “Vero cytotoxin-producing E. coli” (VTEC), which refers to the obersvation that these strains produce a toxin that is cytotoxic for Vero cells (Konowalchuk et al. 1977). The advantages and disadvantages of the respective nomenclature were discussed by their proponents (Calderwood SB 1996; Karmali M A 1996) , whereby both terms still exist today and are used by the scientific community. STEC strains causing

hemorrhagic colitis and the hemolytic uremic syndrome (HUS) are called enterohemorrhagic *E. coli* (EHEC) meaning that EHEC is a pathogenic subgroup of STEC (Nataro und Kaper 1998; Kaper et al. 2004). STEC strains are found in the gastrointestinal tract of many farm animals and wildlife and ecpsecially ruminants are seen as an important reservoir and main source of infection for STEC in humans. The pathogen is mainly transmitted via contaminated food or water to humans, wherby also a transmission route by direct animal contact or human-to-human transfer was reported in individual cases (Kaper et al. 2004). Although there are more than 400 distinct serotypes of STEC known (Blanco et al. 2004), only less than 10 O-groups caused most of the disease outbreaks with the most significant EHEC serogroup worldwide being O157. Besides O157:H7, the serotypes O26:H11, O45:H2, O103:H2, O111:H8, O121:H19 and O145:H28 are the most isolated once during EHEC diseases in humans (Karmali et al. 2003; Karmali et al. 2010). The largest outbreak of EHEC infections in Germany to date occured in 2011 caused by serotype O104:H4. It was characterized by numerous cases of bloody diarrhea and HUS with being at the same time the largest outbreak of reported HUS patients worldwide. Contaminated fenugreek sprouts are believed to be the cause of the outbreak, whereas it remains unclear how the sprouts came into contact with the pathogen (Frank et al. 2011; Burger 2012; Karch et al. 2012). The key virulence factor for EHEC is Stx, which can be devided in two subgroups Stx1 and Stx2 sharing approximately 54 % amino acid homology and Stx2 being potentially more virulent and associated with HUS (Pal 2015; Golshani et al. 2016). Stxs belong to the family of AB_5 toxins consisting of a single 32-kDa A-subunit, which contains the enzymatic activity and of five identical 7.7-kDa B-subunits, which mediate the binding of the toxin to a specific membrane glycolipid receptor, called globotriaosylceramide (Gb3) (Lindberg et al. 1987; Lingwood et al. 1987; Waddell et al. 1988). Gb3 deficient cell lines and mice are insensitive to Stx (Jacewicz et al. 1994; Okuda et al. 2006). After toxin binding, the toxin/receptor complex enters early endosomes and is transported via a retrograde pathway through the Golgi apparatus and then to the endoplasmic reticulum (ER). The catalytic A_1-subunit is translocated to the cytosol and the A_1 peptide acts as an N-glycosidase that removes a single adenine residue from the 28S ribosomal subunit, whereby the affected ribosomes can no longer associate with elongation factor 1 leading to the inhibition of protein synthesis and ultimately to cell death (Obrig et al. 1987; Endo et al. 1988; Tesh 2012). Besides Stx as

a pathogenicity factor, most STECs carry a pathogenicity island called locus of enterocyte effacement (LEE), which encodes proteins necessary for the formation of attaching and effacing (A/E) lesions by which the pathogen damages the microvilli brush border of the intestinal epithelium (Moon et al. 1983; Clements et al. 2012). The LEE contains genes encoding for a type III secretion system (T3SS), a protein translocation system, an adherence system characterized by the bacterial surface protein intimin and its receptor, named translocated intimin receptor (TIR) (Elliott et al. 1998; Gyles 2007). Although LEE-positive EHEC strains are considered more likely to cause disease, LEE-negative strains have been described in recent years that have caused outbreaks of HC and HUS. These includes EHEC serotype O104:H4, which, as mentioned above, caused one of the largest and most severe HUS outbreaks in Germany in 2011 (Karch et al. 2012) as well as an outbreak caused by the LEE-negative strain O113:H21 in 1998, which interestingly encoded in addition to Stx a toxin called subtilase cytotoxin (SubAB). Although the importance of Shiga toxin in the pathogenesis of STEC diseases is well studied, there is evidence that other toxins like SubAB may be a relevant pathogenicity factor in clinical infections.

1.3 Subtilase cytotoxin

The subtilase cytotoxin (SubAB) was first identified in an EHEC strain responsible for a HUS outbreak in Australia in 1998 (Paton et al. 2004) and subsequently reported in numerous other STEC stranis (Paton und Paton 1998; Cergole-Novella et al. 2007; Khaitan et al. 2007). SubAB is an AB_5 toxin, that unlike other kown AB_5 toxins like cholera toxin (Ctx), pertussis toxin (Ptx) or Shiga toxin (Stx), does not translocate into the cytosol but rather remains in the ER and develops its enzyme activity there (Beddoe et al. 2010). Because of the different mechanism of action, it forms a new subfamily within the AB_5 toxins.

1.3.1 Genetic organization and structure

SubAB was first described in the LEE-negative STEC strain 98NK2 belonging to the serotype O113:H21, which contains the subtilase-coding operon on the large, conjugative virulence plasmid pO113 (Paton et al. 2004). Since than, SubAB-coding genes were found in numerous other LEE-negative STEC strains. Nowadays, SubAB variants are categorized in two types: SubAB1, being encoded on plasmids and SubAB2, being encoded on chromosomes, sharing 90 % sequence identity (Tozzoli et al. 2010).

SubAB2 can be further distinguished in three subtypes: SubAB2-1, SubAB2-2 and SubAB2-3 (Tozzoli et al. 2010; Funk et al. 2013; Nüesch-Inderbinen et al. 2015; Tasara et al. 2017). SubAB2-1 is located on the the subtilase encoding pathogenicity island (SE-PAI), which carrys an integrase gene, a shiA gene (homologous to the shiA gene of the *Shigella flexneri* pathogenicity island SHI-2), a sulphatase, the toxigenic invasion locus A (tia) and the subAB operon (Moss et al. 1999; Michelacci et al. 2013). SubAB2-2 is encoded on an outer membrane efflux protein (OEP) (Funk et al. 2013) and upstream of the SubAB2-1 or SubAB2-2 locus, the SubAB2-3 operon can be found, which is associated with a gene encoding a hypothetical protein (Nüesch-Inderbinen et al. 2015). The genes within the SubAB2 variants are highly conserved sharing 99 % sequence identity (Funk et al. 2013). Comparing the SubAB variants it could be shown that SubAB1 triggers the highest cytotoxicity on Vero cells compared to SubAB2-1 and SubAB2-2 and that SubA1 and SubB1 form a more stable complex than SubA2-2 and SubB2-2 *in vitro* (Funk et al. 2013; Funk et al. 2015; Krause et al. 2019). Investigations regarding the prevelance among the SubAB variants suggest that SubAB2 and in particular SubAB2-2 represents the prevalent allelic variant in SubAB-positive, LEE-negative STEC (Nüesch-Inderbinen et al. 2015; Michelacci et al. 2013).

1.3.2 Toxin assembly

In general, the subtilase cytotoxin consists of an enzymatically active A-domain (SubA) and a separate B-pentamer (SubB), which mediates cell binding and uptake into the host cell. SubA is composed of two domains which are connected by a disulfide bond formed by the residues Cys-288 and Cys-331. The SubA A1 domain contains the proteolytic activity via the catalytic triad (Asp-52-His-89-Ser-272) and the SubA A2 domain interacts with SubB by penetrating into the central pore of the SubB pentamer (Figure 1).

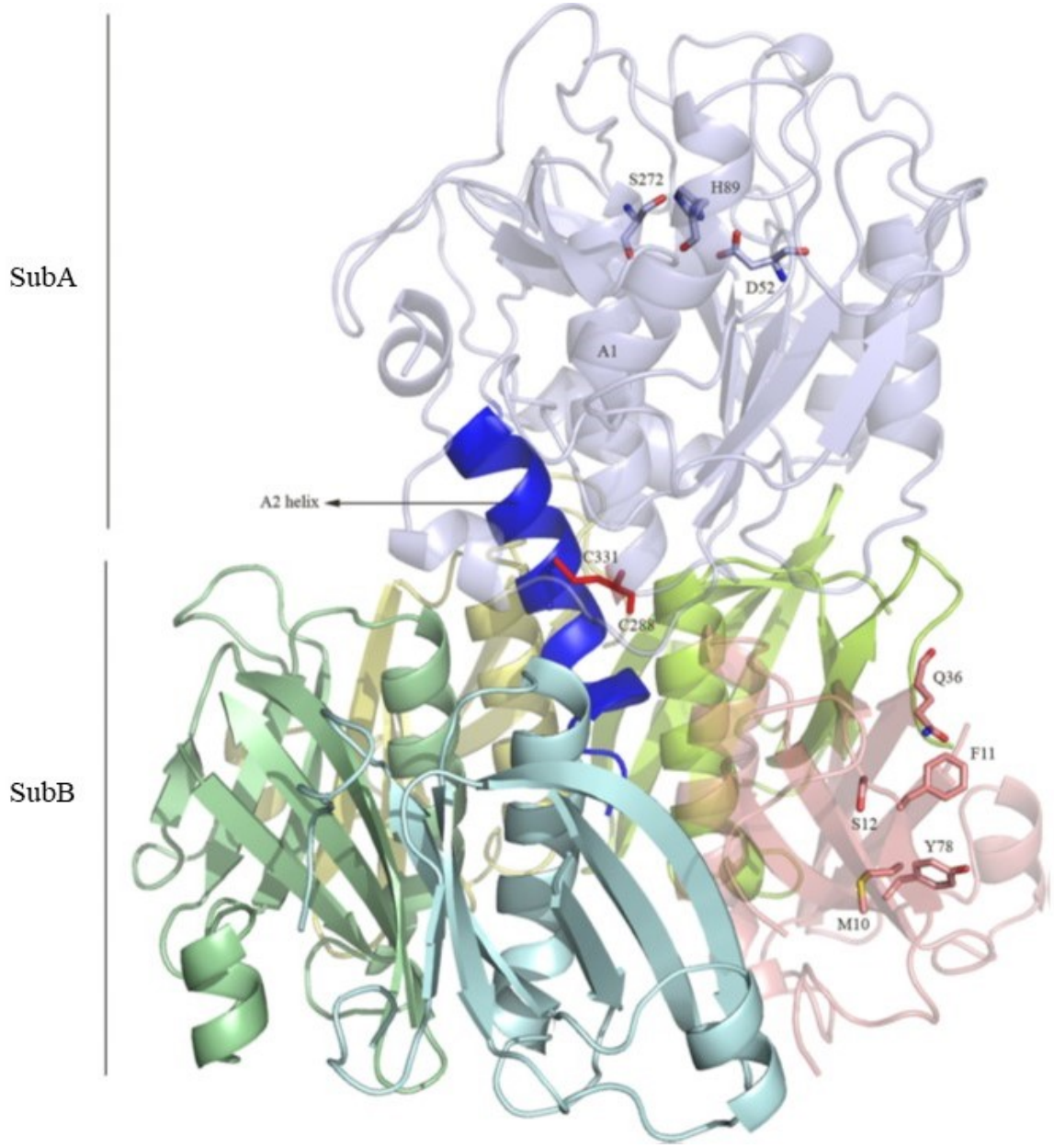

Figure 1 Chrystal structure of SubAB.
SubA is shown in blue with the A1 component in light and the A2 component in dark blue. Both α-helixes are connected via a disulfide bond shown in red. For the SubB pentamer each individual unit is represented differently with the colors cyan, yellow, lemon, salmon, and pale green. (Modified from Le Nours et al. 2013 under CC BY 4.0, https://creativecommons.org/licenses/by/4.0/.)

More precisely, the N-terminal end of the SubA A2 helix (residues Lys-333, Lys-334, Tyr-335, Ile-336, Pro-337, and Val-338) forms mainly hydrophobic interactions with the hydrophobic or neutral residues (Leu-65, Tyr-66, Thr-69 and Thr-70) of the SubB pentamer (Le Nours et al. 2013). Interestingly, mutations in these residues had no impact on the assembly of the B-pentamer or the holotoxin but resulted in reduced cytotoxicity which may be due to decreased stability of the interaction between SubA

and SubB (Le Nours et al. 2013). The main assumption is that AB_5 toxins are already secreted as a homocomplex by cells via specific secretion systems (Weiss et al. 1993; Sandkvist et al. 1997). SubAB assembly with a focus on the formation of active homo- and hetero-complexes of SubAB variants *in vitro* was studied by Krause et al. They hypothesize that SubAB complex formation occurs most likely on the cell surface of target cells rather than in solution because SubAB variants showed different complex formation characteristics (Krause et al. 2019). Additionally it was shown that separately expressed SubA and SubB domains can build active homo- and hetero-complexes, which induce cell death (Funk et al. 2015).

1.3.3 Toxin receptor

In AB type toxins the B domain typically binds to a specific receptor at the cell surface and mediates cell uptake. SubB binds with a high affinity to glycans terminating in a α2–3-linked N-glycolylneuraminic acid (Neu5Gc) and with an approximately 20-fold lower affinity to glycans terminating in α2–3-linked N-acetylneuraminic acid (Neu5Ac) (Byres et al. 2008). Interestingly Neu5Gc is a monosaccharide which is not synthezied by humans, whereas Neu5Ac is the primary sialic acid found in human glycoconjugates (McCombs et al. 2016). Moreover Yahiro et al. identified in immunoprecipitation studies, sialoglycans on glycoproteins, for example, 2β1 integrin, L1 cell adhesionmolecule (L1CAM) and hepatocyte growth factor receptor (Met) as SubAB binding partners (Yahiro et al. 2006; Yahiro et al. 2011). Recently, a genome-wide CRISPR/Cas9 knockout screen showed that SubB not only binds to N-glycans but also to O-glycans and and since in this study L1CAM, Met and Integrin were not enriched, it is hypothesized that no specific glycoproteins serve as SubAB receptors, but rather SubAB binds to various proteins if they have sialoglycans (Yamaji et al. 2019). Recently Funk et al. could show that not only SubB binds to the cell surface of host cells but also SubA showed in Western blot analyses a concentration dependent binding to HeLa cells (Funk et al. 2015). The finding that an A domain, without a B domain, binds to the cell surface of host cells is something completely unexpected and unique among the AB type toxins and was further investigated in this thesis.

1.3.4 Cellular uptake and cytotoxic mode of action

After host cell binding, SubAB is internalized into the cell. Since there are different reports on SubAB uptake mechanisms this can probably happen by several pathways.

A SubAB clathrin-dependent endocytosis is suggested by Chong et al. (Chong et al. 2008), whereas Nagasawa et al. reports an actin- and lipid- dependent, but not clathrin-, caveolin-and dynamin- dependent uptake (Nagasawa et al. 2014). Once inside the cell SubAB is transported via a retrograde pathway through the Golgi apparatus into the endoplasmatic reticulum and unlike other A subunits of bacterial AB type toxins, which transolocate into the cytosol, SubA stays in the ER where it binds to the endoplasmic Hsp70 chaperone BiP/GRP78 (Paton et al. 2006). More detailed investigations on the intracellular transport have shown that SubAB delivery requires several key Golgi resident proteins. SubAB transport is significantly delayed if subunits of the Conserved Oligomeric Golgi (COG) complex are downregulated as well as if the Ras-related protein Rab6 and β-COP proteins are depleted, indicating a COG/Rab6/COPI–dependent retrograde pathway (Smith et al. 2009). Furthermore, it was shown that SubAB delivery requires Golgi SNARE Syntaxin 5 (Smith et al. 2009). Yamaji et al. performed a CRISPR/Cas9 KO screen in which they identified SLC39A9, which is a predicted zinc transporter, as well as KDELRs and jumping translocation breakpoint (JTB) to be critical host factors for SubAB to induce maximal cytotoxicity (Yamaji et al. 2019). Another protein which is suggested to regulate SubAB activity upstream BIP cleavage is the protein disulfide isomerase (PDI). PDI knockdown reduced the SubAB-induced BIP cleavage and bacitracin, a PDI inhibitor, prevented the SubAB-mediated cell death (Tsutsuki et al. 2020b). Once SubAB is successfully transported into the ER, SubA acts as a subtilase-like serine protease which is characterized by a "catalytic triad" of aspartic 52, histidine 89, and serine 272 (Siezen et al. 1991; Paton et al. 2004). SubA specifically cleaves the immunoglobulin heavy chain–binding protein (BiP)/glucose-regulated protein 78 between a dileucine motif (Leu_{416}-Leu_{417}) in the hinge region connecting its N-terminal nucleotide-binding domain (NBD) and C-terminal substrate-binding domain (SBD) and a single amino-acid substitution in the BiP target was sufficient to protect cells from the toxin effect (Paton et al. 2006). BiP is a member of the Hsp 70 chaperon family and plays an essential role in eukaryotic cell survival as it is involved in protein folding and assembly, is maintaining the permeability barrier of the ER membrane, is regulating ER stress signaling and is demonstrating anti-apoptotic properties through interference with caspase activation. In the context of SubAB activity it should be particularly emphasized that BiP is a master regulator of the ER stress response and thus it also regulates the unfolded protein response (UPR) (Lewy

et al. 2017). An accumulation of unfolded proteins triggers BiP to detach from the UPR sensor proteins activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1), and PKR-like ER kinase (PERK) which activates further downstream pathways to re-establish ER homeostasis or if the ER stress is too strong and persistent cells will undergo apoptosis (Boyce und Yuan 2006). SubAB catalyzed BiP cleavage triggers ER stress by activating all three ER stress signaling pathways. Due to a constant SubAB activity BiP cannot be restored,which leads to a continues ER stress response, resulting ultimately in cell death (Szegezdi et al. 2006; Wolfson et al. 2008).

