Thesis for the degree of Philosophiae Doctor

Membrane dynamics in cancer cells

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Simona Kavaliauskiene
Oslo, May 2016

Abbreviations and Lipid Nomenclature

2DG	2-deoxy-D-glucose
Acc1	Acetyl-CoA carboxylase 1
ADP	Adenosine diphosphate
ALPS	Amphipathic lipid packing sensor
AMP	Adenosine monophosphate
ARF	ADP-ribosylation factor
BFA	Brefeldin A
BG	Benzylguanine
BiP	Immunoglobulin binding protein (also called GRP78)
CDase	Ceramidase
CerS	Ceramide synthase
CERT	Ceramide transfer protein
CoA	Coenzyme A
COG	Conserved oligomeric Golgi
COP I	Coat protein I
COP II	Coat protein II
cPLA2	Cytoplasmic phospholipase A2
DAGK	Diacylglycerol-consuming kinase
DRM	Detergent resistant membrane
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
EHD	Eps15 homology domain-containing
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated degradation
FA	Fatty acid
FAPP2	Four-phosphate adaptor protein 2
FB1	Fumonisin 1

FDG	2-fluoro-2-deoxy-D-glucose; fluorodeoxyglucose
FRET	Förster resosnance energy transfer
Gal	Galactose
GalNAc	N-acetylgalactosamine
GARP	Golgi-associated retrograde protein
GCS	Glucosylceramide synthase, UDP-glucose:ceramide glucosyltransferase
GCS	Glucosylceramide synthase gene
GDP	Guanosine diphosphate
Glc	Glucose
GlcNAc	N-acetylglucosamine
GPI	Glycosylphosphatidylinositol
GRP94	Glucose-regulated protein of 94 kDa
GSL	Glycosphingolipid
GTP	Guanosine triphosphate
HEDJ	Human endoplasmic reticulum DnaJ domain-containing protein (also called ERdj3)
HEp-2	Human epithelial type 2
HUS	Hemolytic uremic syndrome
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MDR1	Multidrug resistance gene 1
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
NB-DGJ	N-butyldeoxygalactonojirimycin
PAP	Phosphatidic acid phosphohydrolase
PDI	Protein disulfide isomerase
PET	Positron emission tomography
P-gp	P-glycoprotein
PLD	Phospholipase D
pro-HB-EGF	Heparin-binding epidermal growth factor precursor
Rab6IP2	Rab6-interacting protein 2

RN-tre	Related to the N-terminus of tre (also called USP6NL, ubiquitin-specific protease (USP6) N-terminal like)
RTN-1C	Reticulon-1C
SA	Sialic acid
shRNA	Short hairpin RNA
SIRT1	Sirtuin 1
SMase	Sphingomyelinase
SNARE	Soluble *N*-ethylmalemide-sensitive factor attachment protein receptor
SNX	Sorting nexin
Sp1	Specificity protein 1
STEC	Shiga toxin-producing *Escherichia coli*
Stx	Shiga toxin
Stx1	Shiga-like toxin 1
Stx2	Shiga-like toxin 2
Syk	Spleen tyrosine kinase
TGN	*trans*-Golgi network
TLC	Thin layer chromatography
TMF	TATA element modulatory factor (also called ARA160, androgen receptor-coactivator of 160 kDa)
UDP	Uridine diphosphate
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor

Abbreviations of lipid classes:

Cer	Ceramide
DAG	Diacylglycerol
GalCer	Galactosylceramide
Gb3	Globotriaosylceramide
GlcCer	Glucosylceramide
LacCer	Lactosylceramide
LPC	Lysophosphatidylcholine

LPE	Lysophosphatidylethanolamine
PA	Phosphatidic acid
PC	Phosphatidylcholine (ether-linked PC is shown as PC O (alkyl) or PC P (alkenyl))
PE	Phosphatidylethanolamine (ether-linked PE is shown as PE O (alkyl) or PE P (alkenyl)
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PIP	Phosphoinositide
PS	Phosphatidylserine
SM	Sphingomyelin
Sph	Sphingosine

Lipid species nomenclature:

In this study, for the annotation of different lipid species we used the nomenclature based on Liebisch and colleagues [1]. The different species of glycerolipids are listed with the two fatty acyl groups separated with an underscore (*sn*-position of the fatty acids is not known) or with a slash (*sn*-position of the fatty acids is known), e.g. PC 16:0_18:1 and PC 16:0/18:1. The N-amidated fatty acyl groups for sphingomyelin, ceramide and glycosphingolipids are shown after the slash, e.g. SM d18:1/16:0.

List of Publications

The thesis is based on the following original publications, included in the second part of thesis. They will be referred to in the text by their roman numerals I-III.

I. Kavaliauskiene S, Nymark C-M, Bergan J, Simm R, Sylvänne T, Simolin H, Ekroos K, Skotland T, Sandvig K. **Cell density-induced changes in lipid composition and intracellular trafficking.** Cell. Mol. Life Sci. 2013; 71:1097-1116.

II. Kavaliauskiene S, Skotland T, Sylvänne T, Simolin H, Klokk TI, Torgersen ML, Lingelem AB, Simm R, Ekroos K, Sandvig K. **Novel actions of 2-deoxy-D-glucose: protection against Shiga toxins and changes in cellular lipids.** Biochem. J. 2015; 470:23-37.

III. Kavaliauskiene S, Torgersen ML, Klokk TI, Lintonen T, Simolin H, Ekroos K, Skotland T, Sandvig K. **Cellular effects of fluorodeoxyglucose: Global changes in the lipidome and alteration in intracellular transport.** Under revision.

Related publications not included in the thesis:

Sandvig K, Bergan J, Kavaliauskiene S, Skotland T. **Lipid requirements for entry of protein toxins into cells**. Prog. Lipid Res. 2014; 54:1-13.

Klokk TI, Kavaliauskiene S, Sandvig K. **Cross-linking of glycosphingolipids at the plasma membrane: consequences for intracellular signaling and traffic**. Cell. Mol. Life Sci. 2016; 73:1301-16.

Abstract

In this study we have focused on the role of lipids in intracellular transport, and how trafficking as well as the cell lipidome are regulated in response to various treatments. First, we have elucidated some of the changes that occur in cellular lipid composition during cell crowding in culture, and have suggested a potential link between these changes and the effects on intracellular transport of Shiga toxin. Next, two glucose analogues, 2-deoxy-D-glucose and fluorodeoxyglucose, have been investigated for their potential effects on cellular lipids and intracellular trafficking. We have revealed new effects of these drugs on glycosphingolipid metabolism, as well as on intracellular transport of Shiga toxin. Importantly, our data suggest that these drugs might have novel potential applications both in research and in the clinic.

Introduction

Cell membranes are composed of both proteins and lipids. However, the lipids were long neglected because of lack of adequate methods to analyze them and probe their role in cellular functions. But now with the introduction of new methodologies, such as lipid analysis using mass spectrometry (MS), single molecule tracking and others, lipids are becoming more accessible and their role in cellular functions is being increasingly explored. With the exception of cholesterol, many lipids share a similar structure with having a polar head group and hydrophobic hydrocarbon chains (both phospholipids and sphingosines). Lipids are grouped into classes according to their head group and whether they are derivatives of glycerol or sphingosine. Most studies have been focused on lipid classes, but each class is made of different species of the lipids with varying acyl groups, which highly increases the diversity of the lipids. The number of molecular species of lipids present in any particular cell membrane is much greater than would be required to simply maintain a physical bilayer structure, indicating that lipids perform a wide variety of functions in the cells, in addition to being a barrier for making cellular compartments. There is still a vague understanding how the metabolism of different lipid species is regulated, and importantly, what are the functions of different species of the lipids existing in the cells. It is clear that the cell lipidome is dynamic and cellular lipid composition adapts to changing extra- and intra-cellular signals, however, there is still a huge gap in our understanding of the different signaling pathways regulating lipid metabolism. Importantly, changes in cellular lipid composition have been related to multiple human diseases including cancer, Alzheimer's disease, type 2 diabetes and allergy, among others (for review see [2, 3]). Thus, there is a demand for better knowledge of lipid metabolism and its regulation in the cellular and organism level, which would increase our understanding of disease progression and might suggest novel approaches for prevention, diagnosis and/or treatment.

The endoplasmic reticulum (ER) is the main site for lipid synthesis, but many other organelles also contribute to the generation of the lipid spectrum in cells. Some lipids, such as sphingosine and sphingosine-phosphate, can exist both in lipid and in aqueous environment allowing their distribution between different cellular compartments through the cytosol. However, most lipids are water insoluble, and the newly synthesized lipids can be distributed between cellular membranes via direct lipid transfer across contact sites

between organelles, via membrane traffic, or they are transported by specific lipid transfer proteins (some examples of different lipid transport mechanisms will be given in the section “Sphingolipids”). This ensures the establishment and maintenance of a specific lipid composition that is characteristic to a certain cell type and its variety of membranes. It is starting to be clear that lipid composition and specific distribution is tightly regulated in the cell, however, it remains poorly understood how this is achieved. In addition, there is still a lack of methods to isolate organellar membranes in high enough purity so that their lipidomes could be analyzed.

Structure and organization of cellular membranes

Cellular membranes are composed of lipids and proteins which together form a dynamic membrane bilayer where both lipids and proteins can move laterally. In addition, lipids can be flipped by specific flippases from one leaflet to the other to maintain asymmetric distribution of the lipids between the two layers of the membrane [4]. The fact that lipids are not equally distributed within the cell suggests that lipids are involved in regulating the functions of different cellular organelles. For instance, the early secretory pathway, which includes the ER, nuclear envelope and the *cis*-Golgi, is enriched in lipids with monounsaturated acyl chains and is low in cholesterol and sphingolipids. This is thought to maintain a disordered membrane phase in these organelles, which leads to more defects in the lipid packing, and thus can accommodate polypeptides with various chain lengths [5, 6]. These properties are important for the translocation and folding of diverse proteins in the ER and for their transport into the Golgi, the next station in the secretory pathway [6, 7]. In addition, lipid packing is suggested to be one of the physical membrane parameters recognized by specific protein domains. For instance, based on mutagenic studies and molecular-dynamic simulations, protein ALPS (amphipathic lipid packing sensor) motifs are suggested to sense lipid packing defects in the membranes (for review on lipid packing defects and ALPS motifs see [6, 8]). It is suggested that during membrane association the bulky hydrophobic residues of the ALPS motif become inserted into the membrane, and the overall lipid packing, rather than the presence of specific lipids, is important for such insertion [8]. The ALPS motifs have been found in proteins associated with membranes of the early secretory pathway (e.g. ArfGAP1 in the *cis*-Golgi [9]), indicating that lipid packing defects may lead to preferential association of specific proteins with these membranes.

The late secretory pathway, *trans*-Golgi network (TGN), endosomes and plasma membrane, is characterized by lipids that form more tightly packed and ordered bilayers. In general, there is a gradient of increased amounts of lipids with saturated hydrocarbon chains towards the plasma membrane, and negatively charged phosphatidylserine (PS), phosphoinositides (PIPs), cholesterol and sphingolipids are enriched in the plasma membrane [6]. Furthermore, lipids are not equally distributed between the two lipid layers, with very clear differences observed in the plasma membrane and, to some extent, in all other cellular membranes. For example, PS and PI(4,5)P_2 (phosphatidylinositol 4,5-biphosphate) are primarily located to the cytosolic leaflet of the membrane [10-12], while the majority of complex sphingolipds are at the outer leaflet [13, 14]. In addition, most glycerolipids have saturated or monounsaturated fatty acyl chains of 16 and 18 carbon atoms, and the ones with 20 and more carbons are polyunsaturated and may not reach into the other lipid bilayer (cross the middle line between the two lipid leaflets) [5]. In contrast, sphingolipids often have long saturated fatty acyl chains which may allow interdigitation between the two layers of the membrane [15], and they may thus contribute to signal transduction across the membrane.

Proteins and lipids inevitably interact in the membranes, and it is now clear that lipids modulate protein functions. Thus, to understand the organization and functions of the cellular membranes it is necessary to study not only proteins, but also lipids and how they interact with each other. There is a growing awareness of the role of specific molecular species of lipids that are involved in intracellular transport, cell cycle control and cell-cell interactions, among other cellular processes (for review see [16]). For example, sphingomyelin (sphingomyelin and other sphingolipids will be introduced in the next section) has been found to regulate retrograde transport via direct interaction with one of the proteins in the transport machinery. The COP I (coat protein I) vesicles transport cargo retrogradely within the Golgi and from the *cis* end of the Golgi to the ER (anterograde protein transport is associated with the COP II complex). First, it was discovered that isolated COP I vesicles were enriched in sphingomyelin 18:0 [17]. Later, by using Förster resosnance energy transfer (FRET) Contreras and coworkers revealed that the COP I machinery protein p24 directly and specifically interacts with a fluorescently labeled analogue of endogenous sphingomyelin 18:0 (pentaenoyl-sphingolipid SM18:5) [18]. Binding of SM18:5 induced p24 dimerization and affected COP I-dependent protein transport. The interaction was found to depend on both the headgroup and the backbone of

the sphingolipid, and on a signature sequence in a transmembrane domain of p24. It is now suggested that sphingomyelin regulates the equilibrium between an inactive monomeric and an active oligomeric state of the p24 protein, which in turn regulates COP I-dependent transport.

Sphingolipids

Sphingolipids are a diverse group of lipids containing a long chain aliphatic base, sphingoid (e.g. sphingosine). Ceramide is the N-amidated form of sphingosine, and is the key intermediate molecule for the synthesis of complex sphingolipids. Ceramide can be synthesised *de novo* from serine and fatty acids – fatty acids enter the reaction bound to coenzyme A (FA-CoA) (Fig. 1). Ceramide can also be rapidly formed by the breakdown of more complex sphingolipids, such as sphingomyelin (SM).

Sphingolipid biosynthesis

De novo synthesis of ceramide is thought to occur on the cytosolic side of the ER. However, some experimental data is still missing to clearly show that all of the reactions in the ceramide biosynthesis take place at the cytosolic side of the ER. The active site of the serine palmitoyltransferase, which catalyzes the first reaction in the *de novo* synthesis of ceramide (Fig. 1), has been shown to face the cytosolic side of the ER [19, 20]. Similar localization has also been shown for 3-ketosphinganine reductase [21] and dihydroceramide desaturase [22, 23]. However, ceramide synthase family enzymes (CerS1-6 in mammals), which produce dihydroceramide (Fig. 1), have been shown to have functionally essential amino acid residues on both sides of the ER membrane [24], raising the question of whether the synthesis of dihydroceramide takes place on the cytosolic or luminal side of the ER membrane. Importantly, each CerS has a different FA-CoA preference in the synthesis of (dihydro)ceramide (CerS can also acylate sphingosine that is produced through the degradation of sphingolipids), and the expression of the six mammalian CerS is tissue-dependent [25], indicating that different species of ceramide (and sphingolipids) have important pathophysiological functions.

