Membrane morphology and homeostasis

An electron microscopy approach

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Cover illustration: Electron micrograph of a HEK293 cell under palmitic acid stress.

Membrane morphology and homeostasis: An electron microscopy approach

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“The difference between the novice and the master is that the master has failed more times than the novice has tried.”

-Koro Sensei

ABSTRACT

Biological membranes allow life as we know it to exist. Primarily assembled from lipids and proteins, their morphology and function can greatly vary between species or even within the same cell. They create physical boundaries, separating different cells from each other and forming functional compartments within the cells. The composition and properties of biological membranes must be continuously attuned to their vast variety functions, and this is achieved within the cell by essential homeostatic processes. This thesis studies membranes from two different perspectives. The first focuses on membranes as a means of transport of biological material between compartments, namely between the nucleus and the cytoplasm. The standard view is that nuclear pore complexes act as the main gateways for passive or active transportation of molecules across the nuclear envelope. We have now shown that nuclear membranes are able to form buds enclosing biological material, thus suggesting a hitherto unappreciated second route for transport in and out of the nucleus. While these nuclear envelope buds (NEBs) are present in unperturbed cells, their frequency increases significantly in response to a variety of stress conditions. We also found that NEBs are evolutionarily conserved and were found in all organisms studied: a protozoan protist, two yeast species, a nematode, and a human cell line.

The second part of this thesis focuses on an important molecular pathway that contributes to membrane homeostasis. Challenging cultured cells with an excess of saturated fatty acids, such as palmitic acid (PA), causes membrane rigidification and endoplasmic reticulum (ER) stress, accompanied by defects in several biological processes, increased accumulation of reactive oxygen species (ROS), and apoptosis/cell death. The protein AdipoR2 acts to counter membrane rigidification and therefore increases the tolerance of cells towards excess saturated fatty acids (SFAs). We used electron microscopy to describe, at unprecedented resolution, specific membrane defects caused by an excess amount of PA in control cells and cells lacking a functional AdipoR2. We found that the ER, mitochondrial membranes, and nuclear envelope are all impacted by PA treatment, with the effects exacerbated in cells lacking AdipoR2. In particular, the ER of PA-challenged cells often presented a unique morphology resembling either straight lines or in some cases spiral-like structures. A conclusion from this work is that AdipoR2 is required to prevent severe deformation of cellular membranes, especially when cells are challenged with exogenous SFAs.

Keywords: nuclear envelope bud, AdipoR2, membrane homeostasis

SAMMANFATTNING PÅ SVENSKA

Biologiska membran tillåter livet som vi känner det att existera. Främst sammansatta av lipider och proteiner, kan deras morfologi och funktion variera kraftigt mellan arter och inom samma cell. Membran skapar fysiska gränser, separerar olika celler från varandra och bildar funktionella fack i cellerna. Sammansättningen och egenskaperna hos biologiska membran måste ständigt anpassas till deras mångfaldiga funktioner, och detta uppnås inom cellen genom homeostatiska processer. Denna avhandling studerar membran ur två olika perspektiv. Det första fokuserar på membran som transportmedel av biologiskt material mellan cellulära avdelningar, nämligen mellan kärnan och cytoplasman. Standardsynen är att kärnporkomplex fungerar som portarna för passiv eller aktiv transport av molekyler över kärnmembranet. Vi visar här att kärnmembran kan bilda knoppar som omsluter biologiskt material, vilket uppmärksammar en hittills ignorerad andra väg för transport in och ut ur kärnan. Medan dessa kärnhöljeknoppar (NEB) finns i ostörda celler ökar deras frekvens avsevärt som svar på en mängd olika stressande tillstånd. Vi fann också att NEB är evolutionärt bevarade och hittades i alla undersökta organismer såsom en protozoan, två olika jästarter, en nematod och en mänsklig cellinje.

Den andra delen av denna avhandling fokuserade på en viktig molekylär väg som bidrar till membranhomeostas. Att utmana odlade celler med ett överskott av mättade fettsyror, såsom palmitinsyra (PA), orsakar membranstelning och endoplasmic reticulum (ER)-stress, åtföljd av defekter i flera biologiska processer, ökad ackumulering av fria radikaler och apoptos/celldöd. Proteinet AdipoR2 verkar för att motverka membranstelning och ökar därför toleransen hos celler mot överskott av mättade fettsyra (SFAs). Vi använde elektronmikroskopi för att med oöverträffad upplösning beskriva specifika membrandefekter orsakade av en överskottsmängd PA i kontrollceller och celler som saknar en funktionell AdipoR2. Vi fann att ER, mitokondriemembranen och kärnhöljet alla påverkas av PA-behandling, med förvärrade effekter i celler som saknar AdipoR2. I synnerhet presenterade ER i PA-utmanade celler ofta en unik morfologi som liknar antingen raka linjer eller i vissa fall spiralliknande strukturer. En slutsats från detta arbete är att AdipoR2 krävs för att förhindra allvarlig deformation av cellulära membran, särskilt när celler utmanas med exogena SFAs.