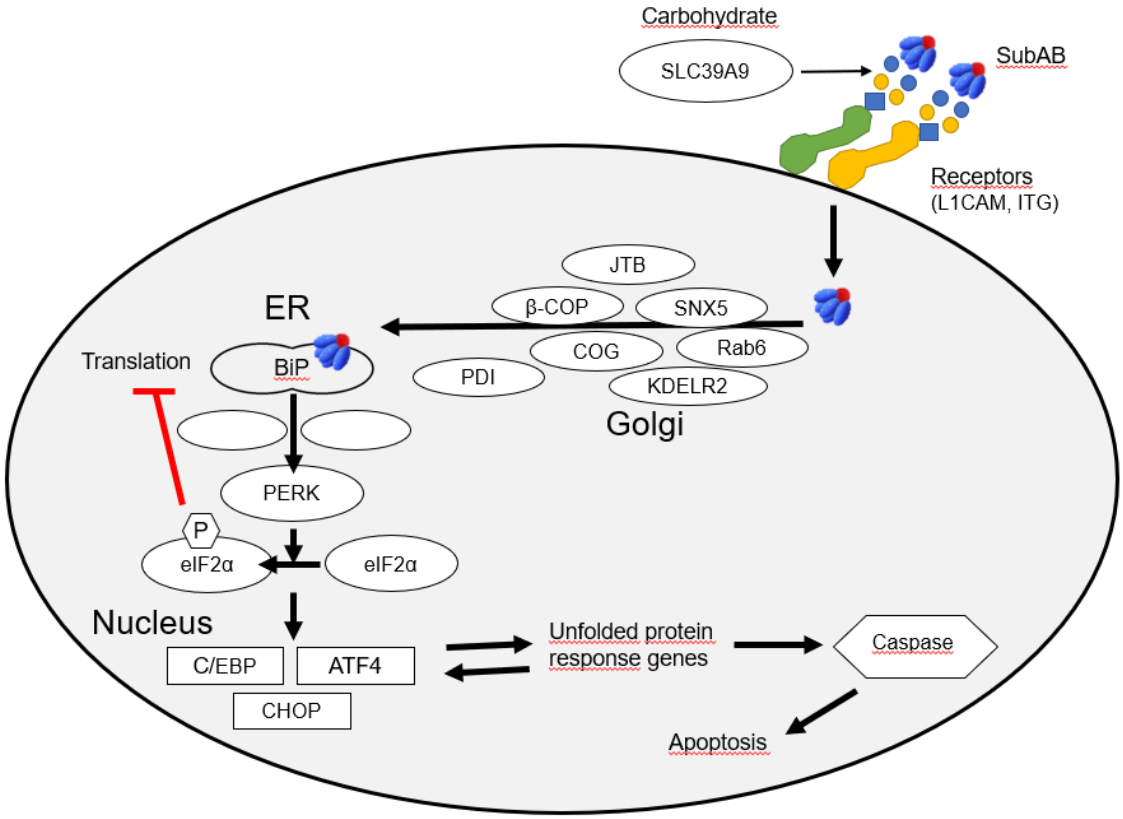

Figure 2 Schematic model of SubAB uptake, transport, and cytotoxic effect. Explanations in the text. (Based on Tsutsuki et al.)

Interestingly, Funk et al. showed that SubA alone applied in higher concentrations than needed for the holotoxin led to the one hand to clear morphological changes in HeLa cells with the formation of round or spindle shapes and on the other hand to a significant decrease in cell number after 72 h. Similar to SubAB, an induction of apoptosis with caspase activation and DNA fragmentation could be demonstrated (Funk et al. 2015) which leads to the hypothesis that not only the holotoxin can lead to

cytotoxicity but also SubA alone can be taken up by cells and harm cells. The underlying meachnisms by which SubA mediates cytotoxic effects in the absence of SubB as well as the pathophysiological relevance of this single SubA effect were characterized in detail at the cellular and molecular level in this thesis.

1.4 Aim of the thesis

SubAB is sectreted in addition to Stx by certain Shiga toxin producing *E. coli* strains (STEC) and can be transmitted to humans mainly via contaminated meat products of wild ruminants, sheep, and goats. Although numerous subAB-positive STEC isolates have been reported the pathogenetic relevance of SubAB is still unclear and the molecular and cellular mechanisms underlying the cyotoxic effect of SubAB are insufficiently understood. This dissertation aimed to characterize the cellular and molecular mechanisms by which SubA triggers cytotoxicity in the absence of SubB in more detail. A fundamental question was whether SubA induced cytotoxic effects are mediated extracellularly or intracellularly. Therefore, SubA was labeled with a fluorescent dye and cell surface binding was analyzed via FACS. To investigate the interaction between SubA and host cells more closely, it was tested wether SubA binding is suturable and competitive. In addition, experiments in which either cells where pre-treated with Pronase, an enzyme that digestes nearly all cell surface proteins into individual amino acids, or SubA binding in the presence of FCS should provide information on whether protein structures serve as SubA binding elements. To clarify if SubA is taken up by cells without SubB being present, SubA uptake was visualized via confocal fluorescence microscopy and SubA indueced BiP cleavage was investigated via Western blot analysis. To gain understanding on how SubA alone is guided to the ER an inhibitor, which induces the breakdown of the Golgi apparatus and interrupts the retrograde transport pathway to the ER was used. Furthermore, a screening with the software scanProsite tool provided information on a potential ER-targeting signal. Therefore, a SubA mutant lacking this ER-targeting signal was generated and further investigated with regards to its cytotoxicity and BiP cleavage. Finally, preliminary experiments with a human intestinal organoid model (“minigut”), which shows all typical morphological and functional characteristics of the human intestinal wall, were performed to see if observations gained in cell lines could be confirmed in an environment more in line with reality. Taken together this thesis

contributes to a better understanding of the mode of action of the subtilase cytotoxin, which is most important for the development of anti-toxin strategies.

2 Materials and Methods

2.1 Materials

2.1.1 Devices

BD FACSCelesta™	Becton, Dickinson and Company (BD) Biosciences, USA
Centrifuge 5415 D	Eppendorf AG, Hamburg
Centrifuge 5417 R	Eppendorf AG, Hamburg
Centrifuge Megafuge 1.0	Heraeus Instruments GmbH, Hanau
Double-gelsystem Twin ExW S (PerfectBlue™)	PEQLAB Biotechnologie GmbH, Erlangen
Electrophoresis Power Supply - EPS 601	Amersham Bioscience GmbH, Freiburg
Freezer HERAfreeze™ 600B HFU B Serie - 86 °C-Freezer	Thermo Fisher Scientific, Waltham (USA)
Glass plate excised 20 x 10 cm	PEQLAB Biotechnologie GmbH, Erlangen
Glass plate normal 20 x 10 cm	PEQLAB Biotechnologie GmbH, Erlangen
Incubator HERAcell 240i	Thermo Fisher Scientific Inc., Waltham, MA, USA
Magnetic stirrer RET basic C	IKA Werke GmbH & Co. KG, Staufen
Microscope Axiovert 40 CFL	Carl Zeiss AG, Oberkochen
Microscope camera Leica MC170 HD DMi1	Leica Microsystems CMS GmbH, Wetzlar
Mr. Frosty Cryo 1 °C Freezing Container	Nalge Nunc International Corporation, USA
Multichannel pipette/Transferpette®-8 (50 – 200 µl)	Brand GmbH & Co. KG, Wertheim
Neubauer Hemocytometer	LO-Laboroptik GmbH, Friedrichsdorf
Pipetboy	INTEGRA Biosciences GmbH, Fernwald
Pipette Reference (0.5 – 10 µl)	Eppendorf AG, Hamburg

Pipettes Pipetman® (2 – 20 µl, 20 – 100 µl, 50 – 200 µl, 200 – 1000 µl)	Gilson Inc., Middelton, WI, USA
Platform shaker Duomax 1030	Heidolph Instruments GmbH & Co. KG, Schwabach
Platform shaker Unimax 2010	Heidolph Instruments GmbH & Co. KG, Schwabach
Safety cabinet HERAsafe	Heraeus Holding GmbH, Hanau
Safety cabinet LaminAir® HA 2448	Heraeus Holding GmbH, Hanau
Scanner Epson Perfection 370 Photo	Epson, Tokio (JPN)
Scale Kern EG 2200 - 2 NM	KERN & SOHN GmbH, Balingen
Semi-dry electroblotter (PerfectBlue™)	PEQLAB Biotechnologie GmbH, Erlangen
Thermomixer comfort	Eppendorf AG, Hamburg
Thermomixer compact	Eppendorf AG, Hamburg
Water bath AQUAline AL 18	Lauda Dr. R. Wobser GmbH & Co. KG, Lauda-Königshofen

2.1.2 Consumables

µ-Slide 8 Well, ibiTreat	ibidi GmbH, Martinsried
Blotting Paper MN 827 B	Macherey-Nagel GmbH & Co. KG, Düren
CryoPure Tube 1.8 mL white	Sarstedt AG & Co. KG, Nümbrecht
Filtropur S 0.2	Sarstedt AG & Co. KG, Nümbrecht
Filtropur S 0.45	Sarstedt AG & Co. KG, Nümbrecht
Medical X-ray film CX-BL+ (13 x 18 cm, 18 x 24 cm)	AGFA HealthCare N.V., Mortsel, Belgium
Nitrocellulose Blotting Membrane Amersham™ Protran™ 0.45 µm NC	GE Healthcare Life Sciences, Sweden
Pasteur glass pipettes	Brand GmbH & Co. KG, Wertheim
Pipette tips	Sarstedt AG & Co. KG, Nümbrecht
Reaction tubes (0.5, 1.5, 2.0 mL)	Sarstedt AG & Co. KG, Nümbrecht
Serological pipettes (5/10/25 ml)	Sarstedt AG & Co. KG, Nümbrecht

Tissue culture dish 100 (d = 10 cm)	TPP® Techno Plastic Products AG, Trasadingen, Switzerland
Tissue culture testplates (12/96 well)	TPP® Techno Plastic Products AG, Trasadingen, Switzerland
Tube 15 mL 120x17 PP	Sarstedt AG & Co. KG, Nümbrecht
Tube 50 mL 114x28 PP	Sarstedt AG & Co. KG, Nümbrecht
Zeba TMSpin Desalting Columns, 7K MWCO	Thermo Fisher Scientific Inc., Waltham, MA, USA

2.1.3 Chemicals

Acetic acid glacial	VWR International GmbH, Darmstadt
Acrylamide (Rotiphorese® Gel 30)	Carl Roth GmbH & Co. KG, Karlsruhe
Ammonium persulfate (APS)	Carl Roth GmbH & Co. KG, Karlsruhe
Bovine serum albumin (BSA)	PAA Laboratories GmbH, Pasching (AUT)
Brefeldin A	Calbiochem Merck (CHN)
Bromophenol blue sodium salt	Sigma-Aldrich Chemie GmbH, Steinheim
Cycloheximide	Merck KGaA, Darmstadt
Dimethyl sulfoxide (DMSO)	AppliChem GmbH, Darmstadt
Dithiothreitol (DTT)	Merck KGaA, Darmstadt
ECL system	EMD Millipore Corporation, Darmstadt, Germany
Ethylenediaminetetraacetic acid disodium salt (EDTA)	Carl Roth GmbH & Co. KG, Karlsruhe
Ethanol	VWR Chemicals, Darmstadt
Fetal calf serum (FCS)	GIBCO® life technologies, Karlsruhe
Glycerol	Sigma-Aldrich Chemie GmbH, Steinheim
Glycine	AppliChem GmbH, Darmstadt
Hydrochloric acid (HCl)	Sigma-Aldrich Chemie GmbH, Steinheim
Isopropanol (2-Propanol)	VWR International GmbH, Darmstadt
L-Glutamine	Biochrom AG, Berlin
Methanol technical grade (MetOH tech.)	University of Ulm, Ulm

Non-essential amino acids (NEAA)	GIBCO® life technologies, Karlsruhe
Penicillin/streptomycin	Thermo Fisher Scientific Inc., Waltham, MA, USA
Ponceau S	AppliChem GmbH, Darmstadt
Potassium chloride (KCl)	Carl Roth GmbH & Co. KG, Karlsruhe
Potassium dihydrogen phosphate (KH2PO4)	Carl Roth GmbH & Co. KG, Karlsruhe
Sodium chloride (NaCl)	Sigma-Aldrich Chemie GmbH, Steinheim
Sodium dodecyl sulfate (SDS)	Carl Roth GmbH & Co. KG, Karlsruhe
Sodium hydrogen phosphate (Na_2HPO_4)	VWR Chemicals, Darmstadt
Sodium pyruvate	GIBCO® life technologies, Karlsruhe
TEMED	AppliChem GmbH, Darmstadt
Thiodiglycol (TDE)	Sigma-Aldrich Chemie GmbH, Steinheim
Tris	AppliChem GmbH, Darmstadt
Trypsin	Roche Diagnostics GmbH, Mannheim
Tween® 20	AppliChem GmbH, Darmstadt

2.1.4 Buffers and solutions

General Buffer

10 x PBS	1.37 M NaCl 27 mM KCl 80 mM Na_2HPO_4 18 mM KH_2PO_4 Diluted to 1 x pH 7.4.
PBS-T	1 x PBS 0.1 % Tween20

SDS Page and Western Blot

Resolving gel mixture (for 12.5 % gels)

Resolving gel buffer (RGB)	1.5 M Tris-HCl (pH 8.8)

Resolving gel mixture (for 12.5 % gels)	8 mL MilliQ H2O 6.2 mL RGB 273 µL 10 % (w/v) SDS solution 10.4 mL acrylamide solution 248 µL 10 % (w/v) APS 25 µL TEMED

Mix all solutions in the order indicated, place in the gel apparatus as quickly as possible and cover the entire gel surface with isopropanol. Allow the resolving gel to polymerize at room temperature, remove isopropanol and then add the stacking gel mixture.

Stacking gel mixture (for 6 % gels)

Stacking gel buffer (SGB)	0.5 M Tris-HCl (pH 6.8)
Stacking gel mixture (for 6 % gels)	8 mL MilliQ H_2O 3.8 mL SGB 150 µL 10 % (w/v) SDS solution 3 mL acrylamide solution 150 µL 10 % (w/v) APS 17 µL TEMED

Mix all solutions in the order indicated, place in the gel apparatus as quickly as possible and insert comb. Allow the stacking gel to polymerize at room temperature and remoce comb. The SDS gel is now ready to use or can be stored in the refrigerator for several days in wet cloths.

10 x Tris-glycine buffer	272 mM Tris base 1.92 M glycine
SDS running buffer	1 x Tris-glycine buffer 0.1 % SDS (w/v)

Towbin blotting buffer	10 % 10 x Tris-glycine buffer 20 % MetOH tech. 70 % H_2O
5 x Laemmli Sample buffer (± DTT) (2.5 x Laemmli Sample buffer)	0.31 M Tis-HCl (pH 6.8) 10 % SDS (w/v) 37.5 % glycerol 0.25 mg/mL bromphenol blue ± 100 mM DTT (for 2.5 x dilute with MilliQ H_2O)
Ponceau S solution	0.2 % Ponceau S (w/v) 5 % acetic acid
Strip buffer A	0.2 M glycine 0.5 M NaCl (pH 2.0)
Strip buffer B	0.5 M Tris (pH 11)

2.1.5 Cell culture medium

HeLa cells	minimum essential medium supplemented with: 100 u/mL penicillin 100 µg/mL streptomycin 10 % FCS 1 mM sodium pyruvate, 2 mM L-glutamine 0.1 mM NEAA

HCT116 cells	Dulbecco's modified Eagle's medium supplemented with: 100 u/mL penicillin 100 µg/mL streptomycin 10 % FCS 1 mM sodium pyruvate, 2 mM L-glutamine 0.1 mM NEAA

Serum-free medium (sf-medium) contains minimum essential medium or Dulbecco's modified Eagle's medium supplemented with only 100 u/mL penicillin and 100 µg/mL streptomycin.

2.1.6 Antibodies and binding proteins

Primary

Type	Species	Dilution	Manufacturer
α-GRP78/BiP	mouse	1:1.000 in 5 % SMP (WB)	BD Biosciences, San Rose, CA USA
GAPDH	mouse	1:2.000 in PBS-T (WB)	SantaCruz Biotechnology, Dallas, TX USA
HSP90	mouse	1:2.000 in PBS-T (WB)	SantaCruz Biotechnology, Dallas, TX USA

Secondary

α-mouse IgG, HRP conjugate	goat	1:10.000 in PBS-T (WB)	Thermo Fisher Scientific Inc., USA
α-mouse IgGκ, binding-protein, HRP conjugate	-	1:2.500 in PBS-T (WB)	SantaCruz Biotechnology, Dallas, TX USA

2.1.7 Labels

DyLight® Amine-Reactive Dyes (DyLight 488 NHS Ester)	Thermo Fisher Scientific Inc., Waltham, MA, USA
ATTO-647N-hydroxysuccinimidyl(NHS)-Ester	ATTO-TEC GmbH, Siegen, Germany

2.1.8 Cell lines

Cell Line	Organism	Tissue	Cell Type	ATCC®
HeLa	Homo sapiens, human	Cervix	Epithelial	CCL-2™
Vero	Cercopithecus aethiops	Kidney	Epithelial	CCL-81
HCT116	Homo sapiens, human	Colon	Epithelial	CCL-247™

2.1.9 Toxins

Commercially purchased toxins

Toxin	Manufacturer
Cholera Toxin subunit B (recombinant), Alexa Fluor™ 594 conjugate	Thermo Fisher Scientific Inc., Waltham, MA, USA

The ALEXAFluor594-labeled CTB was commercially purchased and served as an ER marker, as its ER entrance is well-established for cells (Fujinaga et al. 2003).