More complex sphingolipids (except for galactosylceramide (GalCer)) are synthesised in the Golgi, and thus ceramide needs to be transported between the ER and the Golgi (Fig. 2). Two distinct pathways have been revealed for ceramide transport from the ER to the

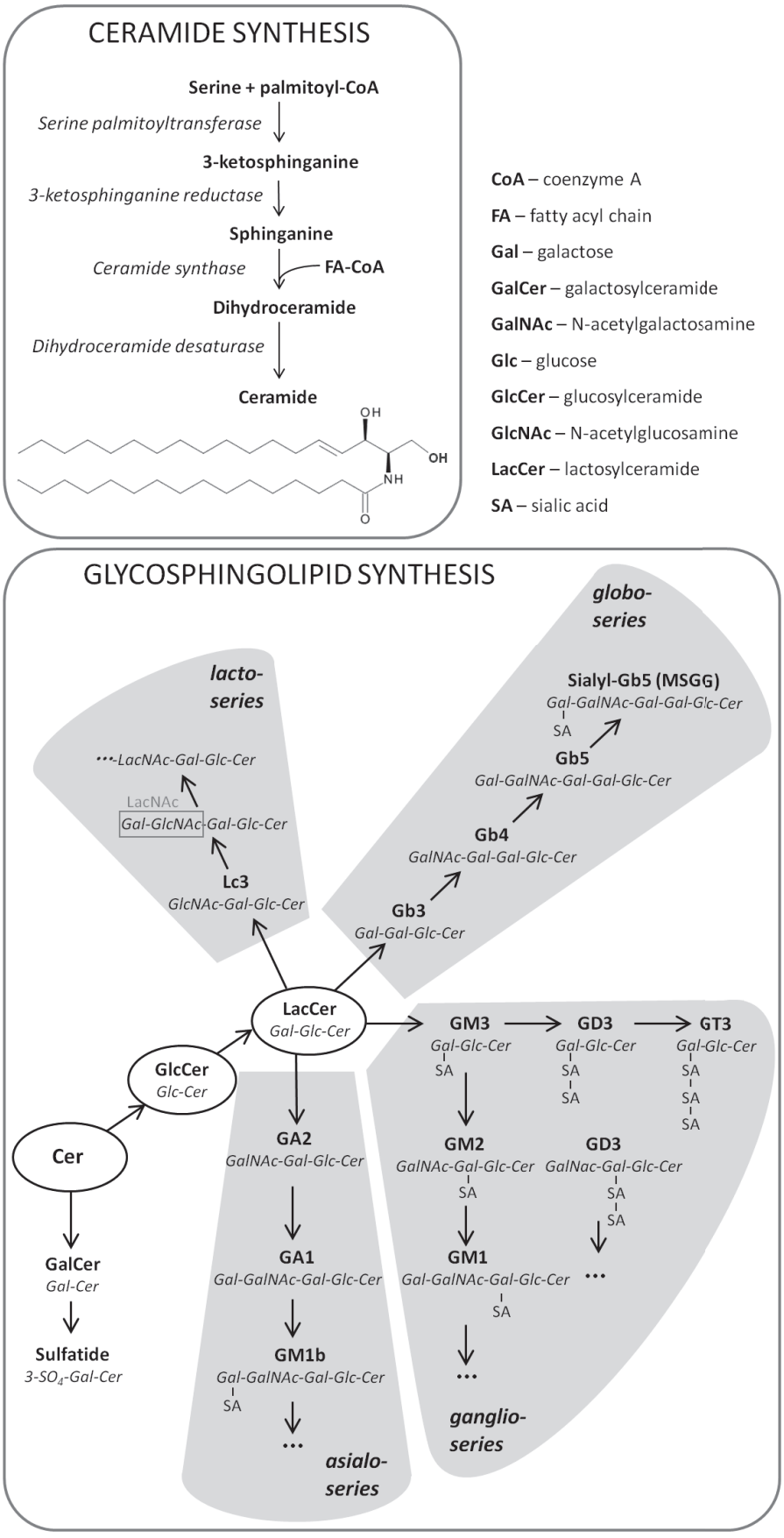

Figure 1. ***De novo*** **biosynthesis of ceramide and glycosphingolipids** (figure legend on the next page).

Figure 1. *De novo* biosynthesis of ceramide and glycosphingolipids. (Top panel) During the first two reactions, sphinganine is synthesised from serine and palmitic acid (C16:0). Sphinganine is then N-acylated by FA-CoA to form dihydroceramide. Finally, the sphinganine part of the dihydroceramide is desaturated in the position 4,5 and ceramide is formed. More complex sphingolipids are synthesised by the addition of head groups such as carbohydrates (glycosphingolipids) or phosphocholine (sphingomyelin) to the ceramide backbone. (Bottom panel) Glucosylceramide (GlcCer) is used as a precursor for the synthesis of majority of glycosphingolipids (GSLs) in the cell, while galactosylceramide (GalCer) is metabolised only to a few GSLs, such as sulfatide [26]. Based on the core structure of the carbohydrate chain, lactosylceramide (LacCer)-originated GSLs are subdivided into four subclasses: globo-, lacto-, ganglio- and asialo-series GSLs [27, 28].

Golgi, one that is ATP- and cytosol-dependent (non-vesicular) and another that is ATP- and cytosol-independent (vesicular). The ATP-dependent pathway is predominantly used for SM biosynthesis and is mediated predominantly via the ceramide transfer protein CERT [29, 30], while the *de novo* glycosphingolipid (GSL) synthesis in the Golgi has been suggested to be CERT-transport independent [30]. CERT-independent ceramide transport from the ER to the Golgi is much less understood. It is thought to be mediated via vesicular transport from the ER to the Golgi, but the ER-Golgi contact sites might also be used to transfer ceramide from ER to the Golgi. The latter suggestion has been based on the discovery that an ER resident protein, reticulon-1C (RTN-1C), interacts with glucosylceramide (GlcCer) synthase (GCS) and promotes GCS activity [31], most likely via formation of the ER-Golgi membrane contact sites.

Sphingolipids and membrane organization

Sphingolipids have longer and more saturated hydrocarbon chains than other membrane lipids. The most abundant sphingolipid species have often C24:0 and C24:1 N-amidated fatty acids [32-34] while in glycerolipids, most fatty acyl chains contain 16 or 18 carbon atoms with none or with a single double bond, and the existing longer fatty acyl chains are often polyunsaturated [5, 16]. It has been suggested that sphingolipids accumulate in specialized transient membrane domains called lipid rafts. The lipid raft concept was originally formulated by Simons and Ikonen [35] and suggested that cell membranes are segregated into cholesterol- and sphingolipid-rich domains which function as sorting and signalling platforms in the membrane. In this model, lipid segregation into such ‘raft’ domains is driven by the intrinsic property of different lipids to partition into liquid ordered and liquid disordered membrane phases [36]. However, the existence and the nature of lipid rafts are still debated. Initially, most of the studies on lipid rafts were based on membrane domain extraction using detergents on and/or labelling with multivalent ligands,

which may cause redistribution and clustering of specific lipids. Further, the partitioning of lipids into different phases was mainly studied in artificial membranes which often lack the asymmetry and the diversity of lipids and proteins present in cellular membranes. The controversies in the field have inspired new efforts and advanced microscopy techniques, which can go beyond the diffraction limit, and improved lipidome analysis have been employed in the field [37-40]. In a recent study, by using single-fluorescent-molecule imaging and novel fluorescent ganglioside analogues, Komura and coworkers showed that GM1 and GM3 were continuously and transiently interacting with the glycosylphosphatidylinositol (GPI)-anchored protein CD59 (and with the clusters of CD59) in the plasma membrane [41]. The interaction between the gangliosides and CD59 was found to be dependent on cholesterol and the GPI-anchor, thus providing strong evidence for the existence of lipid rafts in cellular membranes. In addition, although supporting a general view that raft domains are enriched in gangliosides, this study shows that there is a constant and highly dynamic exchange of the ganglioside molecules between the raft domains and the rest of the membrane. Thus, there now seems to be a general consensus that nano-scale and highly dynamic (lifetime in a range of tens and hundreds of milliseconds) assemblies exist in cellular membranes, although the exact nature and organization of lipid rafts are yet to be understood.

Glycosphingolipids

Glycosphingolipids (GSLs) share a common backbone structure (ceramide) with other sphingolipids, but are distinguished from the rest by carbohydrates attached to the terminal hydroxyl group of the ceramide. The first sugar residue that is attached to the ceramide during GSL biosynthesis is either glucose or galactose. The galactosylation and glycosylation of ceramide take place in two different compartments of the cell: GalCer is synthesized on the luminal side of the ER membrane [42], while GlcCer is synthesized on the cytosolic surface of the Golgi membrane [43] (Fig. 2). GlcCer serves as a precursor for majority of GSLs, except for GalCer-derived glycolipids (GalCer synthesis is restricted to oligodendrocytes, Schwann cells, kidneys and testis) (for review see [26, 28]). GlcCer is synthesized by GCS (full name: UDP-glucose:ceramide glucosyltransferase), which transfers a glucose residue from UDP-glucose to ceramide [44]. Based on structural analysis of the enzyme, GlcCer synthesis is suggested to occur on the cytosolic leaflet of the Golgi membrane [43]. The synthesis of more complex glycosphingolipids occurs on

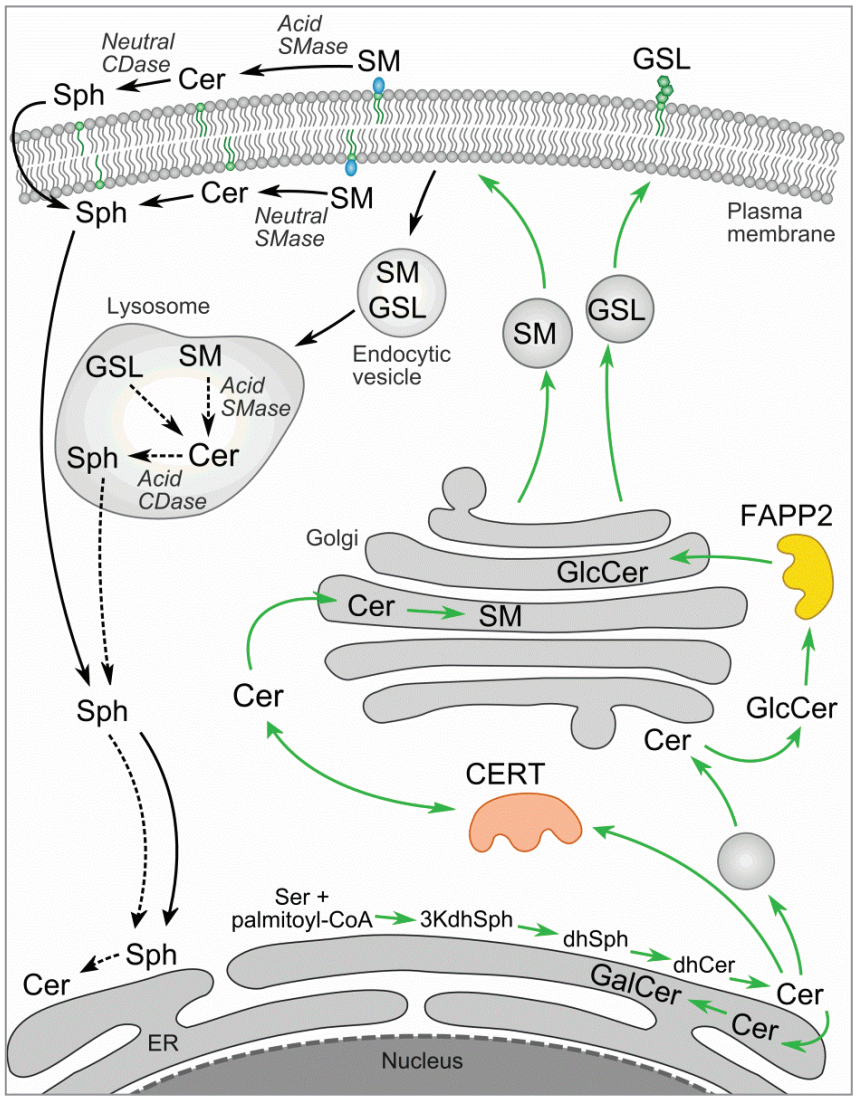

Figure 2. Sphingolipid metabolism. *De novo* synthetic pathway of sphingolipids is depicted by green arrows. *De novo* synthesis of ceramide (**Cer**) occurs on the cytosolic side of the endoplasmic reticulum (**ER**). The synthesis of galactosylceramide (**GalCer)** takes place on the luminal side of the ER membrane [42], while glucosylceramide (**GlcCer**) is synthesized on the cytosolic surface of the Golgi membrane [43]. The newly synthesised Cer is transported to the Golgi by either (i) ATP- and cytosol-dependent (non-vesicular transport), or (ii) ATP- and cytosol-independent transport (vesicular transport, or possibly via ER-Golgi contact sites, not shown). The ATP-dependent transport of Cer is mediated by ceramide transfer protein **CERT** which specifically delivers Cer for the synthesis of sphingomyelin (**SM**) [29, 30]. The Cer transported by vesicles (or possibly via ER-Golgi contact sites) enters glycosphingolipid (**GSL**) synthesis pathway. GlcCer is synthesised on the cytosolic side of the Golgi membrane and transferred to distal Golgi compartments by the flow of Golgi membrane (vesicular transport/cisternal maturation; not shown), or by non-vesicular transport mediated by protein **FAPP2** [45, 46]. Further synthesis of GSLs takes place in the lumen of the distal Golgi compartments. Delivery of Golgi-synthesised SM and complex GSLs to the plasma membrane occurs by vesicular transport. New Cer may also be generated from SM by the action of acid and neutral sphingomyelinases (**SMase**) present in the outer and inner leaflet of the plasma membrane, respectively. SM and GSLs can be endocytosed and reach a lysosomal compartment, where they are degraded by SMase and glucosidases to form Cer. Finally, Cer is hydrolysed by acid ceramidase (**CDase**) to form sphingosine (**Sph**). Sph may be salvaged from lysosomal degradation and recycled back to ER for synthesis of Cer [47]. The salvage pathway is depicted in dashed arrows. The figure is adapted from [47].

the luminal side of the Golgi membrane [48], therefore GlcCer needs to be flipped from the outer into the inner leaflet of the Golgi membrane. However, the mechanism underlying the transbilayer movement of GlcCer and other sphingolipids is not yet understood. On the luminal side of the Golgi membrane, GlcCer is converted to lactosylceramide (LacCer) by the addition of one galactose residue [48], and LacCer is then further glycosylated to more complex GSLs (Fig. 2).

It has been demonstrated that LacCer synthase physically interacts with both GM3 synthase (lactosylceramide α-2,3-sialyltransferase) and Gb3 synthase (lactosylceramide α-1,4-galactosyltransferase), resulting in the relocation of LacCer synthase to different Golgi subregions, depending on the biosynthetic pathway involved [49-51]. GlcCer transport in the Golgi is mediated by two independent mechanisms, either by vesicular trafficking or by the action of the transfer protein FAPP2 [46] (Fig. 2). It has been found that GlcCer that is progressively transported through the Golgi stacks by the vesicular transport is preferentially used by the LacCer synthase/GM3 synthase complex and thus fuels the ganglioside synthetic pathway. On the other hand, GlcCer that is transported directly to the TGN by the action of FAPP2 is used by the TGN-localized LacCer synthase/Gb3 synthase complex and thus fuels the globoside synthetic pathway [46]. Thus, FAPP2 may also act as a regulator of GSL synthesis by modulating the availability of GlcCer for the synthesis of globo- and ganglio- series GSLs.

Functions of glycosphingolipids

Since the discovery of the most common GSLs such as GlcCer, GalCer, sulfatide, LacCer and brain gangliosides in 1960s, extensive effort has been made to understand their role in the context of a single cell and that of a whole organism [27]. GSLs have been found to interact with specific functional proteins such as integrins, growth factor receptors and to be important for the localisation of signal transducers (e.g. Src family kinases [52]). Moreover, GSLs have been discovered to be involved in cell adhesion, signal transduction, phenotype determination, growth, motility and differentiation [27]. Some examples on how these processes depend on GSLs are mentioned below.