Nyckelord: kärnhöljesknopp, AdipoR2, membranhomeostas

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Nuclear envelope budding is a response to cellular stress.**
Panagaki, D.*, Croft, J.* T., Keuenhof, K., Larsson Berglund, L., Andersson, S., Kohler, V., Büttner, S., Tamás, M. J., Nyström, T., Neutze, R., et al. (2021). *Proceedings of the National Academy of Sciences, 118*(30), e2020997118. doi:10.1073/pnas.2020997118

II. **Palmitic acid induced membrane deformations in AdipoR2 depleted cells.**
Panagaki D., Ruiz M., Devkota R., Höög L. J., Neutze R., Pilon M.
Manuscript in preparation

III. **Sphingosine 1-Phosphate Mediates Adiponectin Receptor Signaling Essential for Lipid Homeostasis and Embryogenesis.**
Ruiz, M., Devkota, R., Panagaki, D., Bergh, P.-O., Kaper, D., Henricsson, M., Nik, A., Petkevicius, K., Höög, J. L., Bohlooly-Y, M., et al. (2022). *Nature Communications, 13*(1), 7162. doi:10.1038/s41467-022-34931-0

Other contributions that are not included in this thesis:

I. Striking Membrane Defects in AdipoR-Deficient Cells.
Ruiz, M.*, Panagaki, D.*, Höög, J. L., & Pilon, M. (2023).
Journal of Lipid Research, 64(3).
doi:10.1016/j.jlr.2023.100335

II. Nuclear envelope budding and its cellular functions.
Keuenhof KS, Kohler V, Broeskamp F, Panagaki D, Speese SD, Büttner S, Höög JL.
Nucleus. 2023 Dec;14(1):2178184. doi: 10.1080/19491034.2023.2178184. PMID: 36814098; PMCID: PMC9980700.

**These authors contributed equally to the manuscript.*

CONTENT

ABBREVIATIONS

AdipoR1	Adiponectin receptor 1
AdipoR2	Adiponectin receptor 2
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate-activated protein kinase
ATP	Adenosine triphosphate
AZC	Azetidine-Z-carboxylic acid
CTR	C-terminus
DKO	Double Knock out
EM	Electron microscopy
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
ET	Electron tomography
FRAP	Fluorescence recovery after photobleaching
GPCR	G protein-coupled receptor
HAP1	Near-Haploid human cell line 1
HEK293	Human embryonic kidney 293 cells
HMC-1	Human Mast cell line 1
IMM	Inner mitochondrial membrane
INM	Inner nuclear membrane

LPC	Lysophosphatidylcholine
MEF	Mouse embryonic fibroblast
MUFA	Monounsaturated fatty acid
NEB	Nuclear envelope bud
NEC	Nuclear egress complex
NPC	Nuclear Pore Complex
NTR	N-terminus
OA	Oleic acid
ONM	Outer nuclear membrane
PA	Palmitic acid
PAQR	Progestin and adipoQ receptor
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PINK1	PTEN-induced putative kinase protein 1
PKC	Protein kinase C
PPARγ	Peroxisome proliferator-activated receptor gamma
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
RNP	Ribonucleoprotein
ROS	Reactive oxygen species

RXR	Retinoid X receptor
S1P	Sphingosine 1 phosphate
S1PR	Sphingosine 1 phosphate receptor
SCD	Stearoyl-CoA desaturase
SEM	Scanning electron microscopy
SFA	Saturated fatty acid
sn	Stereochemical numbering
SREBP1	Sterol regulatory element-binding protein-1
TEM	Transmission electron microscopy
TNF	Tumor necrosis factor
U-2 OS	U-2 Osteosarcoma cell line
UFA	Unsaturated fatty acid

INTRODUCTION

This thesis will start with a brief introduction on biological membranes, both in terms of structure and functionality. It will focus on the lipid composition of cellular membranes and how failure in its homeostasis may affect some aspects of lipotoxicity. This work can be divided into two main subjects. The first subject is the formation of nuclear envelope buds as a means of communication between the nucleus and the cytoplasm, whereas the second subject focuses on a molecular mechanism for the detection and remediation of imbalances in membrane composition. This mechanism is dependent on proteins called AdipoR1 and AdipoR2 that will be discussed in some detail. Samples from several model organisms were studied during the course of this doctoral research, ranging from the protist *Trypanosoma brucei* up to human cell lines. In this thesis however, greater attention will be given to the better-established models, namely human cell lines, the budding yeast *Saccharomyces cerevisiae,* and the round worm *Caenorhabditis elegans*.

CELLULAR MEMBRANES

Composition of biological membranes

Biological membranes are fundamental for the formation of what we consider the basic unit of life, the cell. Since the formulation of the Cell Theory around 1839 and the broad acceptance of the fluid mosaic model in 1972, many different theories and models had been proposed to account for the morphology and composition of cellular membranes[1-3]. Modern approaches have gradually given us a very clear understanding of the basic structure and functionality of membranes. Following the broadly accepted fluid mosaic model, cellular membranes are taken to basically consist of lipids, and several proteins either attached to the lipid surface or embedded within the bilayer. Carbohydrates

could also be considered as part of the membrane configuration, as they often attach to proteins of the membranous surface. There are three different categories of lipids that are found in biological membranes, namely phospholipids, glycolipids and sterols with the most abundant ones being the phospholipids[4, 5]. Phospholipids consist of two fatty acid chains linked to a hydrophilic head group of various compositions, most often containing glycerol and a phosphate group, namely glycerophospholipids. Different molecules such as choline, serine, ethanolamine, and inositol can then be attached to the phosphate group creating specific types of glycerophospholipids, named phosphatidylcholine (PC) (Figure 1A), phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) respectively. In the case where the glycerol is substituted by a sphingosine, we then have the formation of a sphingophospholipid, such as sphingomyelin[4, 6, 7].