Provided toxins

Toxin	Working group
Subtilase Toxin subunit A (SubA2-2His)	Prof. Schmidt, Hohenheim
Subtilase Toxin subunit B (SubB2-2His)	Prof. Schmidt, Hohenheim
Inactive mutant of the Subtilase Toxin subunit A (SubA2-2-His$_{S272A}$)	Prof. Schmidt, Hohenheim
ΔC344 mutant of the Subtilase Toxin subunit A (SubA2-2-His$_{\Delta C344}$)	Prof. Schmidt, Hohenheim

ΔC331 mutant of the Subtilase Toxin subunit A (SubA2-2-His$_{\Delta C331}$)	Prof. Schmidt, Hohenheim

Detailed information on the SubA and SubB purification protocol can be found in Krause et al. (Krause et al. 2019). Additionally, to the wildtype toxin, several SubA mutants were used in this study. To get an inactive mutant of the subtilase cytotoxin a single amino acid exchange, serine to an anlaine on position 272, was performed in the A-subunit. In the case of the SubA2-2-His$_{\Delta C344}$ and SubA2-2-His$_{\Delta C331}$ the subtilase A-subunit was truncated from amino acid position 334 and 331 respectively.

2.1.10 Software and data analysis

AxioVision Rel. 4.7	Carl Zeiss AG, Göttingen
BD FACSDiva™ Software	Becton, Dickinson and Company, New Jersey (USA)
Flowing Software 2.5.1	Perttu Terho Turku Centre for Biotechnology University of Turku, Finland
ImageJ 1.52c	National Institutes of Health Bethesda, MD, USA
Microsoft® Office 2016	Microsoft Corporation, Redmond (USA)

2.2 Methods

2.2.1 Culture of mammalian cell lines

Continuous cultivation of cell lines

All cell types were cultivated in 10 cm petri dishes with a total volume of 10 mL of the appropriated culture medium (see 152.1.5) in a humidified atmosphere containing 5 % CO_2 at 37 °C. When the cells reached a confluence of 80 %, they were splited. The split ratio depends on the doubling time of each cell line and is listed in the table below. Here, the splitting procedure is described as an example for HeLa cells. HeLa cells were usually splited 3 times a week (mondays, wednesdays and fridays) using a split ratio of 1:3 for continuous cultivation of 2 days or of 1:10 for continuous cultivation of 3 days. Therefore, the old culture medium was removed using a Pasteur glass pipette,

and the cells were washed once with pre-warmed PBS. Then, 2 ml of trypsin-EDTA solution was added to the cells and they were put back into the incubator until the cells were detached from the surface of the dish (approx. 3 min). Next, the trypsin-reaction was inactivated, by adding 8 ml of pre-warmed culture medium and cells were separated by carefully pipetting the cell suspension up and down. Finally, depending on the split ratio, the corresponding volume of cell suspension was transferred to a new culture dish, taking care to pipette the fresh medium first. Culture dishes were marked with date, name abbreviation, cell type, split rate and split number. Cells were splited until abnormalities were found or until a maximum passage of 25-30. To prevent cells from contamination, all steps were carried out under sterile conditions meaning under a laminar flow cabinet. Furthermore, all utensils used were sprayed with 70 % ethanol before being placed under the laminar flow cabinet and medium and PBS were filtered sterile.

Cell Type	Split rate for 2 days	Split rate for 3 days
HeLa, HCT116	1:3	1:10
CHO	1:10	1:20

Seeding of cells

In order to be able to carry out experiments, the cells were seeded in various multiwell plates. Therefore, cells were detached from culture dishes via trypsin-EDTA solution as described under "continuous cultivation of cell lines" and 10 µl of the cell suspension was applied to a Neubauer hemocytometer. All cells in the four large squares were counted manually and the mean of counted cells was calculated, which corresponds to the number of cells/mL times 10^4. Depending on the desired cell number, the cell suspension was diluted with further medium or concentrated. Seeding of cells was done via a normal pipette or in the case of a 96 well plate a multichannel pipette was used. Which volumes were used for which well format can be seen in the table below. It should be noted that a Master Mix was always prepared, so that approx. the same number of cells per well could be guaranteed.

Well format	Standard Volume/well [µl]	Minimum Volume/well [µl]	Master Mix [µl]
µ-Slide 8 well	300	150	3000
12 well	1000	400	13000
96 well	100	100	13000

Freezing and thawing of cells

For long-term storage of cells, they are kept in a liquid nitrogen tank. The procedure of freezing cultured cells is as follows: cells were detached from culture plates and counted as described before. Afterwards, cells were centrifuged at 500 rcf for 5 min and the cell pellet was re-suspended in an appropriated volume of freezing medium (culture medium supplemented with additional 10 % FCS and 10 % DMSO), so that a cell count of 1 x 10^6 cells/mL was achieved. Now 1 ml aliquots of the cell suspension are applied into CryoPure tubes, which are then placed in the Mr. Frosty Cryo 1 °C Freezing Container and stored at -80 °C. After the CryoPure tubes have been in the -80 freezer for at least 2 days, they can be transferred to the liquid nitrogen tank. It is worth mentioning that for the production of new cryo stocks, only cells that have not been splited for more than 5 times were used.

To thaw the cells, a CryoPure tube is gently swiveled back and forth in a 37 °C warm water bath until only a pinhead-sized piece of ice remains. The cell suspension is then transferred to a 15 ml tube, which has previously been filled with pre-warmed 10 ml culture medium. To get rid of the DMSO the cells were centrifuged at 500 rcf for 5 min and the supernatant was removed. The cell pellet was carefully re-suspended in pre-warmed culture medium and transferred to a 10 cm culture dish.

2.2.2 Cell viability test

The application of toxins and toxin subunits to cells serves to characterize the cytotoxic effects. Therefore, cells were seeded in a 96 well plate, treated with either the holotoxin or the single subunits and further incubated at 37 °C. Toxin was prepared in culture medium and for each treatment triplicates were performed. To keep the deviation within the triplicates as small as possible, a master mix of the toxin was always generated. As negative control, only the buffer in which the toxin was dissolved was added to the medium. The 96 well plate was checked at regular intervals and changes

in the cell number and cell morphology were recorded photographically using a Zeiss Axiovert 40CFL microscope with a Jenoptik ProGres C10 CCD camera.

2.2.3 Flow cytometry

The binding of the subtilase cytotoxin, particularly of the A-subunit, to various cell types was investigated using flow cytometry. A 10 cm dish that had grown confluently with cells was washed once with PBS and cells were then detached by the incubation of 2 ml of 25 mM EDTA/PBS at 37 °C. If the cells were not completely detached after approx. 30 min of EDTA incubation, 8 ml PBS was added to the dish and the cells were mechanically detached by rinsing the dish several times. For pronase assay, cells were incubated with 500 µg/mL pronase instead of EDTA. To get rid of either the EDTA or the pronase, cell solution was collected in a 15 ml tube, pelleted by centrifugation for 5 min at 500 rcf and washed with PBS twice. Thereafter, depending on the solution in which the binding should be investigated, the cells were taken up in PBS, medium or sf-medium and the total amount of cells was calculated using a Neubauer hemocytometer. Cells were adjusted to 1 x 10^6 cells/mL and 200 µl cell suspension was aliquoted in 1.5 ml reaction tubes. To ensure that the cells could no longer perform endocytosis, reaction tubes were kept on ice for at least 5 min prior to toxin treatment. The indicated amount of fluorescently labelled toxin was added to the reaction tubes and the cell-toxin-solution was pipetted up and down once to homogeneously mix everything. The binding of the toxin usually took place for 30 min on ice. Afterwards, the treated cells were pelleted by a prechilled (4 °C) centrifuge for 3 min at 500 rcf. Supernatant was discarded and cells resuspended in 1 mL/sample ice-cooled PBS and again centrifuged with the same settings. This washing step should ensure, that unbound toxin is removed. Finally, the cell pellet is resuspended in 200 µL/sample ice-cooled PBS and subjected to a flow cytometer to measure cell surface-bound fluorescence. In particular, it was used a BD FACSCelesta with an argon-ion laser (488 nm) and the 530 nm band pass filter (FITC). For each treatment 10.000 cells were measured, and data were analyzed using Flowing software version 2.5.1.

In order to make the toxins fluorescent, they were labelled with a Dylight488 NHS Ester according to the manufacture´s manual.

2.2.4 Fluorescence microscopy

Fluorescent microscopy was performed to visualize the uptake of the subtilase cytotoxin in cells. Purified SubA was labeled with ATTO-647N-hydroxysuccinimidyl(NHS)-Ester according to the manufacture´s protocol. After the labeling reaction, excess dye was removed using desalting columns (Zeba ™Spin Desalting Columns 7K MWCO). AlexaFluor594-conjugated CholeraB was obtained from the manufacturer Thermo Scientific. For fluorescence microscopy, HeLa cells were seeded in a µ-Slide 8 well plate and incubated with AlexaFluor594-conjugated CholeraB and/or with either the holotoxin or the single A-subunit of the subtilase toxin for 4 h or 6 h at 37 °C, as indicated. Toxin solution was prepared in sf-medium and the well plate was wrapped in aluminium foil to prevent bleaching of the fluorescent dye during the incubation period. Afterwards, the medium was removed, and each well was washed 3 times with 300 µl ice-cooled PBS. It is important that this washing step is done with caution to avoid rinsing and drying of the cells. Cells were then fixed with 4 % PFA for 20 min at room temperature (RT), again washed 3 times with 300 µl ice-cooled PBS, blocked with BSA for 2 h at RT and kept in 2,2´-thiodieethanol (97 % solution in PBS, pH 7.5) for imaging. Images were taken with a home-built dual-color 3D-STED microscope in confocal mode (Osseforth et al. 2014). Typically, an average power of 1 mW for each excitation beam, 568 nm and 633 nm, respectively, at a repetition rate of 1 MHz was used. Confocal images were captured at a pixel size of 20 nm and a dwell time of 200 µs. Approximately, 100 – 200 counts were the typical peak photon number. Images were analyzed in ImageJ 1.52n. For better visualization, a Gaussian blur $\sigma = 1$ and an intensity threshold of > 10 – 20 counts was applied in each channel.

2.2.5 Western blot

This method was used to detect substrate modification by SubA within living cells. Therefore, cells were seeded in a 12 well with 7 x 104 cells/well. The next day, the cells were intoxicated with the indicated amount of either the single toxin subunit or the combination of both compounds (in a molar ratio of 1:5) for 2 to 24 h. For inhibitor experiments, 10 µM BFA was applied for 30 min prior to intoxication. For protein biosynthesis experiments, HeLa cells were treated with SubA (10 µg/ml) or SubAB (10 µg/ml in a molar ratio of 1:5) for 6 h. After 6 h toxin was removed, cells were washed with PBS and further incubated with MEM culture medium or MEM culture medium

supplemented with 10 µg/ml cycloheximide for further 24 h after initial toxin treatment. After treatment, cells were washed with PBS and lysed in 2.5 x Lämmli sample buffer containing DTT. For protein denaturation, cell lysates were incubated for 10 min at 95 °C. Afterwards, protein separation was performed by running a 12,5 % SDS-Page, fallowed by protein blotting onto a nitrocellulose membrane using a semi-dry western blot transfer system. All componends of the "blotting sandwich" (blotting membrane, nitrocellulose membrane and SDS gel) were previously equilibrated in thowbin blot buffer. After a blotting of 1:25 h, protein transfer was checked by a Panceau S staining of the nitrocellulose membrane. Afterwards, membranes were blocked by 5 % nonfat dry milk in PBS-T for 1 h at room temperature. GRP78 was detected by using anti-GRP78/BiP monoclonal antibody, followed by goat anti-mouse IgG (H+L) HRP conjugate. Primary antibody was used 1.1000 and incubated at 4 °C overnight and secondary antibody was used 1.10.00 and incubated for 1 h at room temperature. To confirm equal amounts of protein, a loading control was always carried out after substrate detection. Therefore, membranes were stripped using strip buffer solution A for 5min and then strip buffer solution B for 10 min. As primary antibodie HSP90 or GAPDH diluted 1:2.000 in PBS-T was used and as secondary antibodie α-mouse IgGκ, binding-protein-HRP conjugate diluted 1:2.500 in PBS-T was used. Both antibodies were incubated for 1 h at RT. Visualization of the signals occurred by enhanced chemiluminescence.

3 Results

3.1 Examination of enzyme activity of SubA and SubA variants *in vitro*

SubAB subunits and SubA variants were provided by the group of Prof. Dr. Herbert Schmidt of the University of Hohenheim (Department of Food Microbiology and Hygiene, Institute of Food Science and Biotechnology). Expression, Purification and subsequent verification of protein structure and oligomerization are described in Krause et al. 2019 (Krause et al. 2019). Since in my PhD project the His-tagged subtilase variants were used, it was ruled out in advance that the His-tag has an influence on the SubA activity. For this purpose, cytotoxcicity assays in camparison with and without His-tagged proteins were carried out on HeLa and HCT116 cells, which exhibitet no differences (Data shown in the Bachelorthesis of Sabrina Noettger, University of Ulm 2021) Furthermore, all SubA variants used in this thesis were checked for their enzyme activity before use. Therefore, HeLa and HCT116 cell lysates were incubated with or without toxin and GRP78 status was visualized via Western blotting (Figure 3).

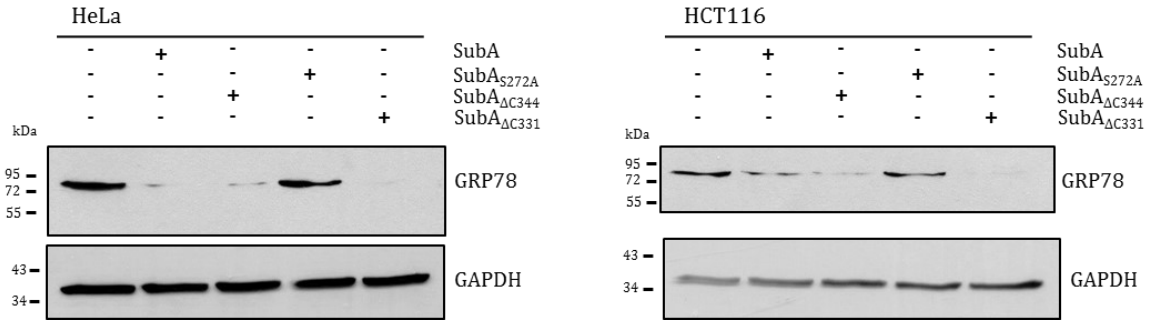

Figure 3 All SubA variants used show enzyme activity.
HeLa and HCT116 cell lysates were incubated with or without 10 µg/ml SubA2-2-His, SubAS272A2-2-His, SubAΔC3442-2-His or SubAΔC3312-2-His in PBS + cOmplete (protease inhibitor) for 30 min at 37 °C. Afterwards, samples were solubilized in Lämmli buffer, subjected to SDS page, blottet and GRP78 cleavage was analyzed by a monoclonal mouse anti GRP78 antibody.

3.2 Characterization of cytotoxic effects exhibited by SubA

So far, the pathogenetic relevance of SubAB, in clinical infections of STEC strains, remains unclear and the molecular and cell biological foundations of the effects of SubAB are insufficiently investigated. In a previous study, we could show that SubA,

binds to human cervix cancer-derived epithelial cells (HeLa) and that SubA, in higher concentrations than required for cytotoxic effects of SubAB, intoxicates cultured HeLa cells (Funk et al. 2015). To examinate the single A-effect in more detail, a concentration dependant cytotoxicity assay was performed, in which HeLa cells were incubated with increasing SubA concentrations and morphological aliterations were documented photographically. After 48 h, the first signs of cell intoxication in terms of cell rounding can be observed for the two highest concentrations (5 µg/ml and 10 µg/ml). This effect became even more prominent after 72 h. Also, a clear reduction in cell number is visible. Lower concentrations up to 1.25 µg/ml did not result in morphological changes after 72 h.