A concept of “glycosynapse”, a membrane microdomain involved in carbohydrate-dependent cell adhesion and signal transduction events, was introduced by Hakomori in 2002, emphasising the role of GSLs in signalling across the cellular membrane [53]. The

concept is based on numerous examples of positive and negative effects on signal transduction which are described for specific GSLs, e.g. (i) ganglioside GM3 interacts with the extracellular part of the epidermal growth factor receptor (EGFR) and inhibits tyrosine phosphorylation without affecting binding of the ligand [54], (ii) binding of Shiga toxin to its receptor, globotriaosylceramide (Gb3), activates Syk kinase and induces signalling which facilitates endocytosis of the toxin [55]. The latter has recently been found to depend on the multivalent cross-linking of the glycosphingolipid at the plasma membrane, which leads to increase in cytosolic calcium levels (most likely via affecting calcium channels at the plasma membrane) and thus mediates intracellular signalling events [56]. The mechanism by which ligand-mediated clustering of the glycosphingolipids initiates intracellular signalling and trafficking events is under investigation in our group at the moment.

The expression of certain GSLs is strictly regulated during embryonic development suggesting their implication in determining cell fate [57]. *In vivo* studies on genetically engineered mice with disrupted genes for specific glycosyl-transferases have revealed that GlcCer, the precursor for majority of complex GSLs, is essential for embryonic development and cellular differentiation. Offspring lacking active GlcCer synthase showed embryonic lethality at a very early stage [58, 59]. Similarly, ablation of the active LacCer synthase also resulted in growth inhibition and embryonic lethality in mice [60]. Taken together these data indicate that GSLs synthesised downstream of GlcCer are collectively required for correct embryonic development. On the other hand, the knock-out of individual glycosyl-transferases involved in synthesis of more complex GSLs displays less profound or no effects in mouse models suggesting functional subdivision among complex GSLs: the functional role of the missing GSL may be taken over by existing GSLs and therefore no well-defined changes may be observed [61-63]. Surprisingly, knock-out mice lacking globo-series GSLs do not have any overt phenotypes, while ablation of either lacto-, ganglio-, asialo- or gala-series GSLs results in specific physiological phenotypes (for review see [61] and [62]). So far, physiological and cellular functions of globo- series GSLs are least understood in healthy organism.

Globotriaosylceramide (Gb3)

Globotriaosylceramide (Gal-α1→4Gal-β1→4Glc-β1→Cer, Gb3) is the first product in the synthetic pathway of globo-series GSLs (Fig. 1). Gb3 is synthesized from LacCer by the

addition of one galactose residue, and the reaction is catalysed by Gb3 synthase (lactosylceramide α-1,4-galactosyltransferase) (Fig. 3). While the sphingosine chain in the ceramide part of Gb3 is relatively invariable (most often it is monounsaturated with 18 carbon atoms, d18:1), the N-amidated fatty acyl chain varies both in length and saturation resulting in multiple Gb3 species. Importantly, the receptor function of Gb3 has been shown to depend on its species composition [64-67].

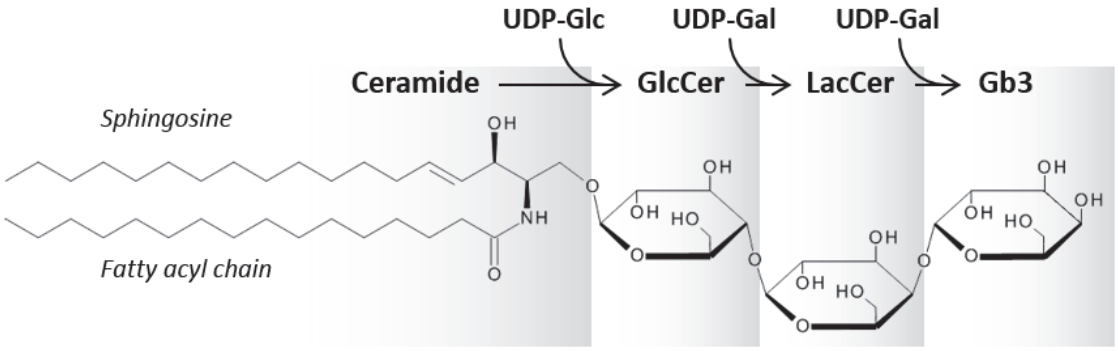

Figure 3. Chemical structure and biosynthesis of globotriaosylceramide (Gb3). Sphingosine most often contains 18 carbon atoms, whereas the fatty acyl chain of ceramide varies both in length and saturation (here shown as C16:0). Gb3 is synthesised from LacCer by the addition of one galactose, and the reaction is catalysed by Gb3 synthase (lactosylceramide α-1,4-galactosyltransferase). The sugar chain for Gb3 is: Gal-α1→4Gal-β1→4Glc-β1→(Cer). The bond α1→4Gal at the core of the sugar chain is a distinct characteristic to all globo-series GSLs.

In the human body, the expression of Gb3 is restricted to only several tissues. Normally, the highest levels of Gb3 are found in the kidney epithelium and endothelium [68-70], in microvascular endothelial cells [71, 72] and in platelets [73]. In the carbohydrate defined P histo-blood group system, Gb3 constitutes the rare P^k antigen present on the erythrocytes [74]. In the immune system, Gb3 represents a lymphocyte differentiation antigen, termed CD77, which is expressed in a subset of germinal centre B lymphocytes [75]. However, the physiological role of Gb3 is still unclear, and it is not known why Gb3 expression is restricted to certain tissues. *In vivo* studies on Gb3 synthase knock-out mice, which displayed a total loss of Gb3 and other globo-series GSLs, showed no changes in birth-rates and no apparent abnormalities over a year of nurturing, with the exception of total loss of sensitivity to Shiga-like toxins as compared to wild-type mice [76]. On the other hand, elevated levels of Gb3 have been associated with several conditions including Fabry disease [77, 78] and a number of cancers [79], such as colon carcinoma [80-82], B cell lymphomas [83-86] and breast cancer [86, 87], among others.

Gb3 serves as a receptor for plant and bacterial protein toxins, such as ricin, Shiga toxin and Shiga-like toxins. Naturally, toxins cause damage to cells and thus lead to occurrence of disease; however, the toxins can also be employed for research and diagnosis/therapy in medicine. The toxins, used in this study and their potential applications are discussed in the last section of the Introduction.

Interestingly, binding of different ligands to Gb3 may trigger different signalling pathways in the cells. It has been shown that the binding of anti-Gb3/CD77 monoclonal antiobodies (mAb) and Shiga-like toxin 1 to Gb3 induces apoptosis in Burkitt's lymphoma cells. However, the induction of apoptosis by these two ligands differs mechanistically: Shiga-like toxin 1 triggers a caspase and mitochondria-dependent apoptotic pathway, while binding of anti-Gb3/CD77 mAb induces caspase-independent and oxidative stress-dependent signalling for apoptosis [88]. This indicates that Gb3 may be involved in several cellular events and a more comprehensive understanding of its functions is needed. In addition, the receptor function of Gb3 is shown to depend on the membrane environment and the composition of Gb3 (different fatty acyl chains in the ceramide part). Examples of how these factors affect Gb3 recognition by Stx are given in the section "Factors regulating Shiga toxin binding and intracellular transport".

Glycosphingolipids and cancer

Changes in the glycosylation pattern are observed in all human cancer types and very often relate to their invasiveness and metastasis: high expression of some glycosyl epitopes correlates with shorter patient survival rates, while other glycosyl epitopes suppress tumour progression and lead to higher survival rates following anti-cancer treatment (for review see [89]). Aberrant glycosylation may occur both in glycoproteins and glycolipids; however, this chapter is focused on aberrant glycosylation in GSLs. Next, several GSLs are discussed in relation to cancer.

Cancer related alterations in GSLs may occur in carbohydrate structure [90] and/or the composition of the ceramide [91]. They can also manifest themselves as up- or down-regulation of certain GSLs. Several GSLs are characterized as human tumour-associated antigens. For example, as mentioned in the previous section, Gb3 has been found to be characteristic for several types of B cell lymphomas [83-86], colon carcinoma [80-82],

breast cancer [86, 87], testicular cancer [92] and acute non-lymphatic leukemia [93], while over-expression of GD3 is characteristic to human melanomas [94, 95].

Other interesting examples are gangliosides GM3 and GD3. GM3 and GD3 constitute the major GSLs expressed during embryonic brain development [96, 97]. During brain maturation, GM3 and GD3 concentrations diminish considerably and more complex gangliosides become predominantly synthesised [96]. However, during malignant transformation, neural cells regain high expression of GM3 and GD3 [98]. On the other hand, metastatic forms of bladder tumours were found to have lower levels of GM3 compared to benign tumours [99]. Moreover, a correlation between virus-induced oncogenic transformation and lowered levels of GM3 has been demonstrated in several cell cultures [100, 101]. Possibly, the ratio between different GSLs might be a more important determinant in the disease progression than up- or down- regulation of only a single GSL.

Increased levels of GCS have been found to be elevated in several human cancers, and importantly, to correlate with cancer multidrug resistance and poor prognosis in cancer patients [102-105]. In agreement, increased levels of GCS have been recognised as a cause of drug resistance in multiple cancer cell lines derived from human breast, ovarian, colon and cervical cancer and leukemia (for review see [106]). Although the understanding of how cancer cells acquire drug resistance during the course of chemotherapy remains incomplete, several studies suggest that ceramide, which is generated in cells exposed to anticancer drugs, actively participates in modulating the expression of genes that contribute to drug resistance. For example, treatment with the anti-cancer drug doxorubicin has been shown to result in increased cellular levels of ceramide and amplified expression of GlcCer synthase gene (*GCS*) in human breast adenocarcinoma (MCF-7) cells [102]. It was also shown that the transcription factor Sp1 was essential for the ceramide-induced upregulation of *GCS* and that the inhibition of ceramide synthesis by fumonisin B1 prevented the transactivation of *GCS* expression by doxorubicin. Moreover, exogeniously added C6-ceramide or sphingomyelinase (SMase) had similar inductive effect on *GCS* expression as doxorubicin [102]. Therefore, it has been suggested that ceramide generated upon doxorubicin treatment activates Sp1, which in turn activates the *GCS* promoter and thus leads to increased levels of GCS. In a later study, Liu *et al.* has also demonstrated that globo-series GSLs, which are synthesised downstream of GlcCer, upregulate expression of multidrug resistance gene 1 (*MDR1*) [107]. *MDR1* encodes ABCB1 transporter P-

glycoprotein (P-gp) which actively exports a wide variety of substrates, including anticancer drugs, out of the cell and thus is an important mediator of acquired drug resistance in cancer [108]. The increase in the levels of ceramide and GCS will consequently increase the concentrations of certain GSLs and thus will alter lipid-lipid and lipid-protein interactions. Liu *et al.* has suggested that it is an increase in the levels of certain globo-series GSLs (in particular Gb3 and Gb5) that activates cytosolic Src kinases, increases β-catenin by diminishing its degradation after phosphorylation, and thus transactivates *MDR1* expression [107]. Taken together, these two studies suggest the mechanism how ceramide and globo-series GSLs may confer to cancer cell resistance: (i) ceramide-induced apoptosis is prevented by rapid consumption of ceramide by GCS for the synthesis of GSLs and (ii) there is an increased drug efflux mediated via GSL-dependent upregulation of *MDR1*.

Glucose analogues and their cellular effects

It is postulated that malignant transformation of cells involves upregulation of glucose uptake and metabolism (Warburg effect), which opens a selectivity window for cancer diagnosis and treatment by glucose analogues [109-111]. Two glucose analogues, 2-deoxy-D-glucose and 2-fluoro-2-deoxy-D-glucose, which were used in this study, are described in this section with the main focus on their cellular effects.

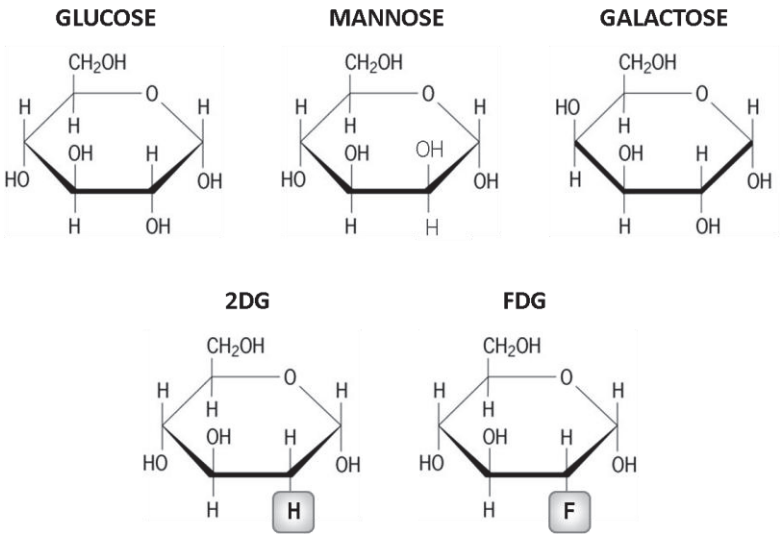

Figure 4. Chemical structure of glucose, mannose, galactose and glucose analogues 2DG and FDG. The hydroxyl group that is present at the second carbon in glucose is substituted with hydrogen and fluorine atoms in 2DG and FDG, respectively (highlighted with the box).

2-Deoxy-D-glucose (2DG)

2-Deoxy-D-glucose (2DG) is a structural analogue of glucose differing from glucose only by the absence of one oxygen atom at the second carbon (Fig. 4). In cells, 2DG becomes phosphorylated [112, 113] and inhibits glycolysis by competing with glucose-6-P for phosphoglucose isomerase [114], and by acting as a non-competitive inhibitor of hexokinase [115]. However, although the inhibition of glycolysis has been a commonly exploited effect of 2DG, the drug has a much broader spectrum of activities.

In addition to inhibiting glycolysis, 2DG inhibits N-linked protein glycosylation [116, 117]. During protein N-glycosylation, an oligosaccharide is first assembled on a lipid carrier, dolichol pyrophosphate, and then transferred onto an acceptor protein. The first sugar residues added onto the dolichol pyrophosphate are two N-acetylglucosamine (GlcNAc) and five mannose residues, before the dolichol-linked oligosaccharide is flipped from the cytosolic to the luminal side of the ER where more sugar residues are added [118]. Structurally, 2DG resembles mannose (Fig. 4), and it has been shown to become incorporated into dolichol-linked oligosaccharides (most likely at the beginning of the oligosaccharide, where mannose residues are normally incorporated) [116, 119]. The addition of 2DG terminates the elongation of the oligosaccharide leading to formation of shortened oligosaccharides. Such immature 2DG-containing oligosaccharides were shown to fail to be transferred onto the proteins [116]. In turn, this leads to accumulation of misfolded proteins and thus triggers the unfolded protein response (UPR) in the ER, leading to ER stress [120, 121].

It has been reported that 2DG induces autophagy, predominantly via ER stress rather than via inhibition of glycolysis [122, 123]. Thus, ER stress is now recognized as the main mechanism by which 2DG induces autophagy [122, 123] and apoptosis [124, 125]. In addition, 2DG has been shown to interfere with cell cycle control [126] and DNA repair [127], and not always by mechanisms dependent on the inhibition of glycolysis or N-linked protein glycosylation [128]. Of particular interest to this study, was the discovery made by Okuda and coworkers showing that 2DG inhibits the expression of the Gb3 synthase and thus reduces cellular Gb3 levels in the cells [129]. Although 2DG has previously been demonstrated to have a repressive effect on gene expression mediated indirectly by inhibition of glycolysis [130], activation of the class III histone deacetylase SIRT1 [131] or

by enhanced *O*-GlcNAc modification of transcriptional factor Sp1 [132], none of these mechanism were found to be involved in the inhibition of Gb3 synthase [129].