Glycolipids on the other hand, contain a glycerol or a sphingosine molecule attached to a sugar molecule, such as glucose, in contrast to the phosphate group found in a phospholipid (Figure 1B). Besides the difference in their head region, the tail region resembles the one of the phospholipids, consisting of two fatty acid chains. In the case of a sphingosine molecule being attached to the sugar molecule, we have the formation of glycosphingolipids such as the glycosylated ceramides lactosylceramides and glucosylceramides[4, 8].

Sterols can also be structurally important components of cellular membranes; in which case they consist of a single polar hydroxyl group attached to a four or five-ring steroid structure and a short hydrocarbon side chain (Figure 1C). The structural importance of sterols is especially evident in the plasma membrane of mammalian cells where cholesterol often amounts to ~45% of total lipids. In contrast, the nematode *C. elegans* does not seem to use sterols

as an important structural component of its membranes, though it must acquire dietary sterols as precursors for signaling molecules[4, 6, 9, 10].

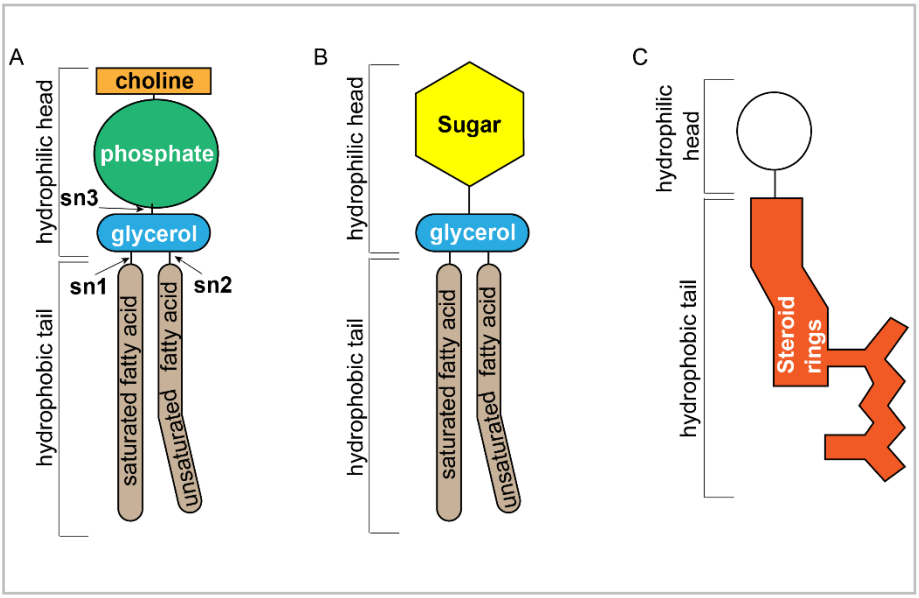

Figure 1. Schematic representation of lipids. (A) Structure of a typical phosphatidylcholine with a glycerol molecule connected to the phosphate group and the choline molecule. The hydrophobic tail shows a typical composition of a saturated fatty acid in sn1 position, an unsaturated fatty acid in sn2 position and a phosphate group in the sn3 position of the glycerol molecule. (B) Structure of a glycolipid with a sugar molecule attached to a glycerol molecule instead of the phosphate group of the phospholipids. (C) Structure of a sterol with a single polar hydroxyl group attached to the steroid rings and the short hydrocarbon side chain.

Small alterations on these three main categories of biological lipids can produce a variety of other lipids such as in the case of lysophospatidylcholines (LPCs) which are the derivative of a PC that lost one of its fatty acid chains[11]. Ceramides are also a specific sphingolipid class consisting of a sphingoid base attached to a FA. If a ceramide gets converted into a sphingosine and a free

fatty acid, further phosphorylation of the sphingosine molecule will result in a sphingosine-1-phosphate (S1P) lipid[12]. Moreover, unique types of phospholipids can also be found in specific cellular compartments such as the cardiolipins which consist of a double phosphate head group attached to four fatty acid chains and is solely localized in the inner mitochondrial membrane (IMM)[13].

For easier navigation and description of the molecules that organize a lipid, the stereochemical numbering (sn) system is used (Figure 1A). As an example, in a typical phospholipid the sn1 and sn2 positions of the glycerol will be occupied by the fatty acid chains whereas the sn3 position will be used for the attached phosphate group[14] (Figure 1A).

All membrane lipids have an amphipathic nature, where their head group acts as a hydrophilic region and the tail as a hydrophobic region. That particular double nature is what initiates the formation of a double bilayer which characterizes biological membranes. As the head group constantly seeks for an aqueous environment and the hydrophobic tails avoid such contact, lipids tend to form liposomes when placed in water, which are basically spheres of lipid bilayers safely enclosing the hydrophobic tails within the bilayer and exposing the head groups to water (Figure 2). Such spontaneous configuration provides the most favorable state for the lipids and is the basis for all biological membrane assembly[4, 6].