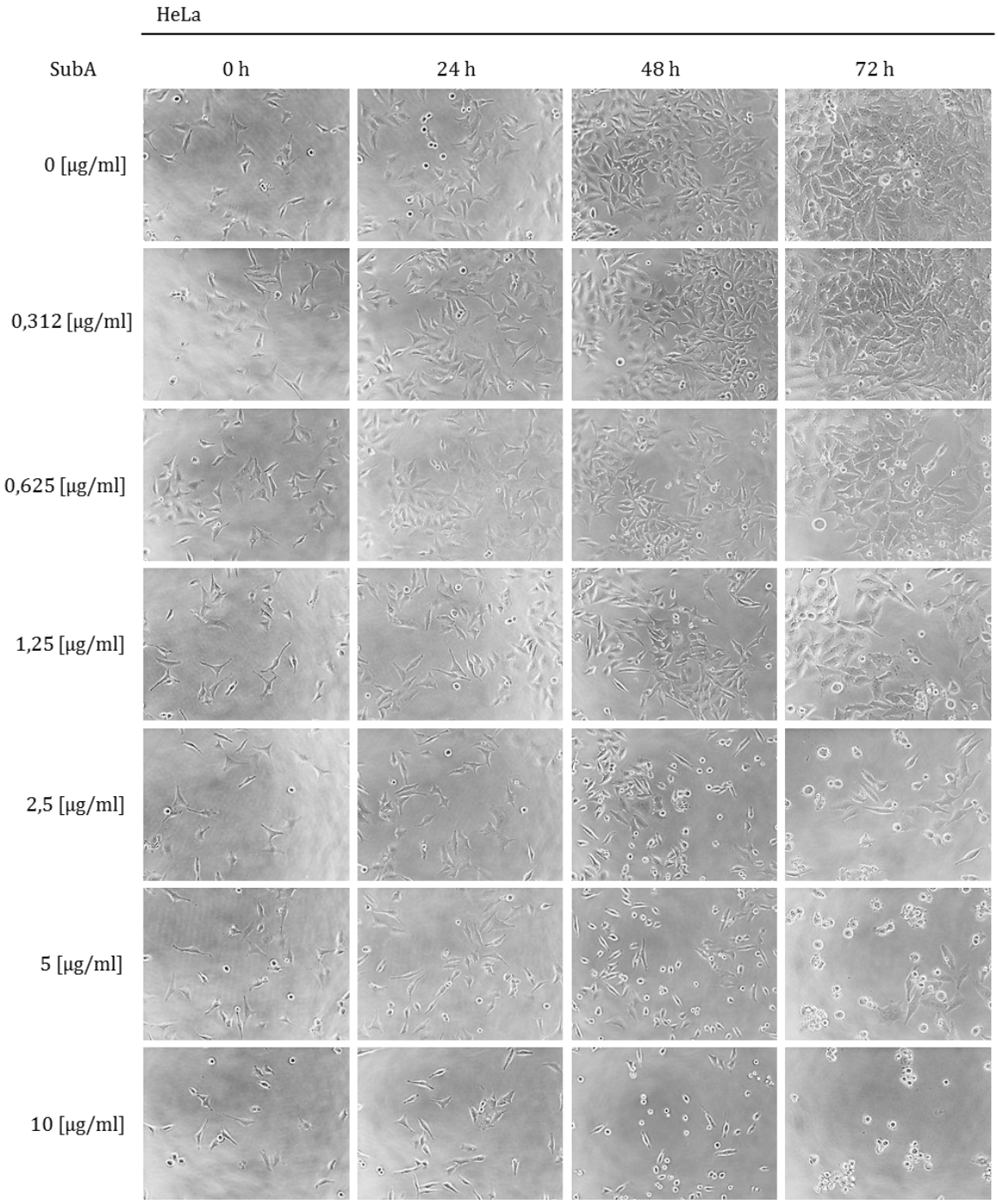

Figure 4 SubA intoxicates HeLa cells in a concentration dependent manner.
HeLa cells were seeded in a 96-well plate and incubated with increasing SubA2-2-His concentrations as indicated or left untreated for control. After 0 h, 24 h, 48 h and 72 h images were taken to visualize morphological changes.

Additionally, the single A-effect was investigated in a second cell line, on human colon epithelial cells (HCT116), because these cell line represents a medically more relevant cell line for an intestinal pathogen than HeLa cells. Therefore, HCT116 cells and for comparison HeLa cells were intoxicated with 10 µg/ml SubA, 10 µg/ml enzymatically

inactive SubAS272A, 10 µg/ml SubAB, 10 µg/ml SubAS272AB, or were left untreated for control and cytotoxic effects were demonstrated in terms of morphological changes. Compared to the negative control, treatment with SubA led to a clear change in cell morphology and decreased cell number on HeLa as well as HCT116 cells, whereas the enzymatically inactive SubA mutant neither alone nor in combination with SubB showed any morphological alterations on both cell lines (Figure 5).

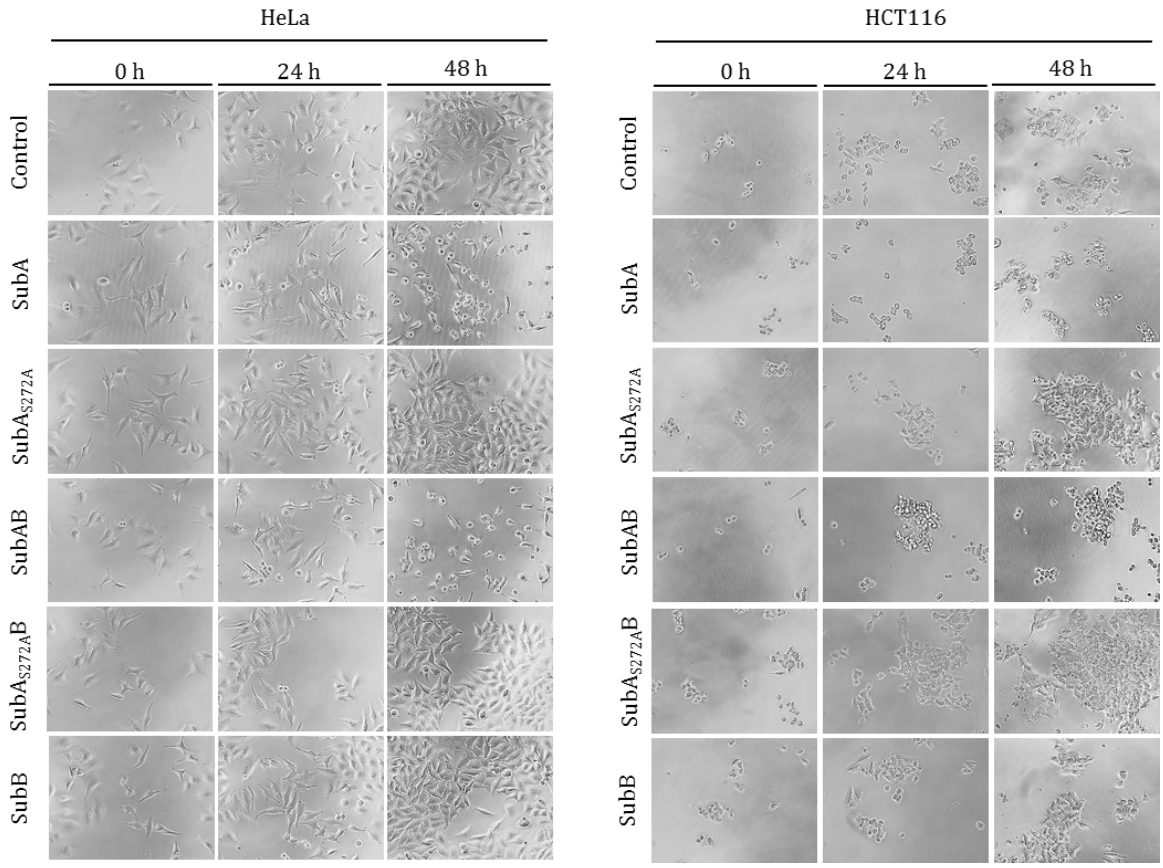

Figure 5 SubA intoxicates HeLa and HCT116 cells.
HeLa and HCT116 cells were seeded in a 96 well and incubated with 10 µg/ml SubA2-2-His, 10 µg/ml enzymatically inactive SubA2-2-HisS272A, 10 µg/ml SubAB2-2-His, 10 µg/ml SubA2-2-HisS272AB, SubB2-2-His for 48 h at 37 °C. For control, cells were left untreated (without toxin). Cell images were taken after 0 h, 24 h and 48 h to visualize morphological changes.

3.3 Investigation of SubA internalization in cells

After showing that several mammalian cell lines are sensitive to SubA alone, the question arose whether SubA mediated cytotoxicity is based on extracellular or intracellular mechanisms. To visualize SubA uptake, HeLa cells were treated with 30 µg/ml ATTO647N-labeled SubA2-2-His, 20 µg/ml (total protein concentration) ATTO647N-labeled SubA2-2-His in combination with unlabeled SubB2-2-His and/or

15 µg/ml of AlexaFluor594-conjugated B-subunit of the cholera toxin (CTB), which represents an established and specific ER marker. Images were taken with a home-built dual-color microscope in confocal mode (Osseforth et al. 2014). The results shown in Figure 6 clearly revealed that not only SubAB but also SubA is taken up by HeLa cells and even more that SubA alone also co-locolaizes with the ER. The result suggests that SubA alone exploits the same uptake pathway in cells as the AB holotoxin.

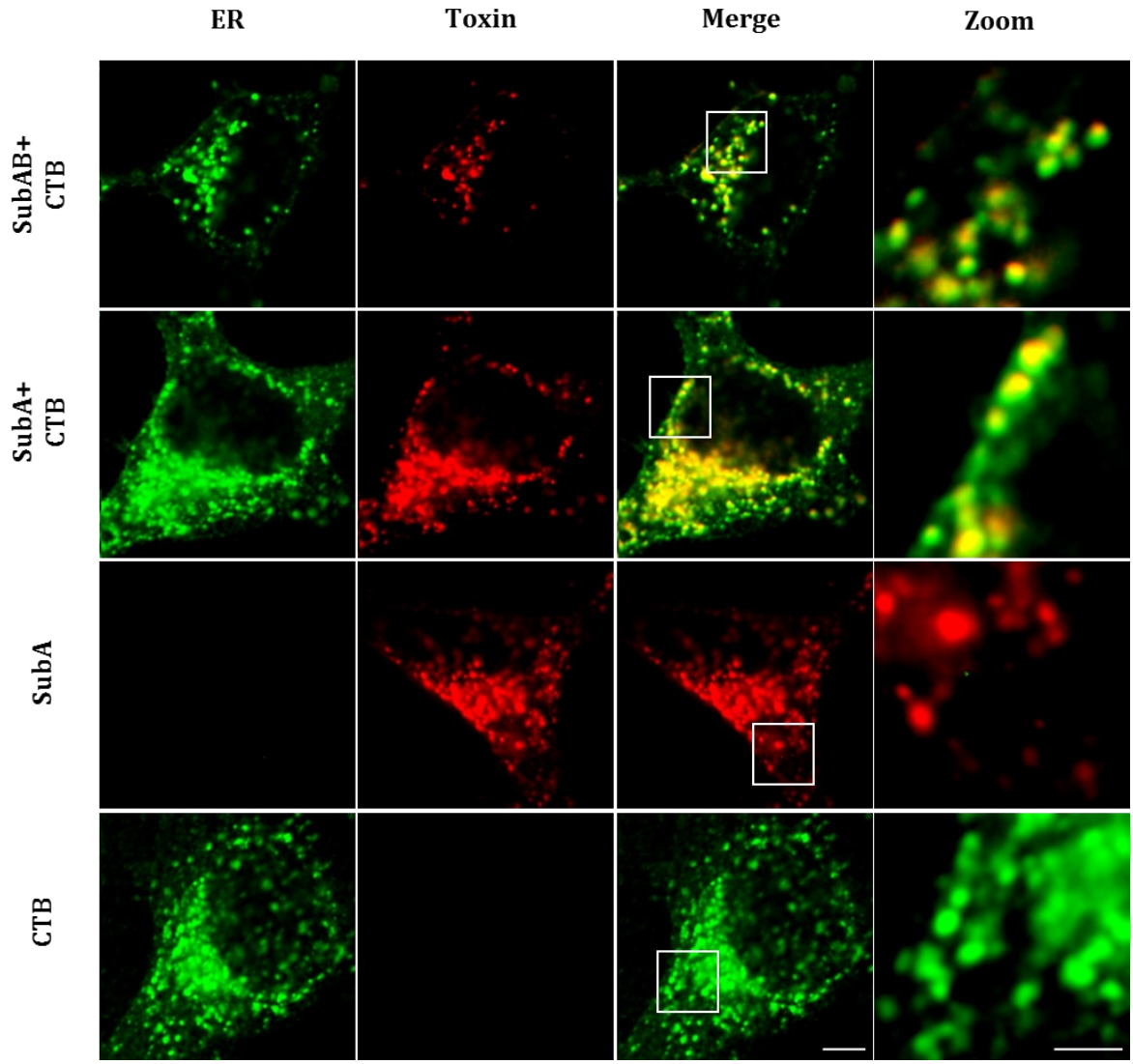

Figure 6 SubA is taken up by HeLa cells and co-localizes with the ER.
HeLa cells were seeded in ibidi 8-well µ-slides and treated with 30 µg/ml ATTO647N-labeled SubA2-2-His (indicated as SubA), 20 µg/ml SubAATTO-647NB2-2His (indicated as SubAB) and/or with 15 µg/ml ALEXAFluor594-conjugated Cholera toxin B subunit (indicated as CTB) as established ER marker. After an incubation time of 6 h at 37 °C, cells were washed with ice-cooled PBS, blocked, fixed, and analyzed via a confocal microscope. Scalebar: 5 µm (Zooms: 2 µm).

To exclude that CTB has any effect on SubA cytotoxicity, cells were incubated with increasing SubA concentrations in the presence or absence of 15 µg/ml CTB and morphological changes were documented photographically.

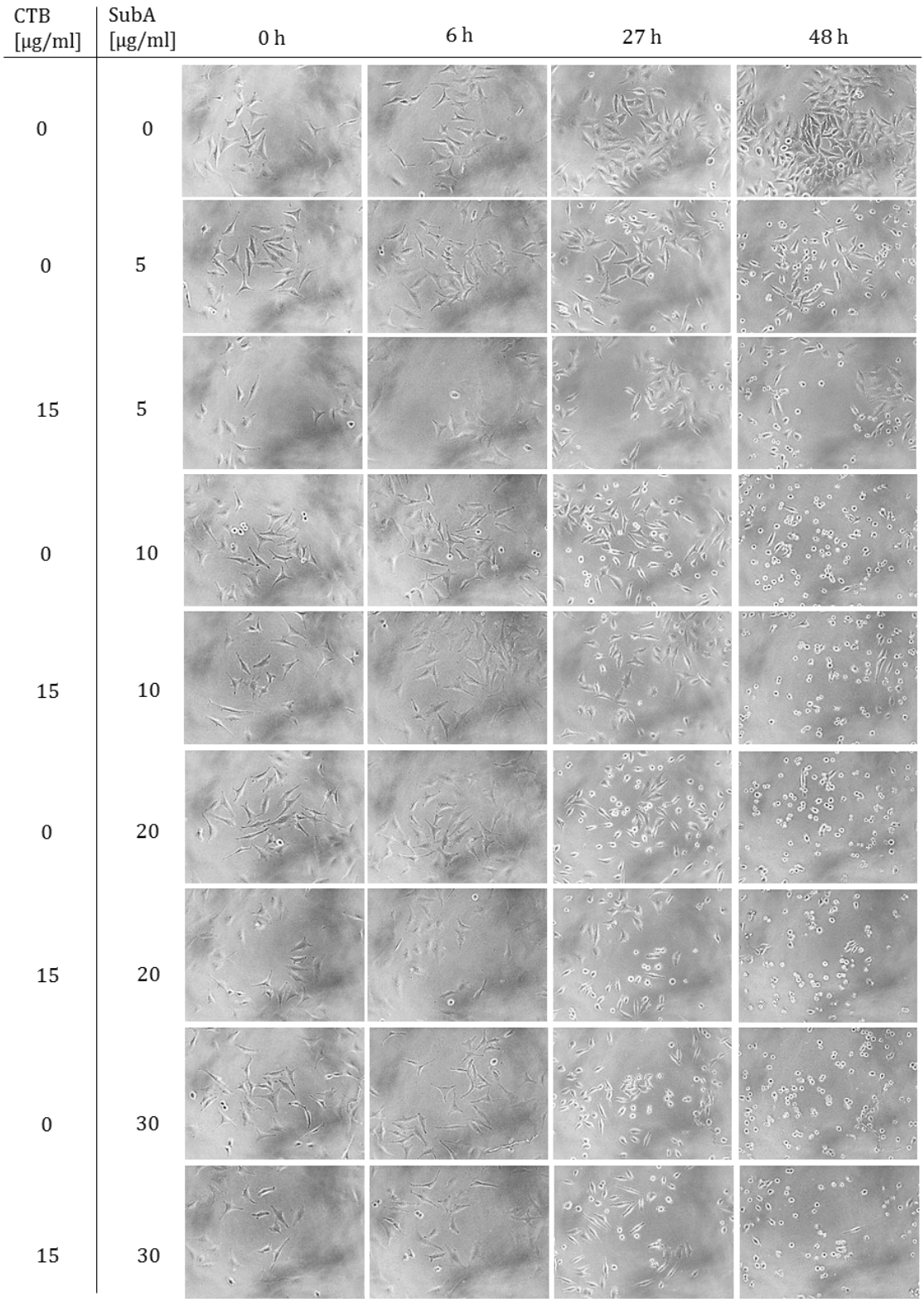

Figure 7 CTB has no influence on SubA cytotoxicity on HeLa cells.

HeLa cells were seeded in a 96-well plate and incubated with 5 µg/ml, 10 µg/ml, 20 µg/ml or 30 µg/ml SubA2-2-His in combination with or without 15 µg/ml ALEXAFluor594-conjugated CTB. Changes in cell morphology were documented after 0 h, 6 h, 27 h and 48 h.

In order to support the hypothesis of SubA uptake into host cells, further investigations regarding SubA induced substrate cleavage in cells were made. Therefore, HeLa and HCT116 cells were incubated on the one hand with increasing concentrations of SubA and on the other hand with a constant SubA concentration but increasing incubation times of SubA and GRP78 cleavage was analyzed via immunoblotting.Figure 8 demonstrates that SubA induced GRP78 cleavage appears concentration (Figure 8A) and time dependent (Figure 8B). To confirm not only SubA uptake but rather retrograde transport of SubA into the ER, HeLa and HCT116 cells were incubated with SubA (10 µg/ml), SubAB (total protein concentration of 10 µg/ml), $SubA_{S272A}$ (10 µg/ml), or $SubA_{S272A}B$ (total protein concentration of 10 µg/ml) with or without pre-treatment of 10 µM brefeldin A1 (BFA), which interrupts the Golgi apperatus and inhibits protein transport into the ER and again GRP78 status was analyzed via immunoblotting. The WB of Figure 8C clearly shows that not only SubAB-induced GRP78 clevage, but also SubA-induced GRP78 cleavage was inhibited by BFA confirming that SubA alone takes the same route to the ER as it does in combination with SubB. In addition to BFA, other inhibitors that were already able to inhibit other toxins were tested. Neither bafilomycin A1 (BAF), which inhibits the endosomal uptake of toxins, nor albumin (HSA), which most likely neutralizes toxins through aggregate formation, nor ambroxol, which is discussed as an inhibitor for pertussis toxin, could show an effect on SubA induced GRP78 cleavage (Figure 9). The results once again confirm the specificity of BFA inhibition and SubA uptake via the retrograde pathway.