2-Fluoro-2-deoxy-D-glucose (FDG)

2-Fluoro-2-deoxy-D-glucose (FDG) is a structural analogue of glucose where the hydroxyl group at the second carbon is replaced by a fluorine atom (Fig 4). Like glucose and 2DG, FDG is transported into cells, where it is phosphorylated by hexokinase to yield FDG-6-P. However, FDG-6-P does not undergo isomerization to fructose and thus cannot be further catabolised, leading to accumulation of FDG-6-P in the cells [133]. Similarly to 2DG, FDG also inhibits glycolysis by (i) competing with glucose-6-P for phosphoglucose isomerase, and by (ii) acting as a non-competitive inhibitor of hexokinase [114, 115, 134]. The binding energy of FDG-6-P for the allosteric site of the hexokinase is lower than that of 2DG-6-P, and closely resembles the energy of glucose-6-P, making it a better inhibitor of glycolysis than 2DG [134]. As a consequence, FDG is more efficient than 2DG in killing hypoxic cells [134].

FDG also interferes with N-linked protein glycosylation [116, 135, 136]. FDG is converted to GDP-FDG and UDP-FDG in cells [136], but it does not compete with UDP-GlcNAc or GDP-mannose for addition onto dolichol-linked oligosaccharides [135]. Thus, FDG has been suggested to interfere with N-glycosylation by (i) competing with mannose and glucose for the formation of GDP-mannose and UDP-glucose, and (ii) the nucleotide diphosphate-linked FDG is suggested to inhibit the addition of carbohydrates from GDP-mannose and UDP-glucose onto dolichol, leading to slower assembly of the dolichol-linked oligosaccharides [133, 135]. In contrast to 2DG, FDG does not become incorporated into dolichol-linked oligosaccharides [135], and thus seems to slow down rather than to prevent the assembly of the oligosaccharide, and thus is a weaker inhibitor of N-glycosylation than 2DG.

[^{18}F]FDG with incorporated ^{18}F radioisotope is a widely employed imaging agent for positron emission tomography (PET). [^{18}F]FDG based PET is used for diagnosis of oncological, neurological and cardiological diseases, with the widest application in oncology where [^{18}F]FDG-PET has been established as a common technique used for staging and monitoring multiple cancers (for review see [111, 137]). The use of [^{18}F]FDG-PET in oncology is based on increased accumulation of FDG in tumor cells. The basis for

specific accumulation of [^{18}F]FDG in tumor cells derives from a number of factors, which include, but might not be limited to, (i) upregulation of glucose transporters (in tumor cells) [138-140], (ii) increased activity of hexokinases [141, 142] and (iii) reduced activity of glucose-6-phosphatases [142, 143], leading to a rapid phosphorylation and slow dephosphorylation of [^{18}F]FDG, thereby trapping [^{18}F]FDG-6-P inside the cell.

Protein toxins

Protein toxins are produced by certain bacteria and plants, and function as autonomous killing devices which target specific cells and modify specific intracellular components. Protein toxins vary in their structure, receptor (and therefore act on different cells), their intracellular target and the mechanism of intoxication. In this study, four different protein toxins, Shiga toxin, Shiga-like toxin 2, diphtheria toxin and ricin, were employed and they are therefore discussed in this chapter. The last part of the chapter is dedicated to a short review on protein toxin applications in research and medicine.

Shiga toxins

Shiga toxins comprise a family of related protein toxins which are similar in their structure and the mechanism of action, but are produced by different types of bacteria. Shiga toxin (Stx) is secreted by *Shigella dysenteriae*, whereas Shiga-like toxin 1 (Stx1) and Shiga-like toxin 2 (Stx2) are produced by certain strains of *Escherichia coli* (Shiga toxin-producing *E.coli*, STEC) and some other bacteria. Stx1 differs from Stx only in one amino acid residue in the catalytic A-moiety of the toxin, whereas Stx2 shares only ~60% sequence similarity with Stx and defines an immunologically distinct subgroup comprised of at least seven variants of Stx2 [144]. Stx2 is much more lethal than Stx1 in animal models [145, 146] and is thought to be the main cause of life threatening infections in humans [147]. Gastrointestinal infection with STEC is highly dangerous to children and adolescents as it may lead to a severe complication, haemolytic uremic syndrome (HUS), which involves kidney damage [148]. However, the large outbreak with Stx2a-producing enteroaggregative *E. coli* strain in Northern Europe in 2011 demonstrated that there are bacterium-toxin combinations that can be as dangerous to adults as to children [149].

Shiga toxins belong to the AB_5 class of protein toxins and consist of an A-moiety (~32 kDa), which is non-covalently attached to a homo-pentameric B-moiety (7.7 kDa per

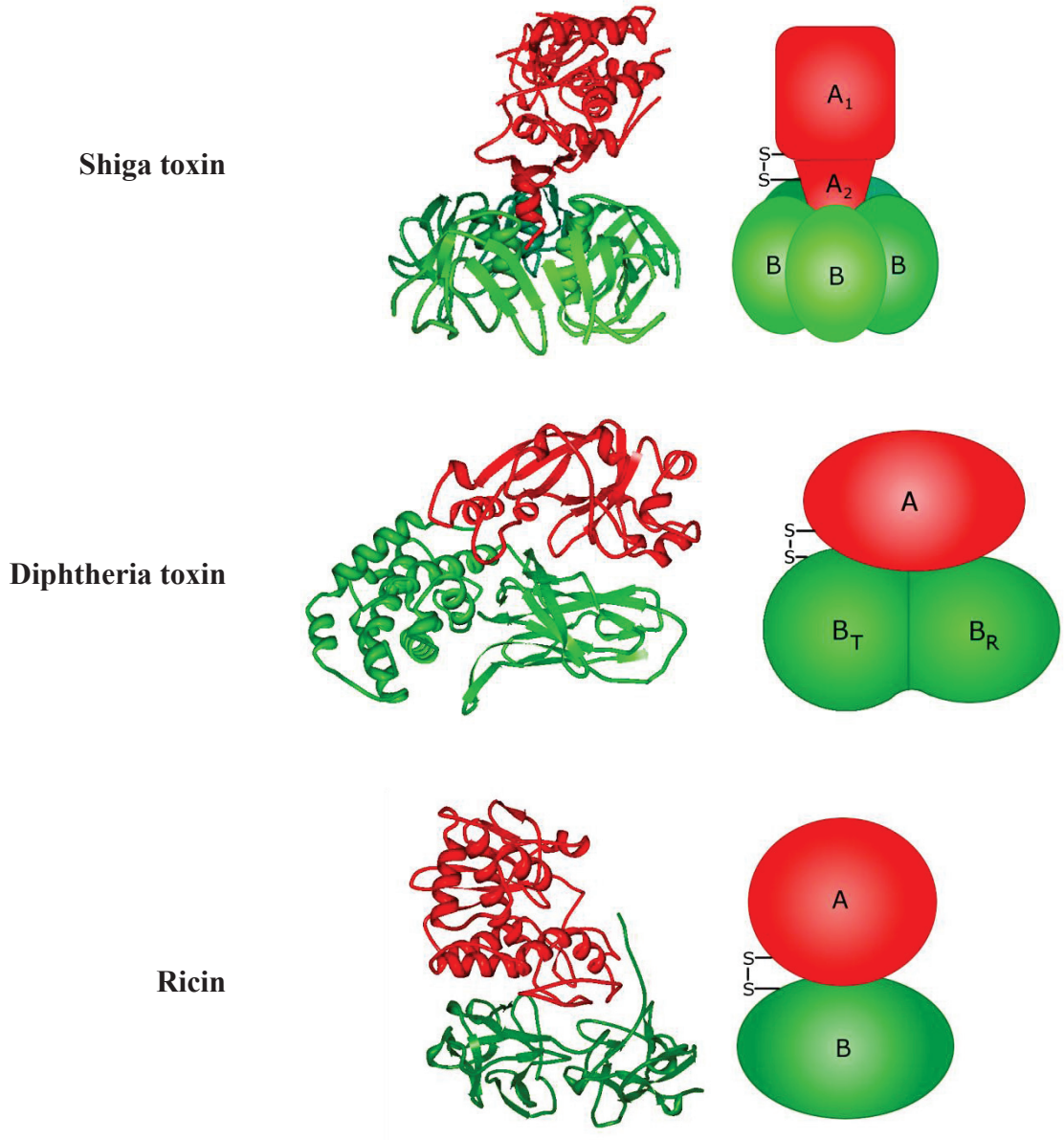

Figure 5. The structural and schematic models of Shiga toxin, diphtheria toxin and ricin. Shiga toxins consist of two non-covalently linked moieties: an A-moiety of ~32 kDa, and a B-moiety, comprised of five 7.7 kDa B-chains [150, 151]. During intracellular toxin transport, the A-moiety is cleaved by the protease furin [152] into two fragments: an enzymatically active A_1 fragment (~27 kDa) and a carboxyl terminal A_2 fragment, which remain linked by a disulfide bond until arrival to the ER [153]. Diphtheria toxin consists of an enzymatically active A-moiety (~21 kDa) and a B-moiety (~37 kDa), which has a transmembrane T-domain and a receptor binding R-domain [154, 155]. The peptide bond between the A-moiety and the T-domain is cleaved by the host proteases, but the A- and B-moieties remains linked by a disulfide bond [156]. Upon endocytosis, low endosomal pH induces conformational change leading to membrane insertion of the T-domain and translocation of the the A-moiety from the endosomes to the cytosol [157-159]. Ricin consists of an enzymatically active A-moiety and a binding B-moiety. Both moieties are of similar size (~30 kDa) and are linked together by a disulfide bridge [160, 161]. The structure images were prepared using PDB ProteinWorkshop 3.9 (PDB protein data bank: Shiga toxin, 1DM0; ricin, 2AAI; diphtheria toxin, 1F0L).

monomer) (Fig. 5) [150, 151]. Nearly all Shiga toxins bind exclusively to Gb3 [162-164] with the exception for one Stx2 variant, Stx2e, which has been shown to bind to Gb4 [165]. Each B subunit harbours three Gb3 binding sites [166], making the toxin capable of binding up to 15 Gb3 molecules on the cell surface. However, not all binding sites have equal affinity for the carbohydrates of Gb3 [167, 168] and, therefore, not all sites might be required for binding to the cell surface, but rather mediate additional recognition and membrane remodelling [168-170].

The B-moiety alone is not toxic to cells (with exception of B cells, where it may induce apoptosis [171]) and functions as a delivery tool for the enzymatically active A-moiety. Upon internalization, which may occur through clathrin-dependent and clathrin-independent endocytic pathways (Fig. 6) [144], the A-moiety is cleaved by the protease furin [152] in two fragments, A_1 and A_2 (Fig. 5), which remain linked to each other by a disulfide bridge between two cysteines until the toxin encounters reducing environment of the ER lumen. In the ER, the disulfide bond between the A_1 and A_2 subunits is reduced and the A_1-subunit is released from the toxin. Finally, the A_1-subunit is translocated across the ER membrane and inhibits protein synthesis by cleaving one adenine residue from the 28S RNA of the 60S ribosomal subunit [144]. However, the action of Shiga toxins in the cells is not limited to the inhibition of protein synthesis and other cellular responses, such as cytokine expression and apoptosis, have been shown to be triggered by the toxin (for review see [172, 173]).

Although the crystal structures of Stx1 and Stx2 share overall similarities, significant differences are found in the C-terminal end of the A-moiety. In Stx1, the amino acids 279-286 of the A-moiety form an α-helical secondary structure in the C-terminus, where it penetrates the pore formed by the B-pentamer, while the last six amino acid residues (288-293) are unstructured and therefore not seen in the crystal structure of Stx1 [151]. In Stx2, amino acid residues 278-285 also form an α-helical structure which locates in the pore of the B-pentamer. However, in contrast to Stx1, the last amino acid residues in the Stx2A C-terminus are structured (Ser289 initiates the final C-terminal α-helix) and project out of the pore in the B-pentamer [174]. Kymre and coworkers have recently shown that the truncation of 6 or 8 amino acid residues from the C-terminus of the Stx1 A-moiety impairs the stability of the toxin, and the removal of even 2 terminal amino acids greatly inhibited release of the Stx1A_1-subunit, indicating that the retrograde transport was impaired in the

truncated mutant. In contrast, Stx2 formed stable holotoxin even after truncation of 8 terminal amino acids in the C-terminus of the A-moiety, and the truncation had only a minor effect on release of the $Stx2A_1$-subunit in the ER [175]. Based on the crystal structure, the C-terminus of the Stx2 A-moiety seems to interact with one of the receptor-binding sites in the B-pentamer [174] indicating that the Stx2A structure might affect binding of the holotoxin. However, the truncation of up to 8 C-terminal amino acid residues from the A-moiety had no effect on Stx2 binding to HeLa cells [175]. Nevertheless, differences in the C-terminal end of the A-moiety and its interaction with the B-moiety might potentially explain the discrepancy in the binding preferences between Stx1 and Stx2 [66, 176, 177].

Factors regulating Shiga toxin binding and intracellular transport

The binding and intracellular transport of Stx are dependent on multiple factors, including species composition and membrane environment of Gb3, various cellular proteins and kinases, cytosolic calcium levels etc. Some of these factors and their effects on Stx binding and transport are discussed in this section.

Lipid composition of the plasma membrane (in particular cholesterol levels) and types of fatty acyl chain in the ceramide moiety of Gb3 modulate the interaction between Stx and the receptor (for review see [32, 144]). Studies based on artificial systems, where Gb3 was immobilized on thin layer chromatography (TLC) or ELISA plates, have shown that Stx has different binding affinities for different Gb3 species [66], although a mixture of various Gb3 was required for the highest binding affinity [67]. In addition, Stx1 and Stx2 have been demonstrated to have different binding affinity for Gb3 [176] as well as different preferences for specific Gb3 species [66, 177]. Stx binding to Gb3 has also been found to depend on cholesterol levels in the membrane. Cholesterol extraction with methyl-β-cyclodextrin increases Stx binding to erythrocyte membranes and sections of human kidney tissue [178], as well as to HeLa and Vero cells [179]. Based on data from molecular simulations, cholesterol induces a tilt in the carbohydrate group of Gb3 and thus affects its access for binding [178, 179]. Moreover, Gb3 might have different localizations in the membrane with a certain fraction of Gb3 being localised in lipid rafts. It is suggested that Gb3 association with such membrane microdomains is required for Stx-mediated signalling [180-182], efficient intracellular transport and cytotoxicity [183-186].