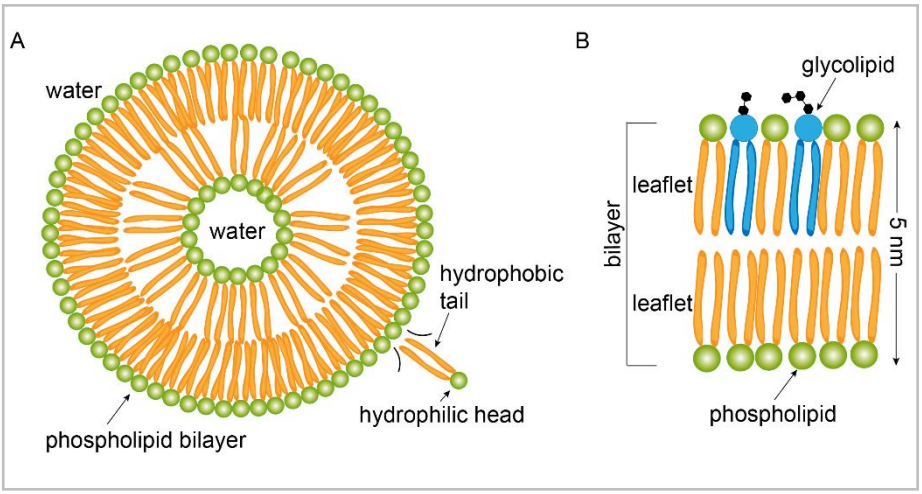

Figure 2. Schematic representation of a liposome and a membrane bilayer. (A) Representation of a spontaneous formation of a liposome. The hydrophilic head groups (green spheres) will always orient towards the aqueous environment whereas the hydrophobic tails (orange tubes) will face inwards away from the water. (B) Structure of a lipid bilayer where different types of lipids form an inner and outer leaflet of approximately a total of 5 nm in width.

Properties of membrane lipids

Phospholipids and glycolipids share a similar structure with a hydrophilic head group attached to their hydrophobic tail. The composition of the head group can vary and can also influence the physical properties of the membrane. PCs and PSs are cylindrical lipids and when packed together, they form a flat lipid bilayer. PEs on the other hand have a smaller head group so when packed together the formed bilayer bends towards the head groups creating a negative curvature in the membrane structure. Lipids such as LPCs which have a larger head group, create an opposite feature where the bilayer positively bends away from the head groups[15].

In addition, the length and composition of the fatty acid chains of the tail strongly influences both the curvature and fluidity state of the membranes[16, 17].

Phospholipids form ester bonds with the two fatty acid chains at the sn1 and sn2 positions of the glycerol molecule. The most common arrangement of a phospholipid would have a saturated fatty acid (no double bonds in their carbon chain) occupying the sn1 position of the phospholipid whereas the sn2 position can be occupied by either a saturated or an unsaturated (one or more double bonds in their carbon chain) fatty acid[18, 19]. The unsaturated fatty acids can be further categorized based on the position and number of double bonds found in their carbon chains. Monounsaturated acids such as oleic acid (OA, C18:1) would have only one double bond in their chain whereas polyunsaturated acids such as eicosapentaenoic acid (EPA, C20:5) could have more than one double bond (Figure 3A)[20, 21]. The presence of such double bonds is what creates pronounced kinks in the acyl chain, which causes stereological obstacles preventing a tightly packed formation of the lipid bilayer thus altering the packing density of such membranes (Figure 3B)[19, 22, 23].

The length of the carbon acyl chain is yet another variant that can influence the properties and structure of the membranes. As a rule of thumb, shorter fatty acid chains can shift from a rigid to a fluid state at lower temperatures compared to longer chains. Thus, phospholipids with short chains tend to keep their fluid state easily whereas membranes containing longer chains tend to form a more tightly packed and thick bilayer[24]. In summary, the composition of the head groups as well as the saturation and length of the fatty acid chains can greatly vary between phospholipids and significantly influence the properties of the membranes such as structure, curvature, and fluidity.

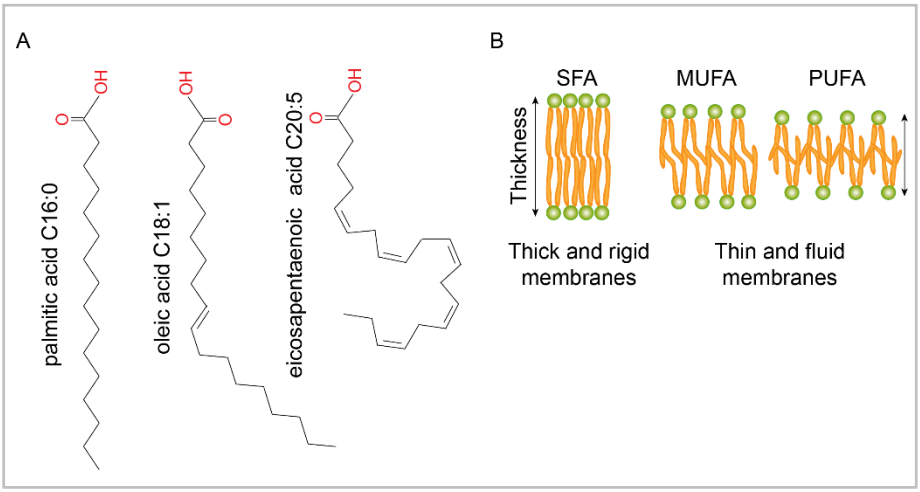

Figure 3. Fatty acid chains and membrane packing. (A) Structure of three representative fatty acids of different saturation levels. First from left is the saturated PA (C16:0) with no double bonds, in the middle is the monounsaturated OA (C18:1) with a single double bond and last is the polyunsaturated EPA (C20:5) with five double bonds. (B) Levels of membrane packing based on the saturation of the phospholipids. Membranes with mainly saturated fatty acids are tightly packed and rigid whereas membranes containing mono- or poly- unsaturated fatty acids are loosely packed, more fluid and form a thinner bilayer.