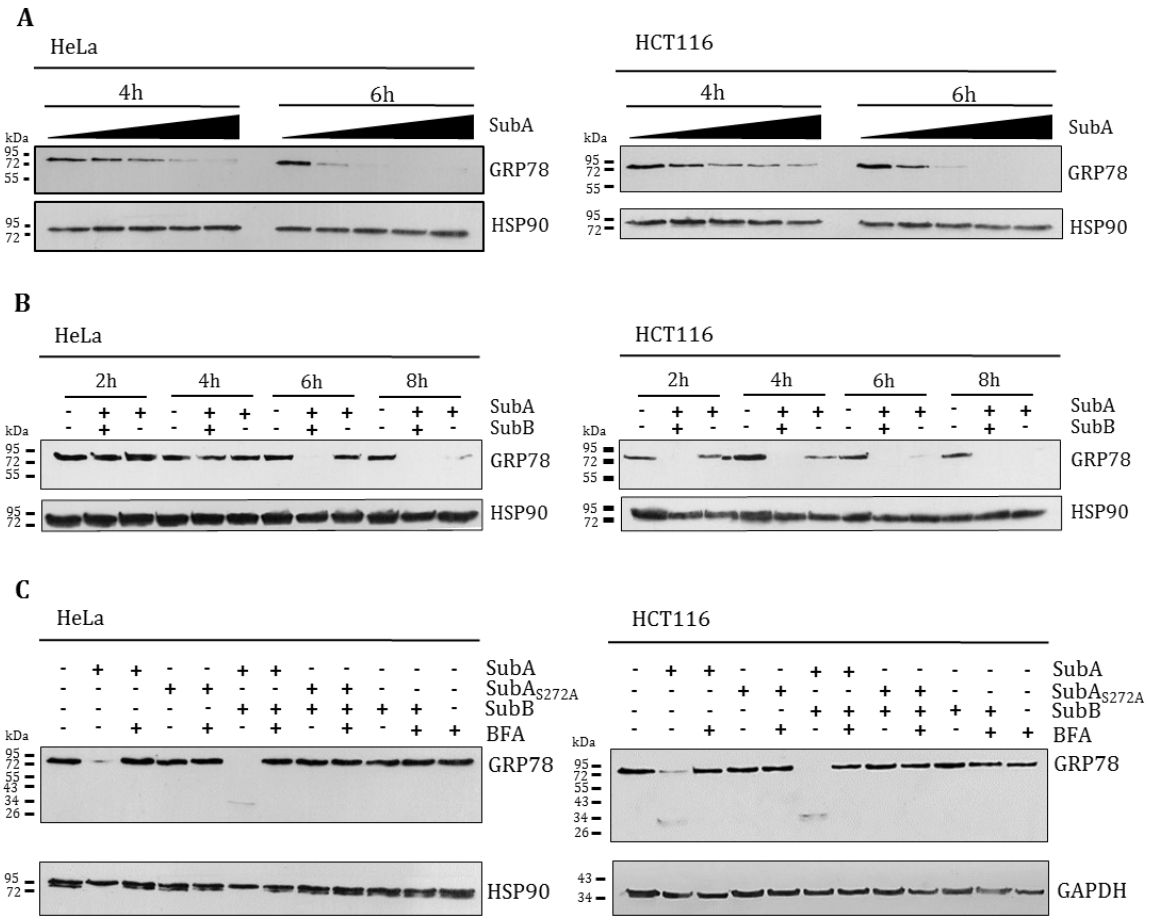

Figure 8 SubA induced GRP78 cleavage is time-and concentration-dependent and is prevented by the pre-treatment of cells with brefeldin A1.

Western blot analyzes of GRP78 cleavage due to toxin treatment via a monoclonal mouse anti GRP78 antibody. HeLa or HCT116 cells were seeded in a 12 well plate, incubated with or without toxin in FCS free medium, solubilized in Lämmli buffer and subjected to SDS page. (A) HeLa and HCT116 were incubated for 4 h or 6 h at 37 °C with increasing SubA2-2-His concentration (3.4 µg/ml, 6.8 µg/ml, 10.2 µg/ml and 13.6 µg/ml blottet from low to high). (B) HeLa and HCT116 cells were incubated for 2 h, 4 h, 6 h, 8 h at 37 °C with 5 µg/ml of either SubAB2-2-His (in a molar ratio of 1:5) or SubA2-2-His alone. (C) HeLa and HCT116 cells were pre-treated with or without 10 µM BFA following incubation with or without 10 µg/ml SubAB2-2-His (in a molar ratio of 1:5), SubAS272AB2-2-His, SubA2-2-His, SubAS272A2-2-His, or SubB2-2-His for 4 h at 37 °C.

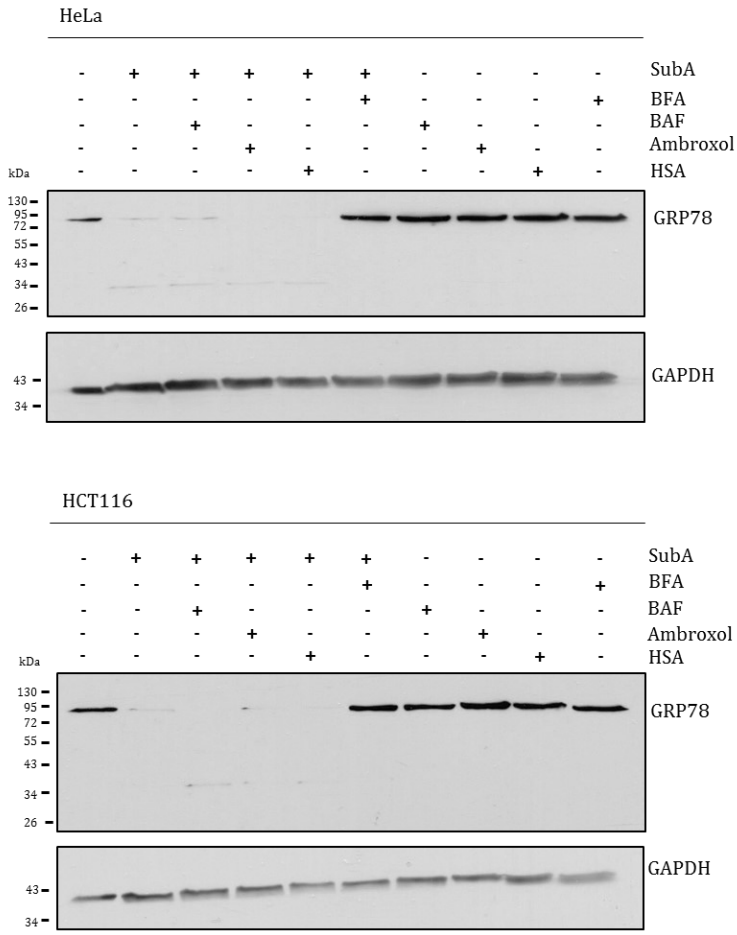

Figure 9 Bafilomycin A1, albumin and amproxol do not inhibit the SubA-induced GRP78 cleavage. HeLa and HCT116 cells were seeded in a 96 well plate and first incubated for 30 min with the inhibitors as indicated, following SubA2-2-His incubation for another 6 h at 37 °C in serumfree medium. Afterwards cells were solubilized in Lämmli buffer, subjected to SDS page and GRP78 status was analyzed by immunoblotting.

3.4 Identification of an ER localization signal within SubA

Since SubA, without its transporter subunit SubB, is taken up into cells via the retrograde pathway obviously, SubA must contain a yet unknown ER localization signal. The software scanProsite™ predicted a C-terminally SEEL-motif as a potential ER targeting signal. Hence, a SubA mutant lacking the C-terminal SEEL motif ($SubA_{\Delta C344}$) was generated and analyzed regarding its cytotoxicity. Interestingly, $SubA_{\Delta C344}$ together with SubB exhibited cytotoxicity, whereas $SubA_{\Delta C344}$ alone showed no morphological changes on HeLa and HCT116 cells (Figure 10).

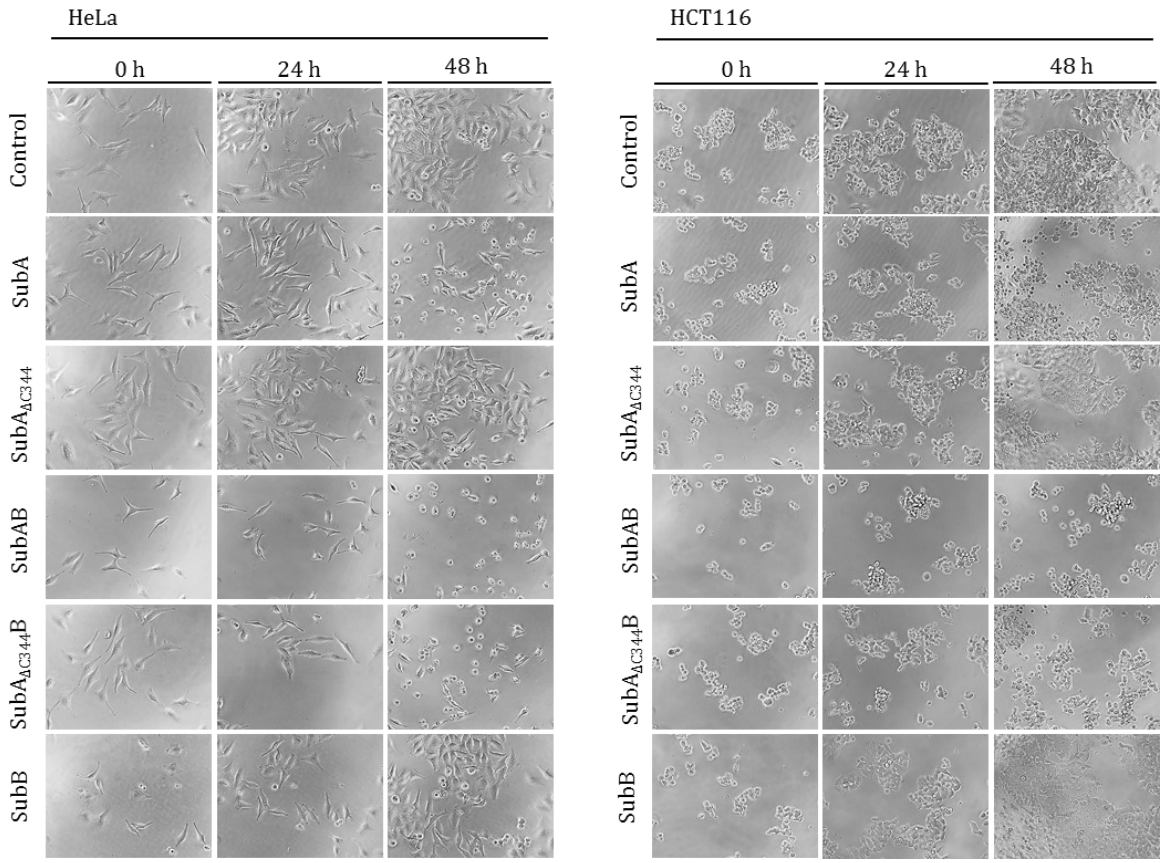

Figure 10 SubAΔC344 exhibits cytotoxicity only in combination with SubB.
HeLa and HCT116 cells were seeded in a 96 well plate and incubated with either 10 µg/ml of the single subunits SubA2-2-His, SubAΔC3442-2-His or SubB2-2-His or a total protein concentration of 10 µg/ml for SubAB2-2-His or SubAΔC344B2-2-His (in a molar ratio of 1:5). Morphological alliterations were documented after 0 h, 24 h and 48 h.

Since SubA$_{\Delta C344}$ alone induces no cytotoxicity, it is conceivable that the toxin is either not taken up at all or is taken up but but does not find its way into the ER. To visualize SubA$_{\Delta C344}$ fluorescence microscopy was performed.

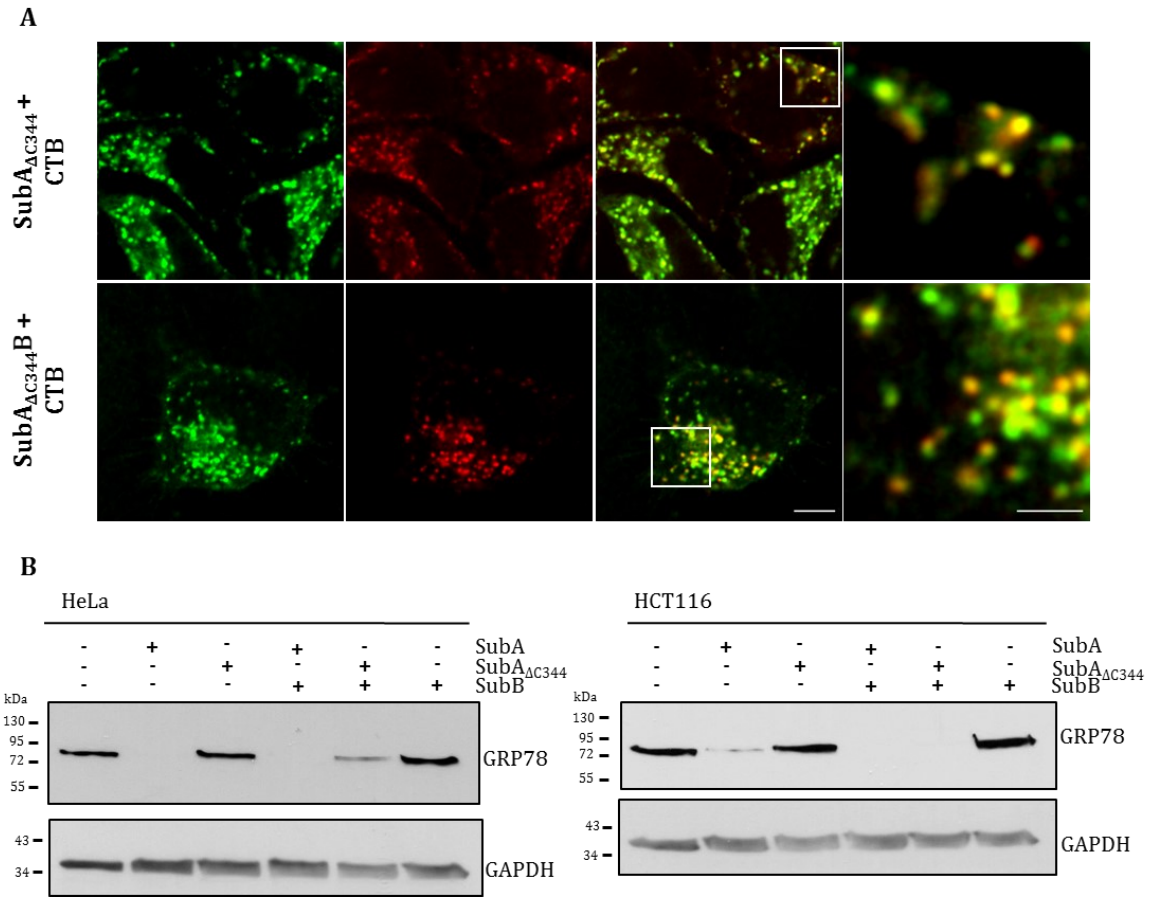

Figure 11 Investigation of SubAΔC344 uptake into cells.
(A) HeLa cells were seeded in ibidi 8-well µ-slides and treated with 30 µg/ml ATTO647N-labeled SubAΔC3442-2His (indicated as SubA), 20 µg/ml SubAΔC344ATTO-647NB2-2His (indicated as SubAΔC344B) and/or with 15 µg/ml ALEXAFluor594-conjugated Cholera toxin B subunit (indicated as CTB) as established ER marker. After an incubation time of 6 h at 37 °C, cells were washed with ice-cooled PBS, blocked, fixed and analyzed via a confocal microscope. Scalebar: 5 µm (Zooms: 2 µm). (B) HeLa and HCT cells were seeded in a 12 well plate and incubated for 4 h at 37 °C with with or without 10 µg/ml SubAB2-2-His (in a molar ratio of 1:5), SubAΔC344B2-2-His, SubA2-2-His, SubAΔC3442-2-His or SubB2-2-His. Afterwards cells were lysed in Lämmli buffer and subjected to SDS page and GRP78 status was analyzed by immunoblotting.

Surprisingly, Figure 11A shows that SubA$_{\Delta C344}$ is taken up into the ER of HeLa cells like wildtype SubA, however further investigations regarding substrate cleavage have revealed that SubA$_{\Delta C344}$ alone induced no GRP78 cleavage, but only together with SubB, when tested on HeLa and HCT116 cells (Figure 11B).

3.5 Characterization of SubA binding to cells

Since our group was able to show that SubA binds to Hela cells via WB (Funk et al. 2015), I verified this cell surface binding using FACS and examined it in more detail. To investigate the interaction between SubA and/or SubB on host cells more closely, I labeled both subunits with a fluorescent Dye (Dylight488-NHS-Ester). For control, it

was initially ruled out, that the labeling procedure had any effect on the cytotoxic activity of the toxin subunits. As can be seen in Figure 12, Dylight488-labeled SubA intoxicated HeLa cells to the same extent as unlabeled SubA. Similar, labeled SubB behaves like unlabeled SubB.