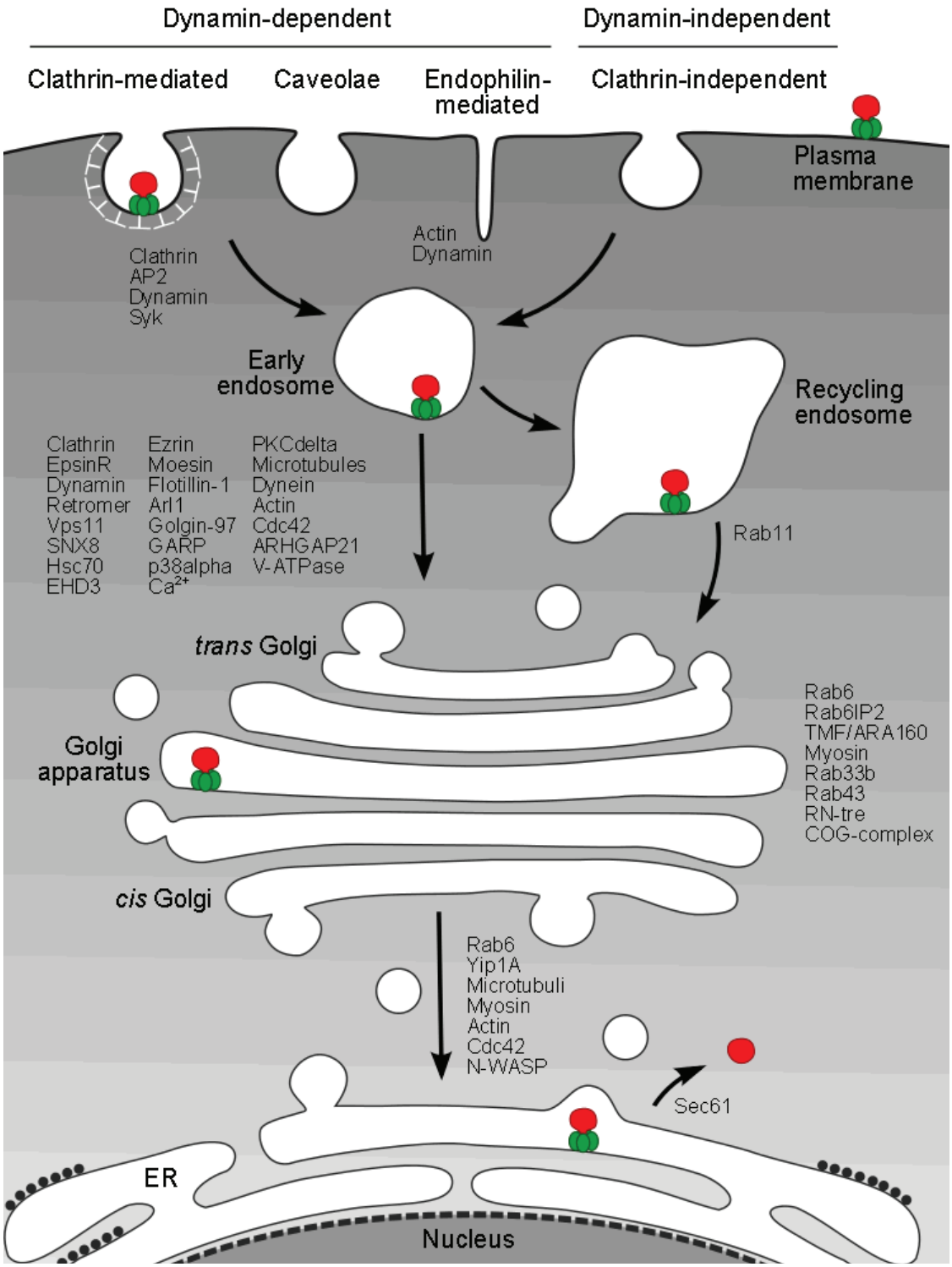

Figure 6. Endocytosis and retrograde transport of Stx. Stx binds to Gb3 on the cell surface and is taken up by various endocytic mechanisms. Following endocytosis, the toxin is transported through early endosomes and recycling endosomes and to the Golgi apparatus. From the Golgi, Stx is transported retrogradely to the ER, where its catalytically active A_1-subunit is released and translocated into the cytosol. Some of the proteins and other factors involved in the various transport steps are listed. The figure is adapted from [144].

Upon binding, the toxin has been found to activate a number of tyrosine kinases, including Syk [55], and the Src kinases Yes [180] and Lyn [181], as well as serine/threonine protein kinase Cδ (PKCδ) [187] and the mitogen-activated protein kinase (MAPK) p38α [188]. Although the exact mechanism of how Stx mediates these signalling is not yet fully understood, a recent study from our group have shown that the activation of Syk depends on the multivalent cross-linking of the Gb3 at the plasma membrane, which in turn leads to increase in cytosolic calcium levels and phosphorylation of Syk [56]. In addition, StxB binding to the cells has been shown to induce release of cytoplasmic phospholipase A2 (cPLA2) form a cPLA2-annexin A2 complex thereby facilitating Golgi transport, which has been shown to be dependent on cPLA2 [189]. Furthermore, binding of the Stx B-moiety has been reported to stimulate remodelling of cytoskeleton components, such as actin, ezrin and dynein [182, 190, 191]. Thus, Stx seems to be able to induce cell signalling and to modulate various cellular components to favour its uptake and intracellular transport.

Stx was the first lipid-binding ligand shown to be internalized by clathrin mediated endocytosis [192]. Later, it was found that Stx can utilize a variety of different endocytic mechanisms, including both clathrin- and dynamin-dependent and independent pathways (Fig. 6). In addition, a recent study by Renard *et al.* has shown that toxin crowding and localized Gb3 enrichment may bend the membrane leading to recruitment of endophilin A and thus Stx internalization through endophilin-mediated endocytosis [193].

After endocytosis, the toxin molecules are transported to the early endosomes from which a large fraction is sorted through the endolysosomal pathway and degraded, or recycled back to the plasma membrane. However, a fraction of Stx is sorted to vesicles destined for the TGN and thus enters the retrograde pathway. A coat like complex called retromer, which includes sorting nexins 1 and 2 (SNX1 and SNX2) and the proteins Vps26, Vps29 and Vps35, has been shown to be important for efficient Stx transport form endosomes to the Golgi [194-196]. In addition, Eps15 homology domain-containing (EHD) proteins EHD1 and EHD3, which are not a part of the retromer complex, are required for retromer stabilization [197, 198], and the knockdown of EHD3 has been shown to inhibit endosome-to-Golgi transport of StxB [198]. Moreover, although clathrin is involved, but not required for endocytosis of Stx, it is, however, together with dynamin and the adaptor

protein epsinR, required for the transport of the toxin from endosomes to the Golgi [199, 200].

Although a range of proteins have been recognized as mediators in retrograde Stx transport (for review see [144]) (Fig. 6), the details of how Stx is actually sorted to the Golgi remain unclear. The Gb3-bound Stx is localized on the luminal side of endosomes, and thus it is unclear how the interaction with the cytosolic sorting machinery occurs (both clathrin and the retromer complex are cytosolic and generally require interaction with the cargo or its receptor for accumulation of the cargo in the transport vesicles). Several studies have indicated that Gb3 association with lipid rafts or detergent resistant membrane domains (DRMs) [183, 184, 201, 202] as well as certain Gb3 species [203, 204] are required for efficient Stx targeting to the retrograde pathway, thus lipid-based sorting might contribute to retrograde Stx transport. In addition, in early endosomes Stx and Stx1 bind the integral membrane protein GPP130, which constitutively traffics between the Golgi apparatus and early endosomes [205, 206]. It has been shown that manganese-mediated downregulation of the GPP130 inhibits Stx transport to Golgi, and the toxin is directed to lysosomes [207]. Although the bulk part of the GPP130 protein is in the luminal side, it has a short cytoplasmic domain which might potentially interact with the cytosolic sorting machinery and thus facilitate the sorting of GPP130-Stx complex. In addition, GPP130 has a longer transmembrane domain than typical Golgi proteins which might promote its localisation in lipid rafts [205]. However, it has been discovered that Stx2 does not interact with GPP130 and its endosome-to-Golgi transport is not affect by downregulation of GPP130 [208], indicating that Stx2 has different requirements for its transport to the Golgi than Stx and Stx1.

In intracellular transport, the final stage of docking and fusion of the transport vesicles is mediated by SNAREs (soluble *N*-ethylmalemide-sensitive factor attachment protein receptors), two of which have also been shown to be involved in Stx delivery to the TGN [209, 210]. At the TGN, the tethering of vesicles is regulated by several Golgi-localized factors, such as Golgin-97 [211], tGolgin-1 (also known as *trans*-Golgi p230 or golgin-245) [212] and GARP (Golgi-associated retrograde protein) [213], which are all regulated by the ARF-like small GTPase Arl1. It has been shown that depletion of Arl1 [211], or its effector proteins Golgin-97 [211] and tGolgin-1[212], prevents transport of Stx to the TGN, indicating that several golgins are required for efficient toxin trafficking. However, several Arl1-independent Golgi factors, including components of the conserved oligomeric

Golgi (COG) complex [214, 215], have also been implicated in mediating Stx-containing vesicle tethering at the Golgi.

Upon arrival to the Golgi apparatus, Stx undergoes further retrograde transport to the ER and the nuclear membrane, and it was the first ligand shown to be transported all the way from the plasma membrane to this compartment [216]. Coat protein complex COP I is one of the most well-characterized components involved in transport within the Golgi and from the *cis*-Golgi to the ER [217]. However, Stx does not have KDEL sequence, a classical ER retention/retrieval signal, and it has been shown that Stx transport from the Golgi to the ER is independent of COP I [218], but rather regulated by the GTPase Rab6a' [218-220]. In addition, the cytoskeletal component actin [221] and an intact microtubule network [222], as well as the motor protein myosin II [223], have been identified to be necessary for proper Stx transport from the Golgi to the ER.

Once in the ER, Stx needs to translocate its catalytically active part into the cytosol to reach its target, ribosome. During the intracellular transport, the A-moiety is cleaved by the host protease furin [152] in two fragments, A_1 and A_2 (Fig. 5), which remain linked to each other by the disulfide bridge. The conditions in the ER lumen enable the reduction of the disulfide bond, thereby releasing the A_1 fragment. The translocation of the A_1 fragment across the ER membrane is believed to occur via the cellular ER-associated protein degradation (ERAD) pathway. It has been proposed that upon the release from the holotoxin, a hydrophobic C-terminal domain of the A_1 fragment is exposed and recognized as a misfolded peptide domain by the ER export machinery [224]. In agreement with this hypothesis, three ER chaperones, HEDJ (also called ERdj3), BiP (also called GRP78) and GRP94 (glucose-regulated protein of 94 kDa), have been reported to bind StxA [225], and thus are suggested to be involved in the release of $StxA_1$ in the ER. In addition, the knockdown of the translocon component Sec61B has been shown to protect cells against Stx intoxication [226], indicating that the Sec61 translocon is utilized by Stx for translocation of $StxA_1$ into the cytosol.

Diphtheria toxin

Diphtheria toxin (DT) is secreted by *Corynebacterium diphtheriae* as a single-chain polypeptide with a molecular weight of ~58 kDa. DT belongs to the AB family of toxins and it consists of two moieties: an enzymatically active A-moiety (~21 kDa), and a B-

moiety (~37 kDa), which has a transmembrane T-domain and a receptor binding R-domain (Fig. 5) [155]. In the first step of the intoxication process, DT binds, via its R-domain, to the heparin-binding epidermal growth factor precursor (pro-HB-EGF) [227] and undergoes receptor-mediated endocytosis in a dynamin-dependent manner [228]. During this step, DT is cleaved by the protease furin, but the A- and B-moieties are kept together by the disulfide bridge [156]. During the transport along the endolysosomal pathway, the low endosomal pH triggers the conformational changes in DT structure leading to insertion of the T-domain into the endosomal membrane, and aid the translocation of the A-moiety from the endosomes to the cytosol [157-159]. At some point, probably during exposure to the cytosol, the reduction of the disulfide bond between A- and B-moieties occurs, and the enzymatically active A-moiety is released to the cytosol [155, 157, 229-231]. The A-moiety catalyzes ADP-ribosylation of the elongation factor 2, resulting in the inhibition of protein synthesis [232, 233].

Ricin

Ricin is a toxic carbohydrate-binding protein present in the seeds of the castor bean plant *Ricinus communis*. It is highly toxic to humans and is regarded as a bioterror threat [234]. Ricin belongs to the AB family of toxins, and consists of two functionally different parts: an enzymatically active A-moiety (~30 kDa) and a receptor binding B-moiety (~30 kDa) [160] (Fig. 5). The two moieties are represented by two individual polypeptide chains, which are linked to each other by a disulfide bridge [161]. Ricin does not have a single receptor, and binds, via its B-moiety, to both glycoproteins and glycolipids with terminal galactose, and thus is efficiently taken up by most cell types [235]. A depletion of GSLs does not protect cells against intoxication with ricin, confirming that ricin exploits multiple receptors on the cells [65, 236]. Upon binding, ricin is taken up by different endocytic mechanisms, i.e. its uptake does not depend on a certain endocytic pathway. For example, ricin is still efficiently taken up by cells after inhibition of both clathrin- and caveolae-dependent endocytic mechanisms [237-239].

The intoxication with ricin is similar to Stx by requiring retrograde sorting to the ER, where the enzymatically active A-moiety is released and translocated to the cytosol [240, 241]. The A-moiety possesses N-glycosidase activity and catalyses cleavage of one adenine residue from the 28S RNA of the 60S ribosomal subunit, leading to the inhibition of protein synthesis [242]. Several components implicated in ricin transport have been

identified by applying a genome-wide shRNA screen. For instance, the depletion of COP II was found to protect cells against ricin, while COP I depletion led to increased cell sensitivity to ricin [243]. However, this seems counterintuitive, since COP II primarily is involved in anterograde Golgi transport and COP I is involved in the retrograde pathway. Thus it might be that the observed effects are due to mislocalization of COP I- and COP II-dependent components that are required for the transport, rather than a direct role of COP II and COP I in ricin retrograde transport. Interestingly, another study has shown that ricin is able to reach the ER even after Golgi fragmentation, indicating that the toxin under some conditions may circumvent the traditional retrograde transport altogether [244]. Thus, although Stx and ricin follow similar routes in the cell, their transport differ mechanistically, as ricin seems to exploit a much larger variety of pathways than Stx (for review see [245]).

Toxin application in research and clinic

Despite the fact that protein toxins are disease causing agents, they have been found to be valuable tools in research and medicine, and several such examples are described below.

In research, toxins are employed to study endocytosis, intracellular transport and signalling pathways. For instance, several ADP-ribosylating toxins target specific GTP-binding proteins. DT targets elongation factor 2 and thus inhibits protein synthesis, whereas cholera toxin targets the α-subunit of heterotrimeric G_s protein, and renders it persistently active, leading to several cellular responses such as increased cyclic AMP levels and protein phosphorylation [246]. Furthermore, studies on transport mechanisms employed by toxins, such as Stx and ricin, help to reveal endocytic and intracellular trafficking processes [247]. Stx was the first lipid-binding ligand shown to be internalized by clathrin-mediated endocytosis [192]. Moreover, Stx was the first ligand shown to be transported all the way from the plasma membrane to the ER [216]. Thus retrograde travelling toxins, such as Stx, have been widely employed to study cellular component facilitating the transport of cargo along this pathway [144].

In addition to their application in research, protein toxins can also be used in medicine: (i) as carriers, which transfer a coupled molecule to specific cells or tissues, (ii) as diagnostic tools, when coupled to an imaging molecule, or (iii) as highly efficient drugs, which are modified to be specifically targeted. The latter approach is based on toxin conjugation

with, e.g. an antibody or a growth factor, which provides specific targeting of the toxin. The first clinically approved toxin derivative (denileukin diftitox) is a recombinant fusion protein of diphtheria toxin (without receptor binding domain) and human interleukin 2, and is used for the treatment of cutaneous T cell lymphoma [248]. For diagnostic purposes, one may explore highly specific non-toxic binding moieties of the toxins. Such moieties can be labelled with an imaging molecule, such as fluorophore (for the optical/fluorescence detection), with positron emitters, such as ^{18}F and ^{11}C, for detection by PET, or gamma emitters, such as ^{99m}Tc, for single photon emission computed tomography (SPECT) [79]. For example, fluorescently labelled B-moiety of Stx has been demonstrated to specifically target human colorectal carcinomas in mice models [249].

Aims of the Study

The overall aim of this study was to investigate the role of lipids in intracellular transport, and to study the potential effects of two glucose analogues, 2-deoxy-D-glucose and fluorodeoxyglucose, on cellular lipids and intracellular trafficking of Shiga toxin. The specific aims of each paper were:

I. **Cell density-induced changes in lipid composition and intracellular trafficking**
   To study how cell lipid composition and intracellular transport changes during cancer cell growth in culture.

II. **Novel actions of 2-deoxy-D-glucose: protection against Shiga toxins and changes in cellular lipids**
   To investigate potential effects of the glucose analogue, 2-deoxy-D-glucose, on cellular lipids and intracellular transport of Shiga toxins.

III. **Cellular effects of fluorodeoxyglucose: Global changes in the lipidome and alteration in intracellular transport**
   To study potential effects of the fluorinated glucose analogue, fluorodeoxyglucose, on cellular lipids and intracellular transport of Shiga toxin.