Membrane homeostasis

Biological membranes not only provide the basis for the structure and compartmentalization of eukaryotic cells but also play an important role in several cellular processes such as vesicular trafficking, maintenance of ionic and osmotic gradients, harboring of receptors, transporters, and channels through which they control the passage of proteins and molecules between compartments and among cells[4, 25, 26]. Their lipid composition provides them with the important properties of fluidity and elasticity. The term fluidity describes the general mobility of membrane lipids and proteins within a

phospholipid bilayer[27]. Under normal conditions, cellular membranes do not particularly show the same fluidity levels throughout the cell but have specific areas of higher and lower rigidity. Such areas include assemblies of higher rigidity, namely lipid rafts, which contain a concentrated amount of cholesterol as well as saturated fatty acids into their phospholipids[28]. Though membranes have this general heterogeneity in their fluidity levels, a proper balance between the saturated and unsaturated ratio in the phospholipids is of great importance. Any extreme situation that would lead to either over-rigidified or over-fluidized membranes could be disastrous for the survival of the cell. Such deviations can jeopardize basic cellular functions, inhibiting protein trafficking and vesicle formation and eventually leading to activation of apoptotic pathways[29-31]. Thus, the maintenance of membrane homeostasis and the existence of a mechanism to detect and regulate such deviations is of obvious importance.

Different factors that can alter the fluidity state of a membrane include temperature deviations, degree of saturation in the fatty acid chains of the phospholipids, length of the fatty acid chains, the presence of sterols in the membrane region as well as incorporation of fatty acids through diet[32]. Several disease conditions, such as Alzheimer's and Huntington's disease, correlate with abnormal composition and properties of cell membranes, suggesting a causal relationship[33]. Recent studies have revealed several sense-and-response proteins that tightly regulate membrane properties. For the purpose of this thesis, we will focus on the mammalian AdipoR proteins and their homologs in *C. elegans*.

THE ADIPONECTIN RECEPTORS

Structure and functionality

The Adiponectin Receptor proteins AdipoR1 and AdipoR2, also known as PAQR1 and PAQR2 respectively, are two of the 11 members of the PAQR (progestin and adipoQ receptors) protein family encoded by the human genome. PAQR proteins share distinct structural characteristics. Firstly, they all contain seven transmembrane domains with their N-terminus being cytoplasmic whereas their C-terminus faces towards the extracellular space (i.e., an orientation opposite to that of G protein-coupled receptors, GPCRs, with which they show no sequence homology). Additionally, PAQR proteins belong to the larger CREST protein family of hydrolases, and specifically can act as ceramidases[34-36]. The crystal structures of AdipoR1 and AdipoR2 have been described and reveal a cylindrical barrel conformation with a large internal cavity open towards the cytoplasm. A zinc-binding site within the seven transmembrane domains and closer to the intracellular surface likely coordinates the structure. Recent updated versions of the crystal structure have also confirmed the presence of a free fatty acid, which was later identified as an OA molecule within the large internal cavity, suggesting that the cavity can potentially accommodate large fatty acids or similar molecules (Figure 4)[37].

The AdipoRs were initially identified as putative receptors for adiponectin, an abundant serum C1Q/Tumor necrosis factor (TNF) type protein secreted strictly by adipocytes [38]. Adiponectin is a protein most widely known for its stimulatory role in glucose utilization. The expression levels of adiponectin in plasma samples have been linked to obesity as there is a reduction of adiponectin in obese humans. Such reduction has further been associated to glucose intolerance and type-2 diabetes[39, 40]. For many years, the AdipoRs have been implicated with the progression of type-2 diabetes due to the belief that

Adiponectin is its natural ligand. Recent studies though have given a new role and functionality to these receptors.

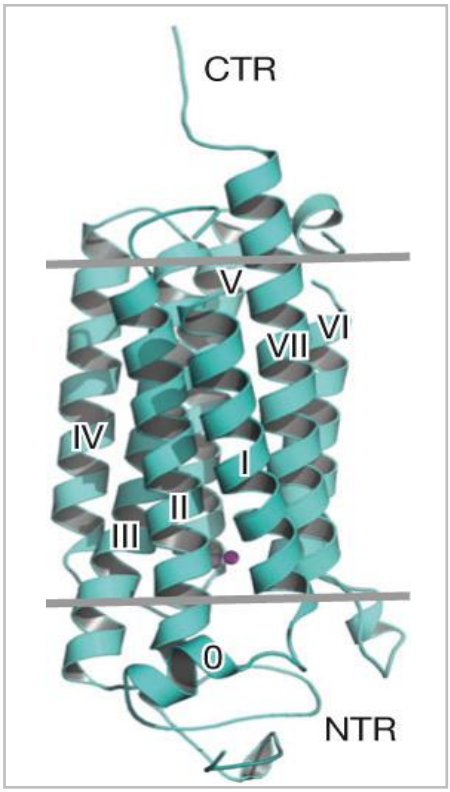

Figure 4. Crystal structure of the AdipoR2. 3D representation of the crystal structure of the AdipoR2 receptor, depicting the seven transmembrane domains (I-VII), the intracellular N-terminus (NTR) and the extracellular C-terminus region (CTR). The Zn atom is presented as a purple sphere. The AdipoR2 structure information was obtained from the following reference [37].