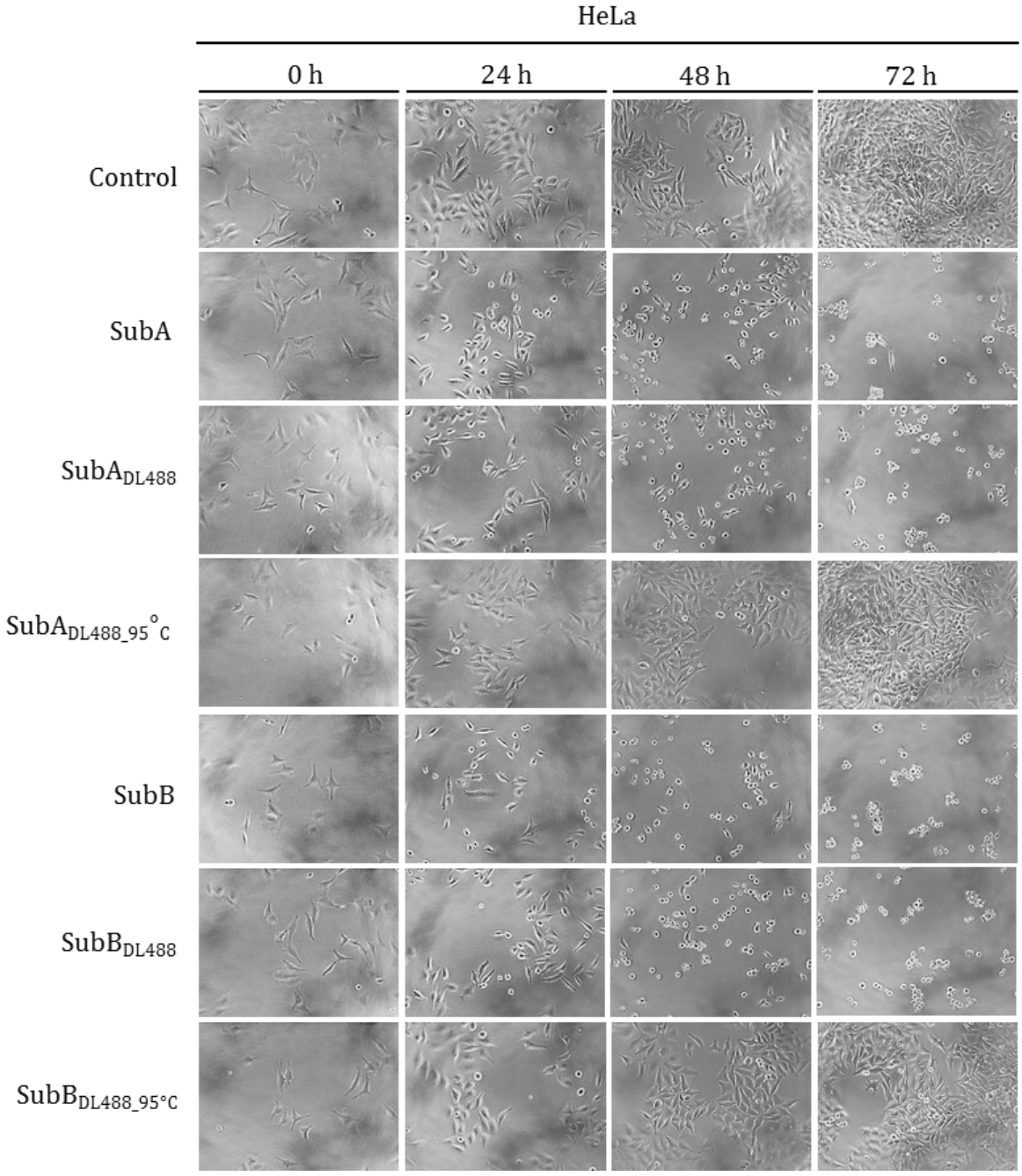

Figure 12 Dylight488 labeling procedure shows in cell culture neither an effect on the activity of SubA nor of SubB on Hela cells.

Cytotoxicity assay. HeLa cells were incubated with unlabeled SubA2-2-His (indicated as SubA), 488-labeled SubA2-2-His (indicated as SubADL488), unlabeled SubB2-2-His (indicated as SubB), labeled SubB2-2-His (indicated as SubBDL488) and labeled SubA2-2-His and SubB2-2-His which was denatured at 95 °C before appling to the cells (indicated as SubADL488_95°C and SubADL488_95°C). In all cases a concentration of 10 µg/ml. Pictures were taken after 24 h, 48 h and 72 h.

Since cell surface binding was analysed by FACS, it was confirmed in advance that the measurements do not show unspecific binding but indeed quantify toxin binding. Therefore, the buffer in which SubA was solubilized was labeled with the same amount of Dylight as SubA and HeLa cells were incubated with or without labeled SubA or labeled buffer for 30 min on ice, washed with ice cooled PBS and cell surface binding was measured via FACS analysis. The result showed that, unlike SubA, the buffer does not bind to HeLa cells, indicating that the measured SubA binding signal is specific and that generally the FACS approach is suitable to characterize SubA binding in more detail.

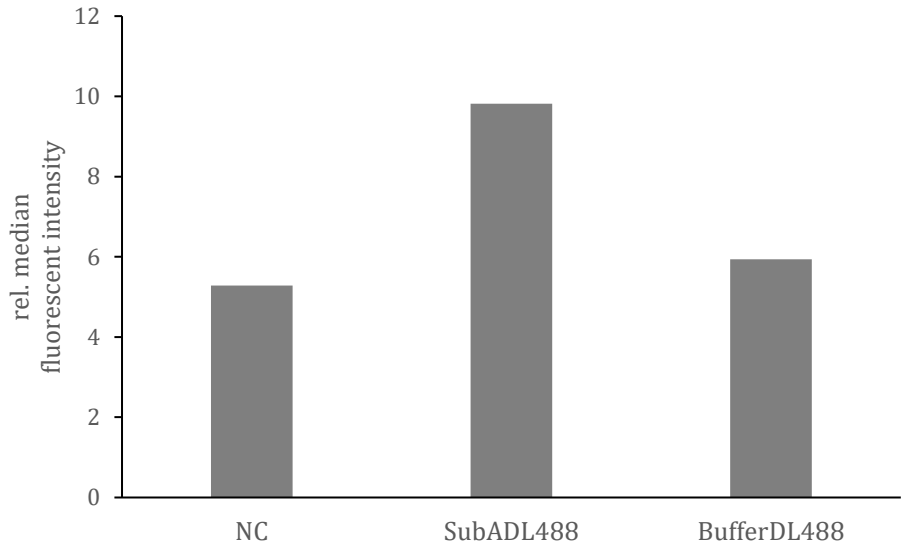

Figure 13 Labeled buffer showed no signal on HeLa cells in FACS analysis.
HeLa cells were incubated with 41 µg/ml labeled SubA2-2-His (indicated as SubADL488) or labeled buffer (indicated as BufferDL488) for 30 min on ice and binding was measured via FACS. The diagram shows the relative median of fluorescence intensity at 488 nm excitation.

3.5.1 Comparison of SubA and SubAB binding to cells

After confirming that SubA binds to cells in the absence of the binding component SubB, the binding value of SubA alone was compared to the holotoxin. Therefore, HeLa cells were incubated with only labeled SubA (30 µg/ml) or two different concentrations of labeled SubA in combination with unlabeled SubB. On time the total concentration of both subunits was 30 µg/ml and the other time 30 µg/ml SubA plus SubB in a molar ratio of 1:5 was used. In the latter case, the result is shown with an asterisk in the figure. At must be noted that the binding in this experiment was

performed in FCS-containing medium. In the further course of the work, the binding was performed in PBS.

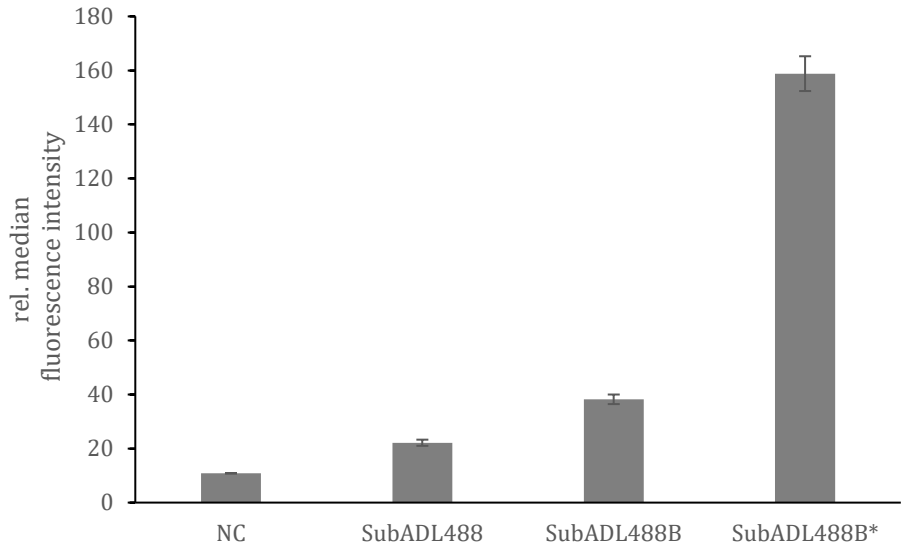

Figure 14 The SubAB holotoxin showed more binding to HeLa cells than SubA alone.
HeLa cells were incubated with 30 µg/ml labeled SubA2-2-His (indicated as SubADL488), 10.2 µg/ml labeled SubA2-2-His plus 19.8 µg/ml unlabeled SubB2-2-His (indicated as SubADL488B) or 30 µg/ml labled SubA2-2-His plus 58.2 µg/ml unlabeled SubB2-2-His (indicated as SubADL488B*). For the holotoxin a molar ratio of 1:5 of the subunits was used. Binding was performed for 30 min on ice and measured via FACS. The diagram shows the relative median of fluorescence intensity at 488 nm excitation and values are given as mean of three technical replicates ± standard deviation (SD).

Figure 14 determines that the binding of SubA alone is much weaker compared to SubAB. Comparing the two bars in which SubA was used at the same concentration only once alone and once with unlabeled SubB, the binding signal increases about 7-fold by the addition of SubB. If the total protein concentration of the holotoxin was the same as SubA alone the binding increased about 30 %.

3.5.2 Influence of different washings steps and media to SubA binding

After the binding of SubA to host cells could be confirmed by FACS analyses, the binding properties were investigated in more detail. The binding strength was studied by different numbers of washing steps (one to three times) post-binding. Additionally, different binding media (PBS, serum-free medium or serum-containing medium) were tried out.

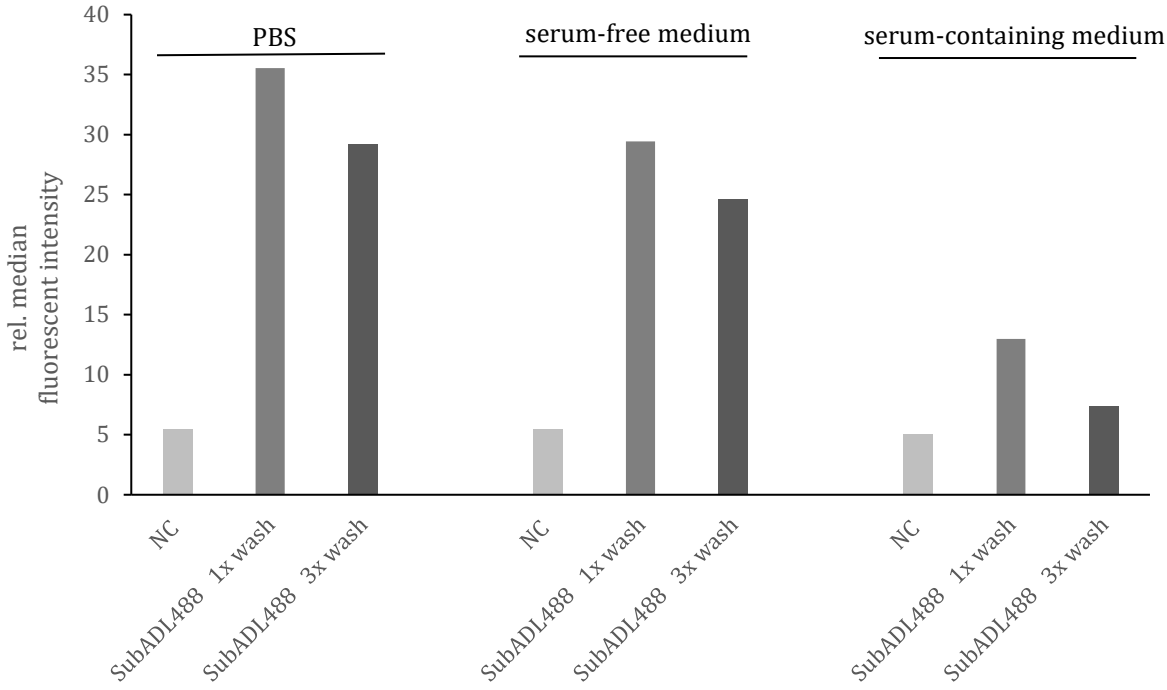

Figure 15 The intensity of the SubA binding to cells is influenced by the number of washing steps post-binding and by the medium used.

HeLa cells were incubated with 41 µg/ml labeled SubA2-2-His for 30 min on ice. The binding was either performed in PBS, serum-free medium or serum-containing medium. After the binding the cells were washed once or three times and analysed via FACS. The diagram shows the relative median of fluorescence intensity at 488 nm excitation.

Figure 15 shows that there is a difference in SubA binding intensity depending on how often the cells were washed after toxin binding. After three washing steps the SubA binding signal is overall weaker compared to the binding signal after one washing step. Due to this result, the cells were only washed once after binding in all further FACS experiments. But not only the number of washing steps had an influence on the binding intensity, also the different media used show differences. Although the same SubA concentration was applied to all samples, the binding signal is decreased in serum-containing medium compared to serum-free medium or PBS. To clarify wether the FCS in the serum-containing medium is responsible for the decrease in binding signal an experiment with medium plus different FCS-concentrations was performed.

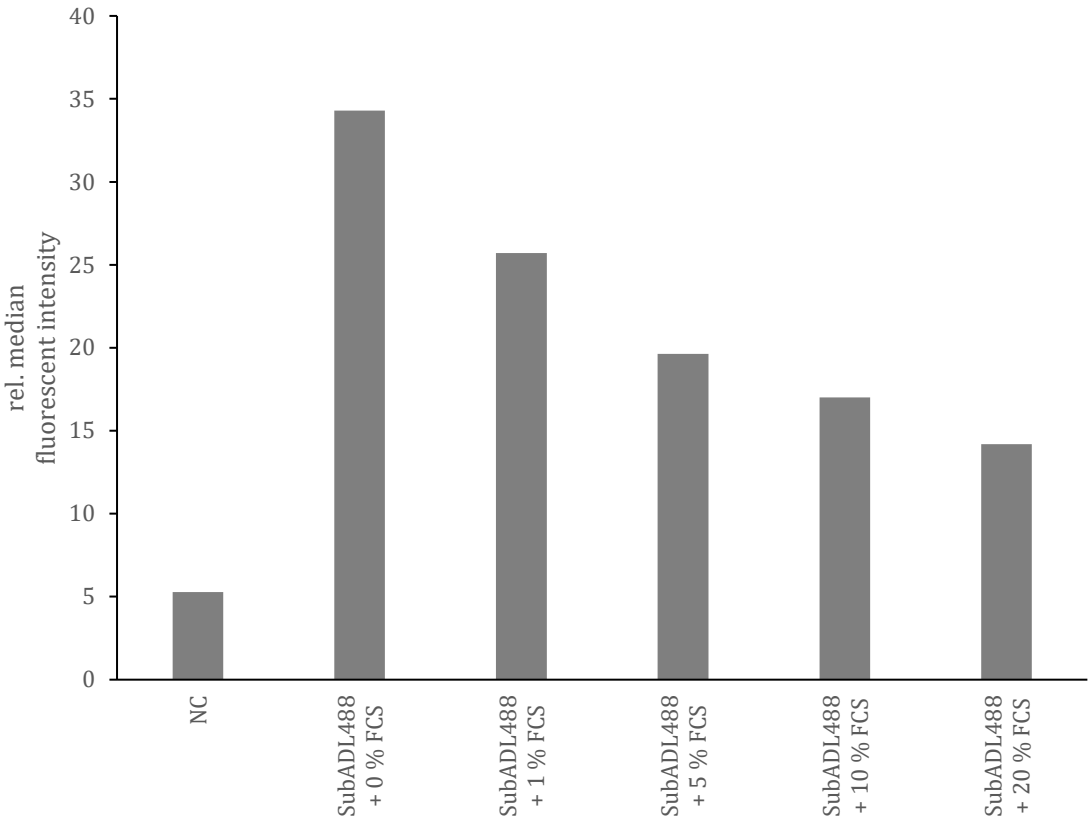

Figure 16 SubA binding to HeLa cells depends on the FCS concentration in the medium.
HeLa cells were incubated with 41 µg/ml labeled SubA2-2-His for 30 min on ice. The binding was performed in PBS with the following FCS concentrations 0 %, 1 %, 5 %, 10 % or 20 % FCS. The diagram shows the relative median of fluorescence intensity at 488 nm excitation.

Results of Figure 16 showe that with increasing FCS concentrations, the binding signal becomes weaker. Comparing the SubA binding in PBS with the PBS plus the highest FCS concentration, the signal deteriorated by more than half. Therefore, it is suggested that FCS most likely intercepts SubA and thus causes a weaker binding signal.

3.5.3 Binding of SubA in combination with a chemical crosslinker

Since the results of Figure 15 indicate that the binding of SubA is not very stable, it was obvious to try out the use of a crosslinker and see if this would improve the binding stability. Therefore, SubA was incubated ± DSS-crosslinker to HeLa cells for 30 min on ice, washed with PBS three times and binding was analysed via FACS.