Summary of Publications

Paper I. Cell density-induced changes in lipid composition and intracellular trafficking

Cell culture is a primary experimental system widely used in cellular and molecular research. However, it has been shown that cell responses to various treatments, including viruses [250], bacterial and plant toxins [251, 252], cytokines [253] and drugs [254], change during cell growth in culture. Having noticed that cell sensitivity to Shiga toxin (Stx), which is widely used in our laboratory, depends on cell density in culture, we set up to investigate the underlying mechanism for this phenomenon. First we observed that high density HeLa and HEp-2 cells were six fold less sensitive to Stx, and we found that this protection was at least partially mediated via reduced Stx association to high density cells. During our attempt to uncover the basis for reduced Stx association to high density cells, we were surprised to discovere that it was only HeLa, but not HEp-2, cells that had reduced levels of the Stx receptor Gb3 in high density culture. This discovery encouraged us to perform detailed lipid analysis of HEp-2 cells to investigate if changes in Gb3 composition and/or membrane environment could be responsible for reduced Stx association to high density HEp-2 cells. We did not observe any significant changes in the Gb3 itself, but found multiple changes in the composition of other lipid classes during HEp-2 cells growth, with the largest effects observed for total levels and/or species composition of diacylglycerol, phosphatidic acid, cholesterol ester and lysophosphatidylethanolamine. In addition, we found that the overall saturation of the lipids changed during HEp-2 cell growth, as we observed an increase in mono-unsaturated and a decrease in poly-unsaturated fatty acyl chains during cell growth. Taken together our data indicate that significant changes occur in cellular lipids during cell crowding in culture. Importantly, such changes are likely to affect multiple cellular processes including cellular transport. To support this, we were able to demonstrate that the release of the enzymatically active Stx moiety ($StxA_1$) in the ER and into the cytosol is reduced in high density HEp-2 cells, indicating that the intracellular transport of Stx is modulated by cell density.

In addition, in this paper we show that the expression of the gene encoding Gb3 synthase (α-1,4-galactosyltransferase), is upregulated during cell growth. Although we did not

analyze the mechanism underlying this phenomenon, it might be of importance to understand how the biosynthesis of Gb3 is regulated in the cells, as Gb3 has been shown to be upregulated in multiple cancers [79], and to be important for development of metastasis [80].

Paper II. Novel actions of 2-deoxy-D-glucose: protection against Shiga toxins and changes in cellular lipids

2-Deoxy-D-glucose (2DG) is a structural analogue of glucose widely used in research as an inhibitor of glycolysis and protein N-glycosylation. 2DG treatment has been shown to affect multiple cellular functions, including autophagy [122, 123], apoptosis [124, 125], gene expression [129] and cell cycle control [126]. Importantly, 2DG has also been explored for its potential to improve the efficacy of cancer therapeutics [255-257]. Thus, in this study we set up to analyze how 2DG affects cell lipid composition and intracellular transport, as such information was lacking in the literature, but might suggest novel applications of 2DG in research and in the clinic.

First, to test whether 2DG affects intracellular transport, we investigated cell sensitivity to three different protein toxins, Shiga toxin (Stx), ricin and diphtheria toxin (DT), which all have different requirements for association to cells and intracellular transport. While ricin toxicity was not affected by 2DG, cell sensitivity to Stx and DT was reduced upon 2DG treatment. However, cell sensitivity to DT, but not to Stx, could be rescued by combined treatment of the cells with 2DG and mannose, which counteracts 2DG-mediated inhibition of protein N-glycosylation, indicating that protection against Stx and DT is mediated via different mechanisms. The binding of DT to its receptor is dependent on the glycoprotein CD9, thus it is very likely that 2DG treatment leads to aberrant glycosylation of CD9 rendering the cells protected against DT.

To elucidate the mechanism by which 2DG protected cells against Stx, we analyzed whether 2DG affected Stx association to the cells, Stx endocytosis or retrograde transport. We did not observe any changes in Stx association to the cells, endocytosis and transport to the Golgi, but found that 2DG blocked the release of the enzymatically active Stx moiety ($StxA_1$) in the ER. 2DG has been previously shown to release Ca^{2+} from the ER in human pancreatic tumor cells (MIA PaCa-2) [123], and as we found, had similar effect in HEp-2 cells. To further test whether ER Ca^{2+} is required for cell intoxication by Stx, we used

thapsigargin (TG), which inhibits ER Ca^{2+} ATPase and thus results in a rapid release of Ca^{2+} from the ER. We found that TG treatment protected cells against Stx, but had no effect on ricin toxicity, which was in agreement with the observation that 2DG also protected cells against Stx but not ricin. Thus, our data indicates that Stx requires normal ER calcium levels for efficient intoxication and that calcium depletion form the ER protects cells against Stx, most likely via inhibiting toxin association with ER chaperones.

Finally, intrigued by our observation that 2DG treatment reduced total Gb3 levels by almost 50% after 24 h without having a significant effect on Stx association to the cells, we performed detailed lipidomic analysis to search for potential effects of 2DG on Gb3 and other cellular lipids. The binding and the intracellular transport of Stx have been shown to depend of certain Gb3 species and on other lipids (for review see [32]). We found that 2DG treatment affects cellular levels and/or species composition of several lipid classes, including phosphatidylinositol, diacylglycerol, cholesteryl ester, ceramide and lysophospholipids, which may potentially change the lipid environment for Gb3 and thus affect its presentation for binding. Moreover, we found that 2DG becomes incorporated into GSLs in the cells, and that 2DG incorporation in the GlcCer ([2DG]GlcCer) does not prevent further addition of the galactose residue to the [2DG]GlcCer resulting in formation of 2DG-containing LacCer.

Paper III. Cellular effects of fluorodeoxyglucose: Global changes in the lipidome and alteration in intracellular transport

Having demonstrated that 2DG protects cells against Shiga toxin and affects cellular lipid composition, we decided to investigate whether a related glucose analogue, 2-fluoro-deoxy-D-glucose (FDG), has similar cellular effects. Moreover, there was little information available on cellular effects of FDG, although the compound is widely used for PET imaging in research and in the clinic. Thus, in this study we investigated whether FDG treatment affects cell sensitivity to Stx, Stx2, ricin and diphtheria toxin. In addition, we also analyzed if cellular lipid composition is affected by the treatment.

We found that FDG efficiently protects cells against Stx and Stx2 without having any affect on cell sensitivity to diphtheria toxin, and giving only a minor protection against ricin. Importantly, FDG appeared to be ten-fold more effective in protecting cells against Stx than 2DG, and the FDG-induced protection remained even after removal of FDG from

the medium and cell growth in a fresh medium for one day, indicating that FDG induces specific and long-lasting changes in the cells.

By quantifying more than 200 lipids from 17 different lipid classes we revealed that FDG treatment significantly changes cellular lipid composition with the largest changes observed for glycosphingolipids, diacylglycerol (DAG), phosphatidic acid (PA) and phopshatidylinositol (PI). Four hour incubation with FDG led to elevated total cellular levels of PA and PI and gave a slight reduction in total DAG. In addition, FDG treatment also changed species composition of these lipids, mainly affecting DAG and PA species with 16:0_18:1 and 16:1_18:1, and PI species with 18:0_18:1 and 18:1/18:1 fatty acyl chains. One day treatment with FDG significantly reduced cellular levels of glucosylceramide (GlcCer), lactosylceramide (LacCer) and Gb3. Importantly, there was a slight reduction in the total levels of GlcCer observed already after 4 h treatment with FDG indicating that GlcCer synthesis was inhibited already at this time point, but it required a longer incubation (24 h) until the existing pool of GlcCer was degraded in the cells. These data indicate that FDG might potentially be used as an inhibitor of GlcCer synthesis, as the observed reduction in total GlcCer after FDG treatment is similar to that reported for PDMP [65], which is a commonly used inhibitor of GlcCer synthesis.

Next, to investigate the mechanism by which FDG protects cells against Stx we have analyzed binding and intracellular transport of the toxin. We found that it was only 24 h, but not 4 h, treatment with FDG that reduced Stx association to the cells. Thus, the reduction in total Gb3 and thereby reduced Stx association to the cells could partially explain the complete protection against Stx following 24 h treatment with FDG, but it could not explain the 13-fold protection which was observed already after 4 h treatment with FDG. Therefore, we investigated whether 4 h treatment with FDG affected Stx endocytosis or retrograde transport and thus interfered with Stx intoxication. While FDG treatment did not affect Stx endocytosis and a subsequent transport step to the Golgi, there was a 50% reduction in toxin transport to the ER, indicating that Stx transport between the Golgi and the ER was inhibited by FDG treatment. Finally, FDG treatment also inhibited the release of the $StxA_1$ moiety in the ER and, importantly, to a significantly higher extent than the transport from the Golgi to the ER, indicating that both the transport from the Golgi to the ER and the release of the $StxA_1$ moiety in the ER were inhibited by FDG treatment. The reduction in $StxA_1$ release in the ER is most likely caused by Ca^{2+} released

from the ER, which is induced by FDG treatment; such Ca^{2+} depletion from the ER is also induced by 2DG treatment and seems to be associated with cell protection against Stx by 2DG [258]. In summary, in this study we show that FDG affects intracellular transport and modulates cellular lipid composition, and thus our data open up for potential novel applications of this drug.

Discussion

The work presented in this thesis has contributed to identification of novel actions of the glucose analogues, 2DG and FDG, on cellular lipids and intracellular transport of Shiga toxin. Importantly, our data suggest new potential applications of these drugs in research and in the clinic. In addition, we have revealed the changes in the cellular lipidome occurring during cell growth in culture, and shown that these changes at least in part modulate cell sensitivity to Shiga toxins. In summary, this work presents original data on how cancer cells regulate their lipid composition and intracellular transport in response to different treatments.

Cell growth in culture: implications of cell density on the molecular level

Cell lines are widely used in molecular biology and genetic studies and have been instrumental in building our knowledge on molecular machinery of the cell. However, one has to keep in mind that such cell lines are derived from multicellular organisms, where cells are meant to interact with their surroundings, receive and send signals, and modify their metabolism based on all this information. Thus, it is not surprising that various factors, such as growth conditions, passage number and cell-to-cell interactions, will modify the signaling and molecular machinery of an individual cell in culture. Nevertheless, the impact of cell density on the experimental results obtained is often neglected, and thus might lead to wrong interpretation of data.

In paper I, we analyzed how cells modify their lipidome during growth in culture and what implications such changes might have for other functions of the cells, including cellular transport. In this paper, we have used two cell lines, HeLa and HEp-2. Although HeLa and HEp-2 cells are considered to be alike, due to contamination of HEp-2 cells with HeLa at some point [259], we found them to react very differently to increasing cell density in culture. Thus, one needs to be very careful when comparing data obtained even with such similar cell lines.

Interestingly, we have also performed additional experiments with HEp-2 cells, which were not included in the paper I, to test whether conditioned medium collected from high density cells may modulate responses in low density cells. Indeed, when low density HEp-

2 cells were incubated with such conditioned medium for 1 day, they were 2 fold less sensitive to Stx than low density cells grown in fresh complete medium. Furthermore, we also observed an increase in the expression levels of Gb3 synthase gene in low density HEp-2 cells treated with conditioned medium for 2 hours, and the mRNA levels for Gb3 synthase were similar to those in high density cells (our unpublished data). Thus, the effects presented in paper I seem to be mediated both via cell-to-cell contacts and via soluble factors released by the cells into the medium. In addition, Snijder *et al.* [250] has shown that cell response to virus infection and the rate of endocytosis highly depend on cell position in the culture (edge or middle of the colony, and the size of the colony that the cell belongs to).

One interesting observation in this study was the reduction in total Gb3 levels in HeLa cells during cell growth, which was opposite to that for HEp-2 cells, which seemed to have a slight increase in total Gb3 during growth. There are several other reports showing that the biosynthesis [117, 260, 261] and the levels of Gb3 [117, 262] increase with increased cell density in several primary cell cultures, but that this up-regulation is lost upon virus-induced transformation of the cells. As suggested in paper I, the reduction in total Gb3 in HeLa cells seems to be mediated via depletion of Gb3 precursors, rather than reduction in Gb3 synthase activity. However, additional analyzes are required to understand the molecular mechanism underlying this phenomenon. One possibility is that HeLa cells upregulate the synthesis of other GSLs, such as gangliosides, rendering a reduction in LacCer and a subsequent depletion of Gb3. The shift from one series of GSLs to another is usually observed during cell differentiation or metastatic transformation (for review see [27, 62]) thus it also might happen during cell crowding in culture.

By analyzing 17 different lipid classes and their species, we discovered that cellular lipid composition changes during HEp-2 cell crowding in culture. One of the most interesting observations in the lipidome was the alteration in species composition of phosphatidic acid (PA) and diacylglycerol (DAG). The levels of two species, 16:0/18:1 and 16:1/18:1, were oppositely affected for PA and DAG during cell growth, indicating that the changes in these lipids are least in part caused by interconversion between these two lipids. Importantly, both PA and DAG (and their specific species) have been associated with specific functions in the cell, which are discussed next in respect to our data.

The acyl chain at the sn-1 position of the glycerolipids is either palmitoyl (16:0) or stearoyl (18:0) for most organisms. It has recently been proposed that the relative ratio of the lipids with 16:0 and 18:0 at sn-1position is regulated by PA. It has been shown that transcriptional repressor Opi1 binds strongest to PA species with saturated fatty acyl chains of 16 carbons (PA 16:0/16:0), while the binding is weaker to PA species containing an unsaturated chain (PA 16:0_16:1 and PA 18:0_18:1) or to PA species with saturated but longer fatty acyl chains (PA 18:0/18:0) [263]. It is suggested that in cells, Opi1 binds to PA containing C16 fatty acyl chains in the ER, which in turn prevents the translocation of the Opi1 into the nucleus. As a consequence, Opi1 does not repress the transcriptional activator INO1, that promotes the expression of enzymes required for fatty acid synthesis. If PA species with C18 fatty acyl chains predominates, Opi1 translocates to the nucleus and represses INO1 activity. One of the enzymes whose synthesis is regulated by this system is acetyl-CoA carboxylase 1 (Acc1). Acc1 is involved in the *de novo* synthesis of fatty acids with the specificity for longer acyl chains, thus action of Acc1 increases the ratio C18/C16 of fatty acids in the cells. This is suggested to provide a feedback regulation of the relative amounts of sn-1 C16 and C18 lipids in the cell [263]. We have observed that the levels of PA 16:0/18:1 decrease during HEp-2 cell growth, while PA 18:1/18:1 increases at the same time. In addition we have also observed that the relative content of lipids, when quantified as pmol lipid per μg protein, increased during cell growth (by 23% after 3 days). When taken together with data from Hofbauer *et al.* [263], this indicates that there might be an overall upregulation of lipid biosynthesis during cell crowding in culture.

Both PA and DAG have been associated with several intercellular transport steps, including COP I-dependent and COP I-independent retrograde protein transport from Golgi to the ER [264, 265]. DAG has a relatively small and electrically neutral polar head, and thus is expected to facilitate membrane bending and formation of highly curved membranes, which is required for transport vesicle formation and fusion. Interestingly, different species of DAG and/or location of its synthesis might determine its function in the cell. For example, decreased levels of Golgi-associated DAG have been demonstrated to inhibit retrograde (Golgi-to-ER) but not anterograde (ER-to-Golgi) transport [264, 265], and the effect on the retrograde transport depended on the inhibitor that was used to block DAG production. The most profound effect was observed after propanolol treatment, which inhibits DAG generation form PA, and a smaller effect by inhibitor U73122, which blocks DAG generation from phosphoinositides PI4P/PI4,5P_2, but no effect observed after

treatment with fumonisin B1 (FB1), the inhibitor of ceramide synthase, which leads to lowered SM levels and reduced DAG generation from SM [264]. These data suggest that only certain DAG pools might be involved in regulating retrograde transport. It has also been proposed that DAG facilitates the COP I-dependent protein transport via recruitment of ADP-ribosylating factor GTPase-activating protein (ARF1GAP1) at early Golgi compartments [264]. ARF1GAP1 constitutes a structural component of the COP I coat and is required for COP I coated vesicle formation on Golgi membranes [266]. In addition, ARF proteins have been shown to activate PC-specific phospholipase D (PLD), which generates PA from PC [267]. As a result, PA has also been implicated in Golgi vesicle formation and budding [268].