A growing number of studies suggest that the molecular function of the AdipoRs is to sense and remedy membrane composition defects[41]. As mentioned already, phospholipids typically consist of a saturated and an unsaturated fatty acid in their tails. The amount of saturated and unsaturated fatty acids can vary among the length of the membrane, and this strongly

influences membrane properties such as fluidity, used here as a general term encompassing lateral mobility, packing density and deformability. Though specific areas of high or low fluidity in the membranes are normal and contribute to a membrane's functionality, the overall composition of the membrane must be maintained within a certain acceptable range to avoid excess fluidity or excess rigidity[42]. Any serious disturbances in the rigidity levels of the membranes must therefore be detected and rectified; the AdipoRs play a significant role in this homeostatic process. Specifically, the AdipoRs detect excessive rigidity caused by high levels of saturated fatty acids (SFAs) in the phospholipids and signal to stimulate expression of fatty acid desaturases that can then convert SFAs into unsaturated fatty acids, restoring membrane homeostasis[36, 41, 43].

The AdipoRs signaling pathway

Though both AdipoR1 and AdipoR2 receptors are involved in the maintenance of membrane homeostasis, it has been shown that AdipoR2 has a stronger influence in this pathway in comparison to AdipoR1. Silencing of the AdipoR2 receptor often results in an increase in membrane rigidity, slower cell growth, and lower tolerance to SFA supplementation. Similar defects become apparent but less severe in the AdipoR1 depleted cells whereas silencing of both receptors contributes to a greater effect[36, 44, 45].

Growing evidence is pointing out a previously undiscovered ceramidase activity of AdipoR1/2[46]. It is believed that the AdipoRs can act as a ceramidase that catalyzes the hydrolysis of ceramides producing sphingosine and free fatty acids. The formed sphingosine molecule can then be phosphorylated by sphingosine kinases to create the S1P signaling molecule[47]. Furthermore, S1P separately activates the SREBP1 and PPARγ transcription factors. Both these molecules have now been shown to increase the expression of fatty acid

desaturases such as the stearoyl-CoA desaturase (SCD) and promote the formation of unsaturated fatty acids which can restore membrane homeostasis[48].

AdipoR2 has also been reported to play a role in the progression of cancer such as in colorectal cancer, human endometrial adenocarcinoma, prostate cancer as well as glioblastoma[49-52]. In these disease models, it has been shown that AdipoR2 may act through the adenosine monophosphate-activated protein kinase (AMPK)/mTOR pathway. AMPK is a sensor of energy homeostasis triggered by imbalances in the adenosine monophosphate (AMP)/adenosine triphosphate (ATP) and the adenosine diphosphate (ADP)/ATP ratios. Overexpression of AdipoR2 can lead to arrest in cell growth and inhibition of cell proliferation in cancer cells but inhibition of the AMPK/mTOR pathway is able to rescue these AdipoR2-induced effects[50].

These observations indicated a downstream role of AMPK in the AdipoR2 pathway but there is no evidence so far of AMPK-related ceramidase activity of the AdipoRs, implying that the adiponectin receptors may activate different pathways depending on the triggering factors and the cell models involved.

HISTORY OF NUCLEAR ENVELOPE BUDDING

Membranes as a dynamic compartment

At a first glance, membranes appeared to be structures necessary for the compartmentalization of the cell. They create a physical barrier around the cell, separating it from the surrounding environment and form specific compartments in their interior space such as the endoplasmic reticulum (ER), mitochondria, the Golgi system, for the eukaryotes, the double nuclear envelope. Often, these compartments share connections to one another through

their membranous nature such in the case of the ER, the nuclear envelope, and the plasma membrane[4, 24, 53].

Besides this fundamental functionality of cellular membranes, they possess further characteristics that have been gradually discovered over the years. Even if their structure seems to be rather stable and robust, membranes are indeed quite a dynamic compartment. They often are decorated with various proteins that either penetrate through their lipid bilayers or bind directly to their surface. These proteins can act as receptors or channels that upon stimulation activate specific pathways and cellular functions. Such membrane bound molecules are often able to move across the phospholipids or even between bilayers. Free cytoplasmic or extracellular molecules of small sizes (about 30 to 40 kDa) can passively penetrate membranes and translocate freely between compartments. In the case of lipophobic molecules, specific intramembrane channels can facilitate the passive transportation of such cargos. For bigger molecules, specific energy-dependent channels exist that could selectively process the passage of these molecules[54-56].

One famous example of such selective transportation is through the nuclear pore complexes (NPCs). An NPC is basically a 120 MDa protein complex consisting of about 30 different proteins, namely nucleoporins or Nups. Due to their size and complex arrangement, it has been proven challenging to determine their structure, but employment of advance techniques such as cross-linking mass spectrometry and cryo-electron tomography have facilitated in the depiction of a near-atomic overall architecture[57]. NPCs are located in the double membranes of the nuclear envelope and are important for both the passive and active nucleocytoplasmic transportation of molecules and ions[58]. NPC related mutations that would hinder the functionality of these channels, have been connected to several human diseases such as cancer, neurological

diseases, and autoimmune diseases, highlighting the importance of these structures[59-61].

First observations of a budding nucleus

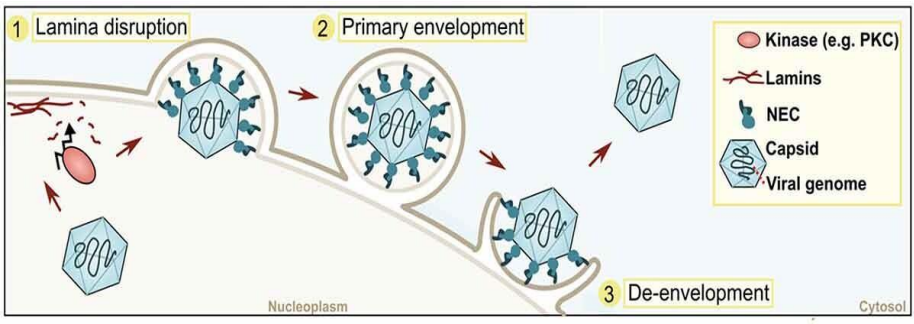

Figure 5. Viral egress of Herpes virus. Schematic representation of the egress pathway for Herpes virus. After breaking through the lamina barrier, the viral capsid goes through an envelopment and de-envelopment process before exiting the nucleus. NEC; nuclear egress complex, PKC; protein kinase C.