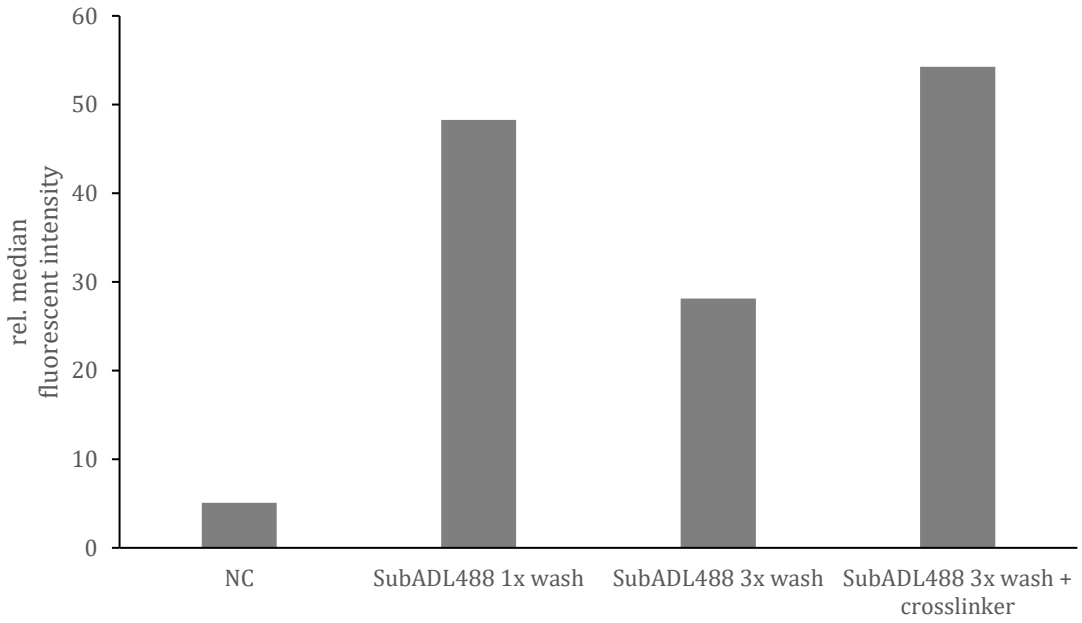

Figure 17 SubA binding to cells is stabilized by the crosslinker DSS.
HeLa cells were incubated with 41 µg/ml labeled SubA2-2His ± DSS-crosslinker for 30 min on ice. After binding cells were washed one or three times with PBS and analysed via FACS analysis. The diagram shows the relative median of fluorescence intensity at 488 nm excitation.

It can bee clearly seen that the use of the DSS-crosslinker stabilizes the cell surface binding of SubA. The decreasing binding affinity with increasing washing steps can be completely prevented with the use of the crosslinker.

3.5.4 Concentration-dependent binding of SubA

To identify further SubA binding characteristics, HeLa cells were incubated with increasing concentrations of SubA. This experiment was designed to provide hinds as to whether a protein serves as a binding partner on the cell surface, as saturation of binding would be expected in this case.

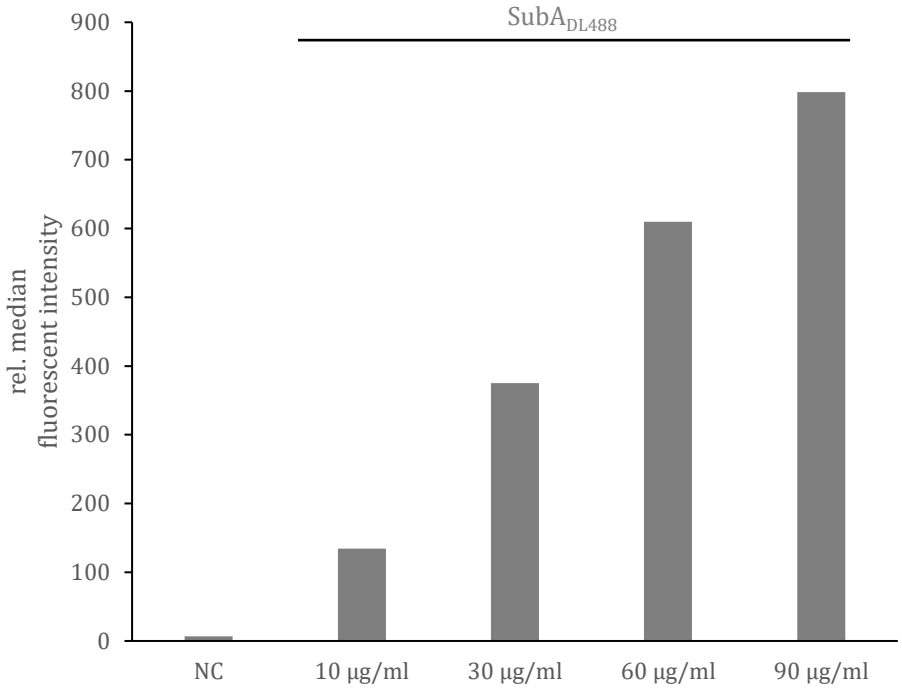

Figure 18 SubA binding to cells is not saturable.
HeLa cells were incubated with increasing concentration (10 µg/ml, 30 µg/ml, 60 µg/ml, 90 µg/ml) of labeled SubA2-2-His at 4 °C for 30 min and SubA binding was analysed via FACS. The diagram shows the relative median of fluorescence intensity at 488 nm excitation.

Figure 18 shows that the binding signal increases with increasing SubA concentrations. Up to 90 µg/ml no saturation of SubA binding is visible, which does not indicate a specific SubA binding with involvement of a protein receptor.

3.5.5 SubA binding after cleavage of cell surface proteins

Since the results so far do not indicate that a protein receptor is involved in SubA binding, a pronase assay should bring clarity. HeLa cells were incubated with 500 µg/ml pronase for 30 min before incubating them with the toxin. Pronase is an enzyme that protolytically cleaves all cell surface proteins. As a positive control the enzymatically inactive diphtheria toxin of *Corynebacterium diphtheriae*, in the form of CRM197 fused to a green fluorescent protein (eGFP_CRM197), was used.

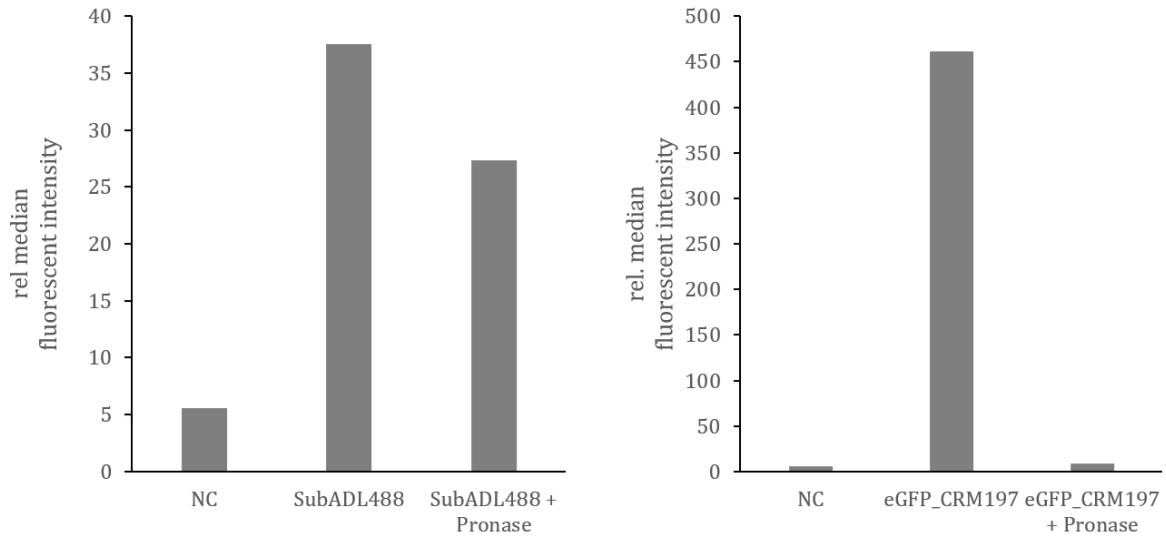

Figure 19 SubA binding to cells is decreased after proteolytic cleavage of cell surface proteins. HeLa cells were pre-treated with ± 500 µg/ml pronase and afterwards incubated with either 41 µg/ml labeled SubA2-2-His or 0.0625 µg/ml eGFP_CRM197 for 30 min. at 4 °C. The diagram shows the relative median of fluorescence intensity at 488 nm excitation.

First of all, the diagram shows that the pronase assay itself worked. The binding signal of eGFP_CRM returns to the level of the negative control after pronase pre-treatment. In the case of SubA, the binding signal decreases by about one third compared to that of untreated cells. This result confirms the previous findings that not exclusively a specific protein might serve as a SubA receptor.

3.5.6 Effect of SubB variants on SubA binding

As descriebd in the Introduction part there are different SubAB variants known. The different variants share over 90 % sequence identity, which suggests that not only the same variant of SubA and SubB can bind to each other but also that different variants can build a holotoxin. In order to investigate wether different holotoxins can be formed, labeled SubA2-2-His was incubated with either unlabeled SubB2-2-His or unlabeled SubB2-1-His and analysed via FACS.

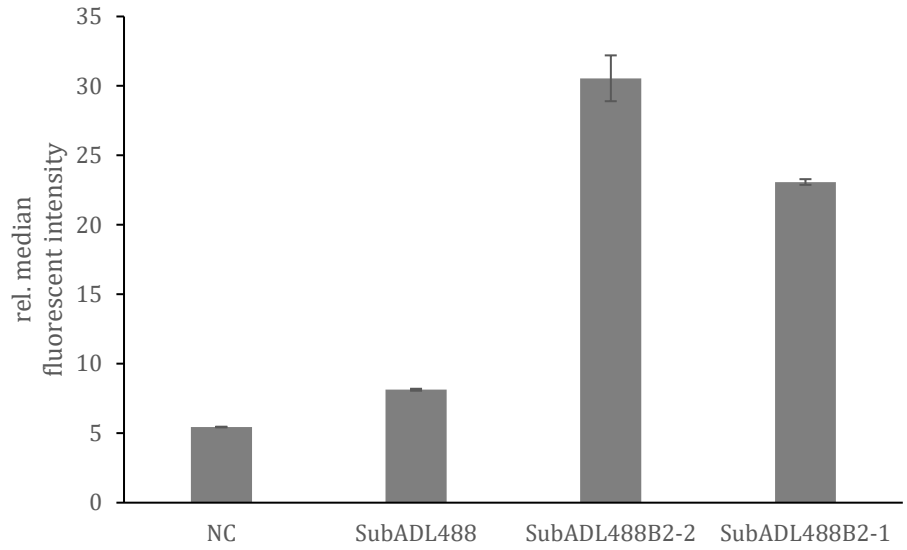

Figure 20 SubA2-2-His + SubB2-2-His shows more binding to cells than SubA2-2-His + SubB2-1-His.

HeLa cells were incubated at 4 °C for 30 min with the following toxin combinations: 3.4 µg/ml labeled SubA2-2-His (indicated as SubADL$_{488}$), 3.4 µg/ml labeled SubA2-2-His plus 6.6 µg/ml unlabeled SubB2-2-His (indicated as SubADL$_{488}$2-2) and 3.4 µg/ml labeled SubA2-2-His plus 6.6 µg/ml unlabeled SubB2-2-His (indicated as SubADL$_{488}$2-1). The diagram shows the relative median of fluorescence intensity at 488 nm excitation and values are given as mean of two technical replicates ± standard deviation (SD).

As expected, the results shown in Figure 20 revealed that most SubA was bound to HeLa cells in the presence of SubB2-2. However, the binding was only about 30 % reduced for the combination of SubA2-2-His and SubB2-1-His.

3.5.7 SubAB complex formation

So far, it is still unclear wether SubAB is secreted by cells as holotoxin complexes or if a complex formation can also happen on the cell surface. This experiment was performed to characterize the mode of assembly of the subunits. Therefore, labeled SubA and unlabeled SubB were either applied directly to the cell suspension or pre-incubated for 30 min. and than applied to cells. Total toxin concentration was 10 µg/ml and subunits were used in a molar ratio of 1:5 to simulate AB$_5$ toxin stoichiometry.

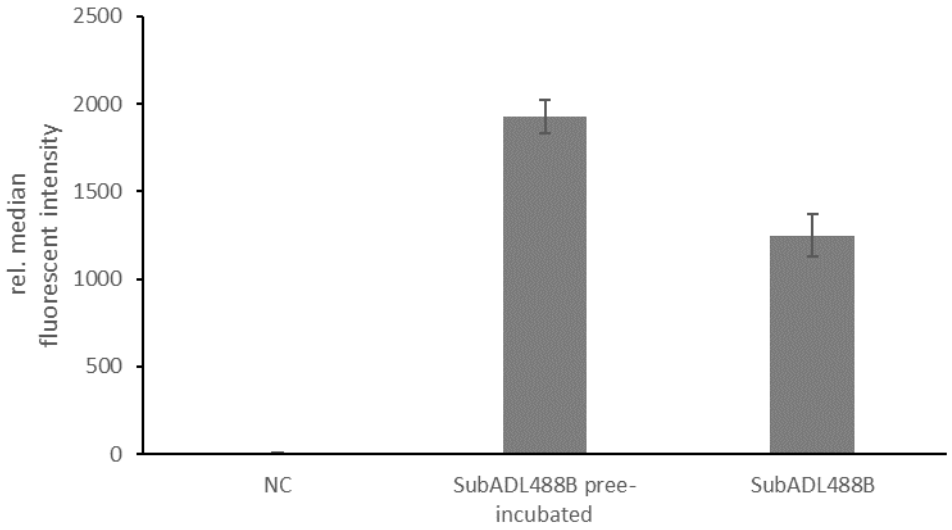

Figure 21 Pree-incubation of SubAB results in more binding of SubAB to HeLa cells compared to direct application of SubA and SubB into the medium.
Labeled SubA2-2-His plus unlabeled SubB2-2-His were either pre-incubated prior to binding or directly applied to the cells. The Subunits were used in a molar ratio of 1:5 with a total protein concentration of 10 µg/mL The diagram shows the relative median of fluorescence intensity at 488 nm excitation. Values are given as mean of three technical replicates ± standard deviation (SD).

The results show that, due to the pre-incubation, the fluorescence signal of the A-subunit was approx. 40 % higher compared to the direct application.

4 Discussion

For bacterial AB_5 protein toxins, the distribution of tasks between their two subunits is clearly defined. The B-subunit is known to be responsible for binding and transport, whereas the A-Subunit keeps the enzyme activity. However, our group together with the group of Prof. Schmidt (University of Hohenheim) made the observation that SubA, in the absence of SubB, binds to and intoxicates HeLa cells (Funk et al. 2015). These findings were completely unexpected due to the commonly accepted distribution of functional roles of the toxin subunits. Additionally, it was believed that an entire AB_5-holotoxin complex is required to induce cytotoxic effects. This thesis aimed to better understand the observations made and consequently, elucidated the underlying cellular and molecular mechanisms of SubA-induced cytotoxicity.

4.1 The intracellular transport and cytotoxic activity of SubA and SubAB are comparable

A series of cell-based experiments were performed to determine the intracellular transport pathway and to characterize the cytotoxic activity of SubA. After confirming the enzyme activity of all purified recombinant SubA proteins *in vitro* (Figure 3), it was necessary to find a suitable concentration for subsequent cytotoxcicity assays. Figure 4 shows that morphological changes, which represent an established specific and selective endpoint to monitor cytotoxicity, were visible for all cells at a concentration of 10 µg/ml after 72 h. These conditions were chosen for further cytotoxicity experiments. In the next step, SubA-induced cytotoxicity was examined on a human colon cancer-derived epithelial cells because HCT116 cells represent a more physiological cell line as SubAB is an intestinal pathogen. Comparing the cytotoxic effect of SubA on HeLa and HCT116 cells, Figure 5 demonstrates that HCT116 cells show a slightly earlier reaction to the toxin at same concentrations but overall, it can be said that there is no big difference in toxin-sensitivity. To answer the question if SubA-induced cytotoxicity is mediated intracellulary or extracellulary SubA uptake was visualized via flurorescent microscopy. The images of Figure 6 clearly reveal that SubA is taken up in HeLa cells and beyond that it co-locolizes with CTB, meaning it reaches the ER even without SubB. As already observed in the cytotoxicity assays, higher concentrations of SubA compared to SubAB were necessary to visualize toxin uptake, indicating that SubA uptake is less efficient than SubAB. Because Paton et al. showed that SubAB cytotoxicity is based on a specific single-site cleavage of BiP/GRP78 in the hinge region connecting its N-terminal ATPase and C-terminal protein-binding domains, BiP/GRP78 cleavage was used to confirm SubA uptake into the ER. The Western blot analysis showed that SubA induced GRP78-cleavage in living cells is time and concentration dependent. Furthermore, Figure 8 supports the hypothesis that SubA uptake and/or the transport is less efficient than SubAB, because SubA induced GRP78-cleavage occured slower compared to SubAB.

In the further course, the intracellular transport route of SubA was considered in more detail. Therefore, SubA-induced BiP/GRP78 cleavage was studied in combination with various inhibitors. Figure 9 shows that only BFA, which is an inhibitor of the ADP-ribosylation factor (ARF), thus inhibits protein translocation from the ER to the Golgi

apparatus (Klausner et al. 1992), had an effect on SubA-induced BiP/GRP78 cleavage. All other inhibitors used, had no effect. Neither the inhibitor Bafilomycin A1, which is a potent V-ATPases inhibitor and thus blocks endosomal acidification (Sandvig et al. 1991; Werner et al. 1984), nor Ambroxol, a licensed muco-lytic drug, which neutralizes intravesicular pH levels as it is a weak base accumulating in acidic vesicles (Fois et al. 2015), nor human serum albumin, an abundant protein in human blood plasma, which reduces cytotoxicity of Clostridium difficile toxins (Di Masi et al. 2018). These results fit to the previous findings that SubA alone is not translocated into the cytosol, as acidification of endosomal vesicles is a fundamental process of toxin transport into the cytosol but that it rather acts in the ER. Moreover, the effect that BFA prevented BiP/GRP78 cleavage in cells treated with SubA, strongly suggests that SubA alone is also transported via a retrograde pathway into the ER similar to SubAB.