The levels of PA and DAG can be modulated by PA−DAG interconversion, where DAG is formed by phosphatidic acid phosphohydrolases (PAPs), and PA results from the activity of the DAG-consuming kinases (DAGKs). In addition, PA and DAG belong to so called PI-cycle (PI → $PI(4,5)P_2$ → DAG → PA → CDP-DAG (cytidine diphosphate diacylglycerol) → PI), through which the cells are suggested to regulate their PI (and maybe PA and DAG) species composition [269]. Some of the enzymes in this cycle have preferences for specific fatty acyl groups, which leads to enrichment of certain PI species (e.g. 1-stearoyl-2-arachidonoyl-PI (18:0/20:4)) in mammalian cells [269]. In addition, different PA species have been shown to be generated by different lipid metabolic pathways: the PA species generated by the action of PLD (phospholipid → PA) are different from those formed in the PI-cycle by the action of phospholipase C (PIP_2 → DAG + IP_3), followed by the action of DAGK (DAG → PA) [270].

Different factors modulating Gb3 receptor function

In this study, we have discovered that both 2DG and FDG reduce total Gb3 levels to the same extent (by approximately 50% after 24 h treatment), but it is only FDG that also inhibits Stx binding to HEp-2 cells at this time point. The fact that reduced Gb3 levels do not give significant effect on Stx binding in 2DG treated cells seems surprising, but there might be several explanations for this phenomenon. First, it is possible that only intracellular Gb3 is depleted upon 2DG treatment, and that the levels of plasma membrane-localised Gb3 are unchanged (lipid analyses have been performed on whole cell lysates). Second, as Stx binding depends on the species composition of Gb3 (for review see [32, 144]), the reduction in the total level of Gb3 could have been counteracted by a change in

the species composition of Gb3. However, we did not observe any significant changes in Gb3 species following 2DG treatment, indicating that this is not the case. Another possibility is that there are two distinct pools of Gb3 at the plasma membrane, and that 2DG treatment depletes only a certain fraction of Gb3 that is not required for efficient Stx association with the cells. The hypothesis that there exist two distinct pools of Gb3 is supported by several experimental observations. By analyzing glycosphingolipid composition in the cells after inhibition of GSL synthesis by PDMP (inhibits GlcCer synthesis) and FB1 (inhibits ceramide synthesis) Raa *et al.* [65] has shown that there is a fast reduction by approximately 50% in total Gb3 during the first 24 h of treatment. However, a double increase in the incubation time with the inhibitors gave only a slight additional reduction in total Gb3, and a remarkable fraction of Gb3 (of approximately 30-40%) remained in the cells even after 48 h incubation [65], indicating that there are two distinct pools of Gb3 with different degradation times. In addition, the study by Mahfaoud and coworkers has indicated that there are two distinct pools of Gb3 present at the plasma membrane, one that is available for binding by Stx, and another that is masked by the interaction with cholesterol. Interestingly, inclusion of GlcCer or GalCer into the Gb3- and cholesterol-containing vesicles increased Stx binding to such vesicles, indicating that GlcCer and GalCer could counteract cholesterol-mediated masking of Gb3 [179]. The fact that 2DG treatment results in changed total levels and/or species composition of several lipid classes suggests that Gb3 availability for binding might be increased by the treatment and thus might compensate the reduction in total levels of Gb3. Finally, as 2DG is more potent inhibitor of protein N-glycosylation than FDG [116, 271], one may expect that cell treatment with 2DG should lead to reduced levels of glycoproteins on the cell surface. The reduction in the total amount of glycoepitopes on the cell surface might increase Gb3 availability for binding and thus compensate for reduced total amount of the receptor.

In the paper I, we have also found that Stx binding per cell to high density HEp-2 cells is reduced down to 30-40% compared to low density cells, while there is a small increase in total Gb3 in HEp-2 cells during growth, showing that there is a non-linear relationship between cellular levels of Gb3 and Stx association to the cells. All together, our data support the idea that Gb3 presentation for binding is strongly regulated by other factors, such as overall lipid composition of the membrane, and the relationship between Stx association to the cells and Gb3 levels is much more complicated than first anticipated.

Shiga toxin transport modulation by lipids

Efficient intracellular transport of Gb3-bound Stx has been suggested to depend on Gb3 association with detergent resistant membranes (DRMs) [183-186]. For instance, it has been reported that cell treatment with N-butyldeoxygalactonojirimycin (NB-DGJ), an inhibitor of GCS [272], reduces Stx association to DRMs and inhibits the retro-translocation process in the ER [185]. Thus, it has been hypothesized that cellular GlcCer is required for the localization of Gb3-bound Stx to lipid rafts and that such localization is necessary for the efficient intoxication process. However, there are large deviations in the lipid data for LacCer and Gb3 upon NB-DGJ treatment [185], thus one cannot exclude the possibility that it were other factors, such as changes in total Gb3 and/or its species, that gave the observed effects. In another study, where StxB association to HeLa and monocyte-derived cells was compared, it was found that Stx fails to associate with DRMs in the monocyte-derived cells and thus the toxin is sorted for degradation in lysosomes rather than retrogradely to the Golgi [183]. The hypothesis that Stx requires association with DRMs for its retrograde sorting was further supported by the data showing that the destabilization of DRMs by cholesterol extraction also inhibits StxB transport from endosomes to the Golgi [183]. In this work we have discovered that it is only FDG but not 2DG that reduces cellular levels of GlcCer. Thus, if GlcCer is indeed required for Gb3 association with DRMs and efficient Stx intracellular transport, the reduction in total GlcCer levels observed upon FDG, but not 2DG, treatment might partially explain the observed differences between the effects of FDG and 2DG on Stx binding, endocytosis and toxicity after 24 h treatment. However, since 4 h treatment with FDG gave only a very small reduction in total GlcCer, it is unlikely that changed Gb3 association with lipid rafts was responsible for reduced Stx transport from the Golgi to the ER that was observed after 4 h treatment.

In addition to directly affecting Gb3 presentation and localization in the membrane and thus its cellular functions, changes in other lipids might also modulate Stx transport by altering membrane properties, vesicle formation, protein recruitment to specific membrane sites, etc. In response to 2DG treatment, it was only total levels of lysophospholipids (LPLs), namely lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE), that were reduced both by 4 h and 24 h treatment, demonstrating that lysolipid metabolism is significantly affected by 2DG. LPLs are known to change mechanical properties of the

membrane [273] and thus might modulate Gb3 diffusion in the membrane and/or its localization in specialized membrane compartments. In addition, LPLs are suggested to be involved membrane bending, fusion and fission processes [273-275], thus changed levels of LPLs might affect vesicular transport. However, additional studies are required to understand the mechanism of how 2DG treatment modulates LPL levels in the cell and whether it has direct consequences for membrane properties, Gb3 availability for binding and/or intracellular transport. It must be noted that we did not observe any effect of FDG on LPLs, indicating that this effect is specific to 2DG or that higher doses (the studies on FDG were performed with 10 fold lower concentrations than used for 2DG) are required to induce the effect.

Finally, in all three papers we have reported that total levels and/species composition of PA, DAG and PI change upon HEp-2 cell growth in culture and after cell treatment with 2DG or FDG. Both PA and DAG have been associated with several intercellular transport steps, and how these changes might affect intracellular transport has already been discussed in the section “Cell growth in culture: implications of cell density on the molecular level”. Changes in the levels and/or species composition of PI might also have a great impact on intracellular transport, since certain phosphoinositides (phosphorylated intermediates of PI) function as specific markers and docking sites recognized by dedicated transport proteins [276]. However, it remains unclear how 2DG and FDG treatment affects cellular levels of PA, DAG and PI, and if these changes might be directly related to observed changes in Stx transport.

Potential applications of 2DG and FDG in the clinic

More than 80 years ago, Warburg observed that tumor cells have increased glycolysis even under normal oxygen conditions [277], and the increase in glucose metabolism is now recognized as one of the cancer hallmarks [278]. Increased uptake and metabolism of glucose in cancer cells open a natural selectivity window for cancer cell targeting with glucose analogues, such as 2DG and FDG, and is the basis for tumor visualization by PET. However, in early clinical trials, 2DG alone was not effective in cancer treatment (for review see [125]). Nevertheless, it was later found that 2DG improves the efficacy of several cancer chemotherapeutic agents in animal models [255, 256]. Therefore, safety and feasibility of 2DG administration has been tested in early clinical trials in cancer patients, as a single agent [257, 279], in combination with chemotherapy [257] or with radiation

therapy [280]. The idea of combining 2DG with chemotherapeutic agents is mainly based on the rationale that the latter drugs usually effectively target rapidly growing tumor cells that are located in areas with normal oxygen tension while 2DG may preferentially target the hypoxic cancer cells. A dose escalation phase I clinical trial has revealed that 2DG could be safely administered in combination with the chemotherapeutic drug docetaxel in patients with advanced solid tumors [257]. The recommended daily dose of 2DG in combination with docetaxel was 63 mg/kg, which resulted in median maximum plasma 2DG concentrations of 0.7 mM, and caused tolerable adverse effects, such as sweating, dizziness and nausea, mimicking the hypoglycemic symptoms expected from 2DG administration. In addition, there was evidence of clinical benefit of this novel combination [257]. 2DG has also been tested in combination with radiotherapy for treatment of glioblastoma [281], and the promising results have led to initiation of phase II and III clinical trials to further test if 2DG could significantly improve the efficacy of radiotherapy of brain tumors [282].

Although 2DG has primarily been explored for its potential to inhibit glycolysis and to target hypoxic cancer cells, it has recently been found to effectively reduce tumor angiogenesis in animal models via inhibition of protein N-glycosylation rather than the inhibition of glycolysis [271, 283]. In cell cultures, 2DG was found to be significantly more toxic to microvascular endothelial cells (HUVEC and HMVEC-L cells) than to cancer cells (HT-29, CAKI-1, MDA-MB231, 786-0 and HT-1080) and normal human renal epithelial cells [271]. The endothelial-specific toxicity was later associated with 2DG-induced hypoglycosylation of vascular endothelial growth factor (VEGF) receptor and thereby reduced AKT and ERK phosphorylation in endothelial cells [283]. Importantly, the inhibition of AKT and ERK phosphorylation was found to be independent of the growth factor used to stimulate endothelial cells [283], suggesting that 2DG may overcome acquired anti-VEGF resistance, which is generally mediated via upregulation of other cell surface growth factor receptors and is often observed in patients treated with anti-VEGF agents [284].

Based on the different efficacy of 2DG and FDG in blocking glycolysis and protein N-linked glycosylation, these two drugs may potentially be used to target different tumor cell populations. While FDG is more efficient in killing hypoxic cancer cells than 2DG [134],

2DG is more efficient in inhibiting tumor angiogenesis [271], and thus even the combination of the two might be potentially explored.

The majority of the studies on 2DG and FDG focus on inhibiting glycolysis or protein glycosylation to increase cancer cell sensitivity to chemotherapeutic drugs. However, other cellular effects of these drugs might also be explored to sensitize certain cancers. For instance, the over-expression of GlcCer synthase (GCS), the enzyme catalyzing the first reaction of ceramide glycosylation, has been associated with drug-resistance and poor response to chemotherapy in a variety of cancers (for review see [106]). In this work, we have discovered that FDG treatment reduces cellular levels of GlcCer indicating that FDG might have inhibitory affect on GCS. Okuda *et al.* has previously shown that 2DG suppresses expression of the Gb3 synthase gene in HeLa and teratocarcinoma (NCCIT) cells [129]. In this work, we showed that 2DG treatment indeed reduced mRNA levels for Gb3 synthase in HEp-2 cells, but neither 2DG nor FDG had any effect on the mRNA levels of GCS. Thus, it seems that FDG does not suppress the expression of the GCS, but rather interferes with the biosynthesis or degradation of GlcCer. FDG might inhibit the synthesis of GlcCer in the Golgi by either a direct inhibition of GCS, or by inhibiting the vesicular transport of ceramide from the ER to the Golgi. In addition, since FDG is converted to UDP-FDG in the cells [136], but does not become incorporated into GSLs (shown in this study), UDP-FDG may accumulate in the cells and thus inhibit GlcCer synthesis via direct competition with UDP-Glc for GlcCer synthesis. However, further investigations are required to reveal the mechanism by which FDG depletes cellular GlcCer levels and to investigate whether FDG might potentially be used as an adjuvant for cancer therapy to target cancer cells with increased GSLs biosynthesis.

In addition to their applications for cancer diagnosis and treatment, 2DG and FDG might potentially be used to treat infectious diseases. For instance, both 2DG and FDG have been shown to inhibit the proliferation of the human malaria parasite *Plasmodium falciparum* [285]. In this work, we have discovered that both 2DG and FDG protect cells against Stx2 intoxication *in vitro*, suggesting that these compounds might have a potential for treating gastrointestinal infection with STEC, as there is still a lack of effective treatment for STEC-infected patients. In this respect FDG is much more promising than 2DG, as the concentration required to inhibit cell intoxication by Stx2 was as low as 1 mM for FDG, and is in a range of achievable plasma concentrations of 2DG shown in humans (the

maximal median concentration of 0.7 mM was reported for patients with advanced solid tumors [257]). Of course, the pharmacokinetics of these two drugs might be different and the applicability of FDG as a therapeutic agent in humans needs to be carefully assessed, but the fact that FDG is already successfully used for PET imaging is highly encouraging.

Experimental Systems

Cell lines

The experiments in the papers presented here have been performed using cultured human cancer cell lines. The majority of the experiments have been performed using HEp-2 cells, while some of the experiments in the papers I and II have also been performed using HeLa (epithelial cell line derived from cervical cancer), HT-29 and SW480 (both derived from adenocarcinoma) cells. HEp-2 cells have been chosen as a main model due to being robust, sensitive to Stx and compatible with all the assays established in our laboratory. HEp-2 cells were originally derived from larynx carcinoma, but have at one point been contaminated with HeLa cells [259]. However, HEp-2 and HeLa cells are not identical, and this was clearly observed in our first paper. While both HEp-2 and HeLa were becoming less sensitive to Stx with increasing cell density in culture, we were surprised to discover that it was only HeLa and not HEp-2 cells that down-regulated Gb3 during cell crowding in culture. Apparently, this makes it difficult to compare data obtained from these two cell lines, and thus for the later experiments we focused on HEp-2 cells only. However, HEp-2 cells are not suitable as a model for a specific tissue, and thus the investigations described in papers II and III, in the future, should be extended to other cell lines, such as human endothelial cells from human kidney or brain, to increase the pathological relevance of the data. It should be noted that the 2DG-mediated protective effect against Stx was observed for all four cell lines tested (HEp-2, HeLa, HT-29 and SW480), indicating that at least some of the 2DG-induced effects presented in this work are not limited to HEp-2 cells only.

Cell-based toxin assays

Protein toxins have different requirements for their binding to the cell surface, internalization and intracellular transport, and thus are useful as tools to investigate changes in various cellular processes. In this work, several protein toxins, including Stx, Stx2, ricin and DT, were used. For example, in paper II we have discovered that 2DG protects cells against Stx, Stx2 and DT without affecting sensitivity to ricin. In addition, the protection against DT, but not against Stx, was found to be mediated via inhibition of protein N-glycosylation by 2DG. These findings suggested that 2DG affects specific routes

in the cell, and thus motivated us to investigate 2DG-mediated effects on intracellular transport. For mechanistic studies, a non-toxic Shiga toxin 1-mutant (Stx1-mut) was used in majority of the toxin assays in this work due to limited availability of Stx, and the greater challenge and restrictions involved in producing the toxic molecule. To our experience, the transport of Stx1-mut is not affected by the two point mutations in the active site which render it non-toxic.