For a long time, NPCs have been considered to be the only gateway allowing molecules to go in and out of the nucleus. Perhaps the first well characterized exception to this belief was the description of the egress pathway for a variety of viruses such as the Herpes virus complex. These types of viruses use a peculiar way of exiting the nucleus of the infected cell by creating small buds that help them escape through the double nuclear envelope (Figure 5)[62, 63]. This pathway has been thoroughly described but always in the scope of a viral infection. It is a common belief though that viruses often copy already existing cellular pathways and use them to their benefit. Thus, the fact that such budding events have actually been previously noted in healthy cells since 1955[64] does not come as a surprise but the limited work that has been performed around them is indeed unanticipated.

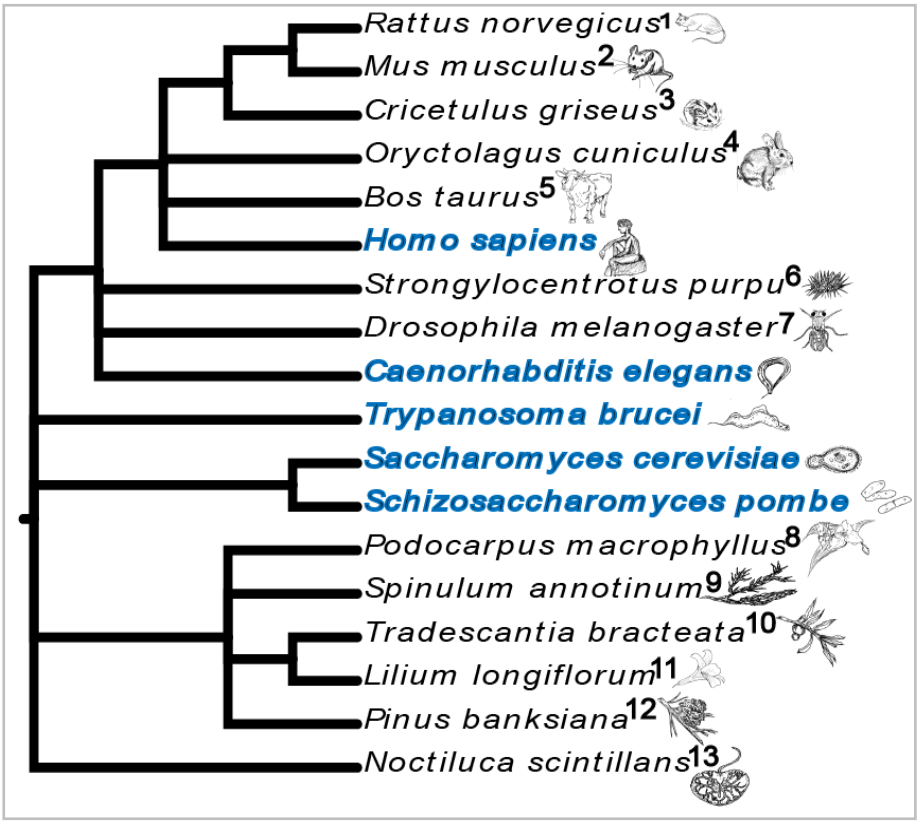

Figure 6. Organisms with observed NEB events. Phylogenetic tree of all organisms where NEB has been noted. In blue are the species that were investigated during my doctoral projects.

Several publications dating back to 1955 have described observations resembling the viral nuclear buds and have appointed a variety of names to these events, from nuclear blebbings to nuclear envelope "outpocketings" (Table 1). Interestingly, all these observations were made in a wide range of model organisms indicating a potential evolutionarily conserved nature of the nuclear budding pathway (Figure 6). The first publication to properly investigate potential functions and the nature of the transported cargo came quite later in 2012 where *Speece et. al*. worked on the fruit fly *D. melanogaster* and discovered that large ribonucleoproteins (megaRNPs) used NEBs to exit

the nucleus of larval muscle cells. These buds appear to follow quite a similar pathway to that of the Herpes virus egress. As a first step, the nuclear lamina must be disrupted, a process involving several phosphorylation events and the recruitment of the protein kinase C (PKC)[65]. Once the cargo is able to approach the INM, a membrane invagination and cargo envelopment stage begins. The transported material can then de-envelope at the ONM and successfully exit the nucleus in order to translocate to the assigned destination[65, 66].

A later study in the budding yeast *S. cerevisiae* demonstrated that aged cells with a higher number of deteriorated and misassembled NPCs, formed similar blebbings between the INM and ONM to that of the previously described NEBs[67]. The formed blebbings were believed to be the result of a hindered NPC assembly and were described as a means of evidence for the age-induced NPC defects. Later experimental studies though have shown these events to be independent and for NEBs to not always represent a misassemble NPC[68].

Table 1. List of organisms where NEB events have been previously observed, presented in a chronological order.