Since so far it was believed that a B-subunit is necessary to transport SubA into the ER, SubA obviously must have its own ER target sequence. In the case of the cholera toxin, which also belongs to the AB_5-type toxin family, a C-terminal KDEL motif in the A-subunit is respomsible for retrograde transport, but this motif is missing in the SubA amino acid sequence (Lencer et al. 1995). However, the scanProsite tool (Castro et al. 2006) predicted a C-terminal SEEL motif as a potential ER-targeting signal, which is why a SubA mutant lacking this motif was generated (SubAΔC344) and analyzed on its cytotoxicity. It was shown that the mutant induced no morphological changes on HeLa cells (Figure 10) and that SubAΔC344 does not cleave BiP/GRP78 when tested under the same conditions as SubA wildtype (Figure 11). On the other hand, Figure 11 visualizes SubAΔC344 uptake into the ER which seems contradictory at first glance. That SubAΔC344 is inactive can be excluded on the one hand by a positive enzyme activity test (Figure 3) and on the other hand because in combination with SubB it behaved like wildtype SubAB. Taken the results of all experiments on SubAΔC344 together, it can be concluded that SubAΔC344 uptake and transport to the ER is much less efficient compared to wildtype SubA meaning that the C-terminal SEEL motif is crucial for guiding SubA into the ER. Based on the current knowledge a scheme of SubA uptake, intracellular transport and cytotoxic effect was created (Figure 22.).

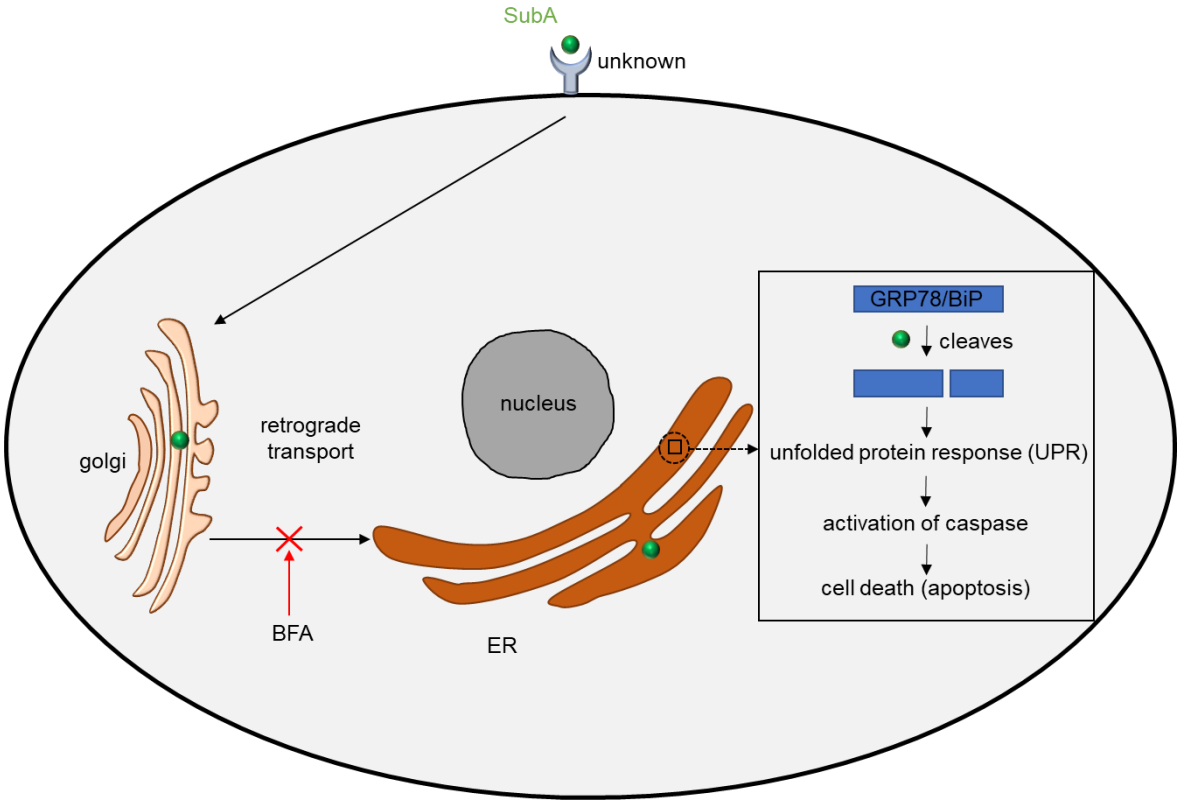

Figure 22 Schematic overview of the current assumption of SubA uptake, transport, and cytotoxic effect in the absence of SubB.
Explanations are given in part 4.1. (Based on Sessler et al. 2022.)

4.2 SubA binding to the cell surface in the absence of SubB seems not to be highly specific and efficient

During host–pathogen interaction a crucial step is entry into the cell. Some bacteria like *Salmonella*, *Shigella, Pseudomonas or Yersinia* directly interact with the host cell and inject their toxic proteins into the cytosol, which prevents contact of numerous proteases in the body fluid (Mota et al. 2005; Galán und Wolf-Watz 2006; Galán et al. 2014). However, STEC strains do not have a direct injection mechanism but rather secret their toxins into the surrounding body fluid and host cell entry is ensured by receptor-mediated endocytoses (Waddell et al. 1988; Lingwood et al. 1987; Yahiro et al. 2011). Therefore, successful toxin binding to the eukaryotic cell surface is an essential step in the pathophysiological action and the identification of a toxin receptor an important step in developing anti-toxin strategies. Since Funk et al. showed for the first time that SubA is able to bind to HeLa cells, the binding characteristics of SubA to the cell surface were studied in more detail via FACS anaylsis. First of all, it should be

noted that SubA binding is significantly weaker than SubAB. Furtehrmore it was shown that SubA does not bind with a high affinity to the cell surface, because with increasing washing steps post-binding the binding signal became weaker and weaker. The low binding affinity together with the fact that SubA binding is non-saturable suggests that SubA binding is not very specific and thus does not bind to a protein receptor. This hypothesis is supported by the finding that SubA binding is reduced after proteolytic degradation of cell surface proteins but not completely abolished. It would therefore be conceivable that SubA binds to sugars or lipid structures that occur both independently and on proteins on the cell surface.

To identify the SubA binding element several approaches could be considered. One possibility would be to perform a classical affinity-based co-precipitation assay. However due to the low binding affinity of SubA to the cell surface this method might be challenging as the limiting factor of this method is the receptor-ligand stability as it must survive cell lysis and precipitation. Figure 17 shows that SubA binding can be stabilized via a DSS- crosslinker which is why the use of a crosslinker is highly recommended for a co-precipitation assay. A completely different approach for receptor identification would be via the he clustered regularly interspaced short palindromic repeat (CRISPR)/-Cas9(CRISPR associated) system. A genome-wide CRISPR /-Cas9 knockout screen can be performed to create cells which do not express the SubA binding element. SubA would not be ableto induce morphological changes in these cells and therefore, it would be easy to collect these cells for further mass spectrometry analysis.

4.3 Potential clinical relevance of the single SubA-effect

In the cases of STEC infections reported to date, the shiga toxin is thought to have a much higher clinical significance than SubAB. Nevertheless, in-vivo studies showed that SubAB injected intraperitoneal in mice caused microangiopathic hemolytic anemia, thrombocytopenia, and renal impairment (Wang et al. 2007), which on the one hand is a pathology that is comparable to animal studies with purified Stx (Richardson et al. 1992; Siegler et al. 2003; Yamamoto et al. 2005) and on the other hand illustrates parallels to human cases of STEC associated HUS (Inward et al. 1997; Proulx et al. 2001). Looking at all the results of this thesis, it can be summarized that SubA alone

showed similar intracellular and molecular mechanisms like SubAB on cultured cells, however due to a less efficient SubA uptake and/or transport to the ER, it was less toxic compared to the holotoxin (Sessler et al. 2021). The B-subunit of the subtilase cytotoxin is therefore not mandatory to induce cell death but it acts as an amplifier and makes it possible for the toxin to cause effects at lower concentrations. So far, the distribution of SubA and SubB released by the bacteria is unknown, why it would be possible that excess SubA might enhance the pathogenic potential of STEC. At the moment, *in-vivo* studies on the single SubA- effect are missing, which is why experiments on human miniguts would be of enormous interest. Miniguts represent a model for the human intestine tissue as they form a crypt-like structure and architecture of a polarized intestinal epithelium (Hohwieler et al. 2016), thus being a good test system for an intestinal pathogen. Besides that, it should be noted that a recent study reported a single A-effect also for the Stx. The A-subunit of the Stx, without the B-subunit, induced comparable cytotoxic effects as the recombinant Stx2a-holotoxin on HeLa cells and even higher cytotoxic effects on Vero cells (Heinisch et al. 2021). Since both toxins are sectreted by the same bacteria, the single A-effect should not be underestimated and be investigated also for other AB_5 toxins.

4.4 SubA as a potential transporter protein for delivery of therapeutic molecules into cells

By developing therapeutic proteins many models struggle with overcoming cellular barriers. As bacterial toxins are autonomously taken up by cells and exploiting cellular pathways for protein transport, they represent a highly attractive model for a delivering system for biopharmaceuticals. For example the inactive form of the Anthrax toxin was already fused to over 30 cargo molecules including peptides, other toxin effector proteins, non-canonical peptides, antibody-like molecules, and oligonucleotides and analyzed with regards to there cellular uptake (Dyer et al. 2015; Liao et al. 2014; Rabideau und Pentelute 2016) . Not only for the anthrax toxin also the diphtheria toxin represents a well studied toxin-based transport system (Wesche et al. 1998). So far, there are not many studies which use SubAB or SubAB as a delivery system, however recently Harada et al. fused Poly(D,L-lactide-co-glycolic) acid (PLGA) nanoparticles to SubAB to target macrophages and to examine anti-inflammatory effects (Harada et al. 2020). Another subtilase based transport system would be

conceivable via the already established biotin/avidin technology (Fahrer et al. 2010), whereby the inactive mutant of SubA is fused to avidin so that biotinylated cargo molecules can bind and be delivered into the cell. An outstanding feature of a subtilase-based transport system to other toxins would be its ER specificity, which allows completely other therapeutic applications.

5 Summary (English)

The subtilase cytotoxin is secreted by shiga toxin-producing *E. coli* strains and is mainly transmitted to humans through contaminated food and water. The toxin belongs to the family of AB_5 toxins with a typical structure of a SubB pemtamer, which is responsible for toxin binding and uptake, and an A-subunit, which represents the enymatically active toxin moiety. Research of our group together with that of Prof. Schmidt (Hohenheim) could show for the first time that SubA induces cytotoxic effects in the absence of the B-domain. This discovery was completely unexpected, as it was previously assumed in expert circles that an intact holotoxin was necessary for this. In my PhD. thesis I investigated the cellular and molecular mechanisms underlying the single SubA effect in more detail. Since the previous work was mainly done with human cervical cancer ephithelial cells (HeLa), I first investigated whether human colon cancer-derived epithelial cells (HCT116), are target cells for the cytotoxic effects caused by SubA alone. These cells represent a more physiological cell line for an intestinal pathogen to study the autonomous effects of this toxin component. Having confirmed the cytotoxic effect of SubA, without SubB, on HCT116 cells, the question arose whether these effects were mediated intracellularly or extracellularly. Autonomous SubA uptake could be visualized by fuorescence microscopy and showed that SubA accumulates in the endoplasmic reticulum even in the absence of SubB. This observation was confirmed by Western blot results. These showed that SubA alone is capable of cleaving and thus inactivating the substrate GRP78/BiP in a time- and concentration-dependent manner. Furthermore, a series of inhibitor experiments showed that SubA, like SubAB, enters the ER through a retrograde transport pathway. These results suggested that SubA must carry an independent ER target sequence and indeed a sequence at the C-terminus, the SEEL motif, was identified as a critical motif for ER transport. Taken together, SubA shows similar cellular uptake and cytotoxic mode of action on eukaryotic cells as SubAB holotoxin. However, it should be noted that uptake of SubA and/or its transport into the ER is less efficient in the absence of SubB. This suggests that SubB is not essential for causing cytotoxicity, but that the presence of SubB enormously enhances the cytotoxic effect, which may allow the toxin to act at lower concentrations. However, the SubA effect alone should not be underestimated, since SubA subunits secreted by bacteria that have not found a SubB

pentamer to form a holotoxin could extend and enhance the pathogenic potential of subtilase-producing *E. coli* strains.

6 Summary (Deutsch)

Das Subtilase Zytotoxin wird von Shigatoxin-produzierenden *E.coli* Stämmem sekretiert und wird hauptsächlich durch kontaminierte Nahrungsmittel auf den Menschen übertragen. Das Toxin gehört zur Familie der AB_5 Toxine mit dem typischen Aufbau von einem SubB-Pentamer, dass für die Toxinbindung und Aufnahme verantwortlich sind, sowie einer A-Untereinheit, die den enymatisch aktiven Toxinteil darstellt. Arbeiten unserer Arbeitsgruppe gemeinsam mit der von Prof. Schmidt (Hohenheim) konnten zum ersten Mal zeigen, dass SubA in Abwesenheit der B-Domäne zytotoxische Effekte auslöst. Diese Entdeckung war komplett unerwartet, da man in Fachkreisen bisher davon ausgegangen ist, dass ein intaktes Holotoxin dafür notwendig ist. In der vorliegenden Dissertation wurden die zellulären und molekularen Mechanismen, die dem zytotoxischen Effekt von SubA zugrundeliegen, eingehend charaktersiert. Da die vorangegangenden Abeiten hauptsächlich mit humanen Ephithelzellen eines Zervixkarzinoms (HeLa) durchgeführt wurden, wurde in dieser Arbeit untersucht, ob Epithelzellen, die von einem humanem Colontumor abgeleitet wurden (HCT116), Zielzellen für SubA darstellen. Diese Zelllinie wäre (patho)physiologisch relevanter, um die SubA-Effekte zu untersuchen. Nachdem der zytotoxische Effekt von SubA in Abwesenheit von SubB auf HCT116-Zellen bestätigt wurde, stellte sich die Frage, ob diese Effekte intrazellulär oder extrazellulär vermittelt werden. Die SubA-Aufnahme konnte durch Fuoreszenzmikroskopie visualisiert werden und zeigte, dass SubA auch ohne SubB sich im endoplasmatischen Retikulum ansammelt. Diese Beobachtung wurde durch Western Blot Ergebnisse bestätigt. Diese zeigten, dass SubA allein in der Lage ist, das Substrat GRP78/BiP zeit- und konzentrationsabhängig zu spalten und somit zu inaktivieren. Darüberhinaus zeigte eine Reihe von Inhibitorversuchen, dass SubA genau wie SubAB durch einen retrograden Transportweg ins ER gelangt. Diese Ergebnisse deuteten darauf hin, dass SubA eine ER-Zielsequenz enthalten muss und tatsächlich wurde mit dem SEEL-Motiv am C-Terminus von SubA eine solche Sequenz identifiziert und ihre Funktion für den ER Transport von SubA in Zellen durch Amoinosäureaustausch bestätigt. Zusammengefasst zeigen die Ergebnisse, dass SubA in Abwesenheit von SubB über

vergleichbare Wege in das ER von Zielzellen aufgenommen wird und durch Spaltung von GRP78/BiP eine vergleichbare zytotoxische Wirkung auf Zellen ausübt wie das SubAB-Holotoxin. Allerdings ist anzumerken, dass die Aufnahme von SubA und/oder sein Transport in das ER in Abwesenheit von SubB weniger effizient ist. Dies deutet darauf hin, dass SubB für die Verursachung der Zytotoxizität nicht unbedingt erforderlich ist, dass aber das Vorhandensein von SubB die zytotoxische Wirkung von SubA verstärkt. Die biologische Relevanz könnte darin bestehen, dass bei niedrigeren Konzentrationen von SubA und SubB dennoch eine zytotoxische Wirkung vermittelt werden kann. Andererseits könnte von Bakterien freigestztes SubA, dass kein SubB-Pentamer für die Bildung eines Holotoxins gefunden hat, auch unabhängig von SubB Zellen vergiften, was das pathogene Potenzial der Subtilase produzierenden *E. coli*-Stämme erweitern und verstärken könnte.

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8 List of Publications

1. **Sessler K**, Schmidt H, Barth H. Novel Aspects of the SubA Subunit of the Subtilase Cytotoxin. Toxins (Basel). 2022 Feb 21;14(2):156. doi: 10.3390/toxins14020156
2. **Sessler K**, Papatheodorou P, Wondany F, Krause M, Noettger S, Bernhard D, Michaelis J, Schmidt H, Barth H. The enzyme subunit SubA of Shiga toxin-producing E. coli strains demonstrates comparable intracellular transport and cytotoxic activity as the holotoxin SubAB in HeLa and HCT116 cells in vitro. Arch Toxicol. 2021 Mar;95(3):975-983. doi: 10.1007/s00204-020-02965-2.
3. **Sessler K**, Blechschmidt V, Hoheisel U, Mense S, Schirmer L, Treede RD. Spinal cord fractalkine (CX3CL1) signaling is critical for neuronal sensitization in experimental nonspecific, myofascial low back pain. J Neurophysiol. 2021 May 1;125(5):1598-1611. doi: 10.1152/jn.00348.2020
4. Krause M, **Sessler K**, Kaziales A, Grahl R, Noettger S, Barth H, Schmidt H. Variants of Escherichia coli Subtilase Cytotoxin Subunits Show Differences in Complex Formation In Vitro. Toxins (Basel). 2019 Dec 3;11(12):703. doi: 10.3390/toxins11120703

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