Stx transport

When working with 2DG and FDG, we have discovered that these drugs interfere with two of our well established assays for Stx transport to the Golgi and ER. To measure Stx transport to the Golgi we commonly use modified Shiga toxin B molecule (StxB) containing sulfation sites. Protein sulfation occurs exclusively in the Golgi, therefore the amount of the toxin that reaches the Golgi can be determined by quantifying the addition of [^{35}S]sulfate to the StxB sulfation sites. However, we have found that both 2DG and FDG reduced total protein sulfation (the mechanism underlying this phenomenon is under investigation at the moment) and thus interfered with the sulfation assay. In addition, both 2DG and FDG are known to compete with mannose for N-linked protein glycosylation, thus they also interfered with our assay for Stx transport to the ER, which is based on protein glycosylation in the ER. Due to these experimental challenges, we explored two other assays for Stx transport to the Golgi and ER in this work, and these assays are discussed in two next sections.

Fluorescence microscopy-based analysis of Stx transport

We have used two different microscopy approaches to study retrograde Stx transport. To study Stx transport to the Golgi, we employed automated widefield imaging using the ScanR system. The main advantage of this approach is the ability to automatically acquire and analyze large datasets from hundreds of cells, while retaining the information from each individual cell. The analysis was cell- and object-based, where single cells were recognized based on the nuclear staining and the objects (Golgi) were recognized based on the immonofluorescent staining of the Golgi-resident protein giantin. The transport of Stx to the Golgi was quantified as mean signal for immunofluorescently labeled Stx within the giantin positive objects. We did not choose to quantify total Stx signal within the giantin positive objects, because such an approach would be much more sensitive to changes in

object size and/or object number per cell, and thus would give false effects when Golgi morphology is affected by the treatment (we observed Golgi vesiculation upon treatment with 2DG, as demonstrated by the redistribution of the Golgi markers giantin, GM130 and TGN46 to vesicular structures). However, one of the drawbacks of a microscopy-based approach is its sensitivity to reduced Stx binding, as the amount of Stx transported to the Golgi will largely depend on the amount of Stx bound to the cell surface. This was partially overcome by adjusting the exposure time during the imaging of the samples from low and high density cells. However, such data is then much more difficult to interpret, and gives only qualitative (if Stx reaches the Golgi and how fast), but not the quantitative (if higher/lower proportion of cell-bound Stx reaches the Golgi) data.

For Stx transport to the ER, we have used confocal fluorescence microscopy. In confocal microscopy, the out of focus light is eliminated resulting in a significantly improved signal to noise ratio compared to conventional widefield microscopy. The ER is much more dispersed than the Golgi, therefore the ScanR microscopy, where the whole cell (not the individual confocal sections) is imaged, does not provide detailed enough images of the ER structures. For ER visualization, we have used immunofluorescent labeling of the ER-resident enzyme PDI (protein disulfide isomerase). Although we did not test the specificity of the PDI antibody, our data indicate that the staining was specific: (i) signal for PDI was markedly increased upon 24 h treatment with 2DG. 2DG has been shown to induce unfolded protein response (UPR) in the ER, and thus upregulate ER chaperones [122]; PDI acts both as an oxidoreductase and as a chaperone [286]; (ii) mannose, which prevents UPR when added in combination with 2DG, also prevented the increase in immunofluorescence signal for PDI. To further show the validity of the assay, we have also analyzed samples treated with Brefeldin A (BFA), which disrupts Golgi and thus prevents Stx transport to the ER [287, 288]. The fact that BFA treatment led to a significant reduction in Stx colocalization with PDI indicated that this approach could be used to analyze transport of Stx to the ER. However, the resolution obtained by confocal microscopy is limited to the diffraction limit of the light used (~200 nm in the lateral dimension). Therefore, by using this approach, we could not exclude the possibility that 2DG treatment prevents fusion of the Stx-positive transport vesicle with the ER membrane, or changes Stx localization within the ER.

SNAP-tag method for studying retrograde Stx transport

To overcome some of the limitations of the microscopy-based assays, we have developed a new biochemical assay for Stx transport to the Golgi and the ER. For this purpose we employed SNAP-tag® technology [289] based on a SNAP-tag enzyme which is a mutant of the DNA repair enzyme O^6-alkylguanine-DNA alkyltransferase. The SNAP-tag enzyme reacts specifically and rapidly with benzylguanine (BG) derivatives leading to irreversible covalent labeling of the SNAP-tag with the probe attached to the BG molecule [289] (Fig.7, top panel). To render SNAP-tag localization to the Golgi or ER, we have fused the SNAP-tag either with the first 120 amino acids of galactosyl transferase (GalT; for Golgi localization) or with hen lyzozyme and KDEL (for ER transport and retention) (Fig. 7, bottom panel). The EGFP was also included in both plasmids and was used for visualization and immunoprecipitation of the constructs. We generated two stable HEp-2 cell lines expressing Golgi localized-SNAP-tag (HEp-2-GalT-GFP-SNAP) or ER localized-SNAP-tag (HEp-2-ER-GFP-SNAP). The correct localization of the constructs to the Golgi and ER was verified by confocal immunofluorescence using giantin and PDI immunolabeling, respectively. Importantly, the localization of the constructs was not affected by FDG treatment, and thus the SNAP-tag assay was used to study Stx transport to the Golgi and ER following FDG treatment in paper III.

The main advantage of this method is that it is quantitative even when Stx association to the cells is affected by the treatment, as the amount of Stx covalently bound to the SNAP-tag can be normalized to total cell associated Stx (both SNAP-tag bound and unbound Stx). However, the labeling of Stx with BG might affect its transport. To test whether BG-labeling affects retrograde Stx transport, we have compared the release of the $StxA_1$ moiety from the ^{125}I-Stx1-mut and ^{125}I-Stx1-mut-BG (with and without FDG treatment), and found no difference (data not shown), indicating that the BG tag did not affect Stx transport. Finally, it should be mentioned that this assay might not be compatible with 2DG treatment. 2DG induces Golgi vesiculation and ER stress, and thus might interfere with this assay by changing the localization of the SNAP-tag constructs (this has not been tested yet).

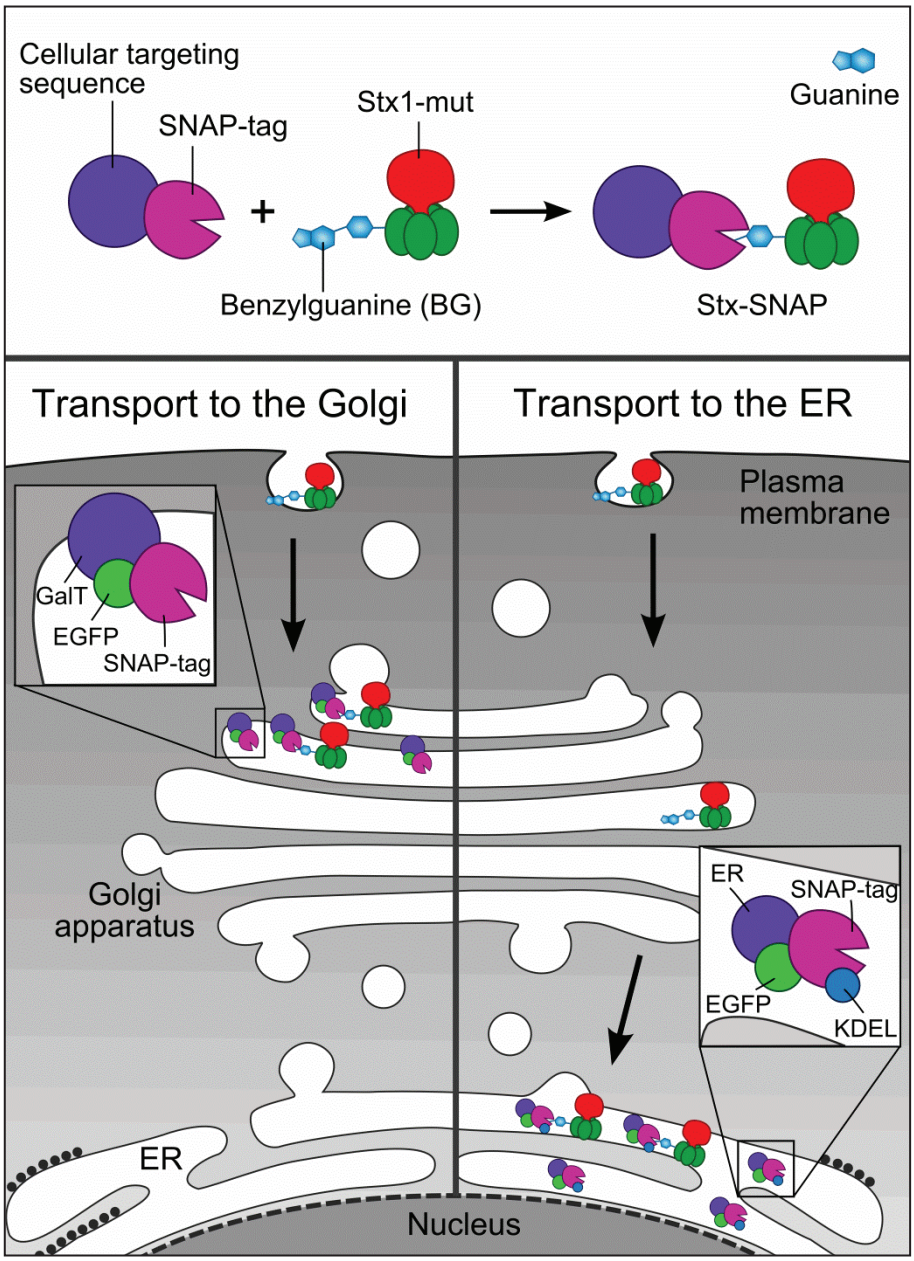

Figure 7. SNAP-tag method for studying retrograde Stx transport. *Top panel* shows the principle underlying protein covalent labelling with the SNAP-tag. The SNAP-tag protein can be targeted to a specific cellular localization by fusing it with a targeting sequence. In addition, other sequences can be added for detection by fluorescence microscopy and/or immunoprecipitation (not shown in this panel). Target molecule (in this case Stx) is labelled with benzylguanine (BG) (the labelling is shown only for the B-moiety of Stx, although both A and B moieties are labelled with BG). The SNAP-tag enzyme reacts specifically with BG leading to formation of covalent bond between the SNAP-tag and the target (Stx) molecule. *Bottom panel* shows how the SNAP-tag method was applied to study Stx transport to the Golgi and ER in this work. For the Golgi localization, SNAP-tag was fused with first 120 amino acids of galactosyl transferase (GalT). For the transport to the ER and retention, SNAP-tag was fused with hen lyzozyme (ER) and KDEL sequences. The EGFP was also included and served for visualization and immunoprecipitation of the constructs. During the intracellular transport, Stx-BG molecules meet the SNAP-tag constructs localised to the Golgi (*left*) or the ER (*right*) and become covalently bound to the SNAP-tag construct. The SNAP-tag labelled Stx is then immunoprecipitated by using GFP-trap and quantified.

Quantitative MS analysis of lipids

The advances in mass spectrometry (MS) technology have made it possible to obtain detailed information of the lipidome, including precise quantification of single lipid species [290]. Quantitative MS analyses of lipids were performed in all three papers included in this thesis. Various MS techniques, including shotgun lipidomics and sequential MS/MS analysis combined with reverse phase ultra-high pressure liquid chromatography, were employed in the analyses of different lipid classes. Deuterium-labeled or heptadecanoyl-based synthetic internal standards were added to samples and used for quantification of the endogenous lipid species. In paper I, MS analysis revealed that HEp-2 cells adapt their lipidome in response to cell crowding in culture, and thus suggested a possible link between changes in lipid composition and other cellular functions, such as retrograde transport. In papers II and III, by performing MS analyses we have discovered that the glucose analogues 2DG and FDG modify the levels and composition of various cellular lipids. Importantly, by this approach we were able to show that 2DG becomes incorporated into newly synthesized glycosphingolipids. However, since the MS analyses were performed on whole cell lysates, the link between the changes in lipid composition and the functions of specific organelles or transport pathways can only be speculative. Although giving very detailed information of the overall changes, MS based analyses lack information of the spatial and temporal distribution (transient local changes) of specific lipids in the cell. The spatial distribution of certain lipids could be obtained by combining MS analysis with advanced isolation methods of different cellular organelles. However, at the moment there are no good enough purification methods developed for isolation of the Golgi or the ER membranes to perform such studies. A small contamination of, for instance, ER in a Golgi preparation, might give misleading results. Therefore, for studying transient and localized changes in lipids one needs to use specific lipid-binding (fluorescent) probes or fluorescent lipid analogues in combination with high resolution live cell imaging [291]. However, an introduction of artificial lipids and/or lipid probes might affect the natural distribution and/or functions of endogenous lipids, and thus such data should be interpreted with care. Microscopy-based approaches to study lipids in cells are now being implemented in our laboratory to study how certain lipids affect specific intracellular transport pathways.

Conclusions and Perspectives

There is a growing awareness of the role of specific molecular species of lipids in intracellular transport, cell cycle control and cell-cell interactions, among other cellular processes (for review see [16]). The MS analysis is now providing increasing knowledge about eukaryotic lipidomes and their flexibility, which may help to understand the complex relationship between certain lipids and essential cellular functions. In paper I, we show that cultured cells change their lipid composition, intracellular trafficking and gene expresion in response to increasing cell density. Thus our study shows that there is a need for a better knowledge of cell density induced molecular changes, since such changes may significantly affect the interpretation of the data acquired in cell-culture based studies.

One of the purposes of this work was to elucidate possible effects of two well known glucose analogues, namely 2DG and FGD, on intracellular transport and cellular lipids, which might then open up for novel applications of these drugs. The results presented in paper III suggest at least two new potential applications of FDG. First, we have discovered that FDG treatment protects cells against Stx, and, more importantly, against Stx2, which is more potent *in vivo* than Stx [145, 146] and is more often associated with life-threatening infections in humans [147, 292]. As there is still a lack of effective treatment for STEC-infected patients, FDG should be investigated for its therapeutic potential against STEC infections, especially keeping in mind, that FDG is already used in the clinic. However, further studies are required to elucidate the exact mechanism by which FDG protects cells against Stx, as this might lead to the development of even more potent agents against Stx and STEC infections. For instance, additional studies using other ligands following the retrograde pathway, as well as the ones that follow the anterograde route, such as vesicular stomatitis virus G protein (VSVG), could reveal the specific steps in the intracellular transport that are affect by FDG (and 2DG). In addition to its effect on Stx transport, we have also discovered that FGD inhibits GlcCer synthesis. An increased expression of GlcCer is often associated with multidrug resistance in cancer, and several GlcCer synthase inhibitors are now being tested in the clinic as an adjuvant treatment for resistant cancers to restore their sensitivity to chemotherapy (for review see [106]). Based on our data, FDG effectively blocks GlcCer synthesis in the cells, however, the underlying mechanism of the inhibition is not yet known. Thus, further studies should be performed to

understand how FDG affects GlcCer synthesis, e.i. does it have a direct inhibitory effect on the enzyme, does it compete with glucose to be transferred onto ceramide, or does it act indirectly by other means. These are some of the questions that should be answered in the future.

Finally, the articles included in this thesis also demonstrate the usefulness of using protein toxins to study intracellular transport and drugs that affect cellular processes.

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