Organism	Cell type	Terminology
D. melanogaster[64]	Salivary gland cells	Membrane "outpocketings"
O. cuniculus[69]	Blastocysts	Nuclear extrusion
N. scintillans[70]	unicellular	Annulated vesicles
C. griseus[71]	Embryo cell line A_1	Nuclear budding
R. norvegicus[72]	Fertilized oocytes	Nucleolar extrusion
B. taurus[73]	Fibroblastic cells	Nuclear buds
T. bracteata[74]	Developing microspores	Membrane bounded bodies
P. macrophyllus[75]	Haploid microspore tetrads	Invaginations of nuclear envelope
L. annotinum L.[76]	Immature spores	Extrusions/bulges
P. banksiana[77]	Post meiotic microspores	Folding of nuclear envelope
L. longiflorum[78]	Young microspores	Membrane-bound inclusions
D. melanogaster[79]	Salivary gland cells, midgut cells	Pod-like infoldings of the nuclear envelope
M. musculus[80]	Zygotes, early embryos, hybrid cells	'Blebbing' of nuclear envelope
D. melanogaster[65]	Larval muscle cells	Nuclear envelope budding
S. purpuratus[81]	Embryonic cells	Nuclear egress
S. cerevisiae[67]	Aging mitotic cells/NPC assembly mutants	Herniations at the nuclear envelope
D. melanogaster[82]	Salivary gland cells	Nuclear envelope budding

Nuclear envelope budding and cellular stress

The first project of this thesis is entirely dedicated to the investigation of NEBs and has added more information on the nature of the transported cargo supplementing the work that has been done so far on these events. It has been proven that these NEBs exist in many different organisms and are part of a normal cellular function as they appear even in undisturbed and healthy cells. The frequency of them though varies significantly between species as human cell lines would show a frequency of up to 9% of cell sections, whereas they seem rarer in the budding yeast *S. cerevisae* (about 2 to 3% of cell sections)[68].

When *S. cerevisae* cells undergo different stress conditions, such as heat shock or arsenite treatment, NEB events become more frequent revealing a correlation between cellular stress and the importance of these events. Further investigation showed that the NEB pathway is linked to the protein quality control system as inhibition of the proteasome machinery can also trigger the appearance of nuclear buds. It has been now recognized that misfolded proteins are also part of the transported cargo that uses NEB as a means to escape the nuclear envelope barrier. In combination to the previous work from *Speece et al*., it becomes apparent that the NEB cargo can be of different nature, size, and composition but most often it appears to include molecules of complex structure and larger size which would probably be incapable of penetrating the NPC pathway[65, 68, 83].

AIM

The aim of this thesis is to investigate morphological changes of biological membranes under different cellular environments with the use of transmission electron microscopy. **Paper I** focuses on the formation of nuclear envelope blebbings, namely NEBs, as a normal cellular occurrence but also in response to cellular stress. These membrane protrusions enclosing cargo of various nature were investigated for their morphology, size, and frequency of appearance in several organisms but especially in the budding yeast *S. cerevisiae*. **Paper II** and **Paper III** shift the focus of this thesis into the study of membrane and lipid homeostasis. Membrane deformations in cells lacking the receptors responsible for sensing and maintaining lipid homeostasis, AdipoR1 and AdipoR2, were observed and studied for their frequency, and possible implications in normal cellular processes. **Paper III** compiles a thorough description of the AdipoR1/2 pathway and sheds light into the downstream molecules that are implicated in the process of restoring lipid imbalances.

METHODS

This thesis is built around one specific technique, namely electron microscopy (EM). There are several different types of electron microscopy that will only briefly be discussed in this section, but the main focus will be given to room temperature transmission electron microscopy.

History of electron microscopy

The invention of the electron microscope by Max Knoll and Ernst Ruska dating back to 1931, gave scientists the first opportunity to break through the limitations in resolution of the widely used optical microscopes[84, 85]. As a general rule, the maximum theoretical resolution of an acquired image depends on the wavelength (λ) of the source of radiation ($r = \lambda/2NA$, where NA refers to the numerical apparture). Therefore, optical microscopes which use light for visualization of a sample, theoretically have a limited resolution of about 200 nm, even though this has now been circumvented by ultra-resolution light microscopy methods. A 200 nm resolution is sufficient for good visualization of tissues, entire cells and organelles but is not enough to reveal intracellular molecular details[86, 87]. To overcome this limitation, the source of radiation had to shift to something with a lower wavelength compared to light. Though the earliest electron microscopes were not able to reach a significantly higher resolution, they managed to prove an important concept: electron beams could be used to provide visible images of matter. This revolutionary accomplishment was awarded the Nobel prize in 1986 and set the goal for achieving higher resolution that now reach Ångströms[88].

The structure of an electron microscope is quite similar to that of a light microscope as it consists of a radiation source, lenses to orient and focus the source, a sampleholder and a detector unit. Instead of light, the source is an

electron beam which can be generated with the use of different filament types such as tungsten filaments, LaB6 crystal filaments or field emission guns. A series of magnetic lenses will then orient and focus the produced electron beam until it reaches the sample. At that contact point, a fraction of the electrons will scatter away from the sample whereas another fraction will manage to penetrate and go through the sample. Finally, specialized cameras will be able to record the desired electrons and produce an image of the examined sample (Figure 7)[89].

If the detection camera records the electrons that were elastically scattered away from the specimen, then the final image will provide information on the surface of the specimen and the technique will be referred to as scanning electron microscopy (SEM). However, if the camera detects the electrons that managed to penetrate through the specimen, the final image will illustrate a detailed representation of the intracellular structure of the sample, a technique referred to as transmission electron microscopy (TEM)[90, 91].

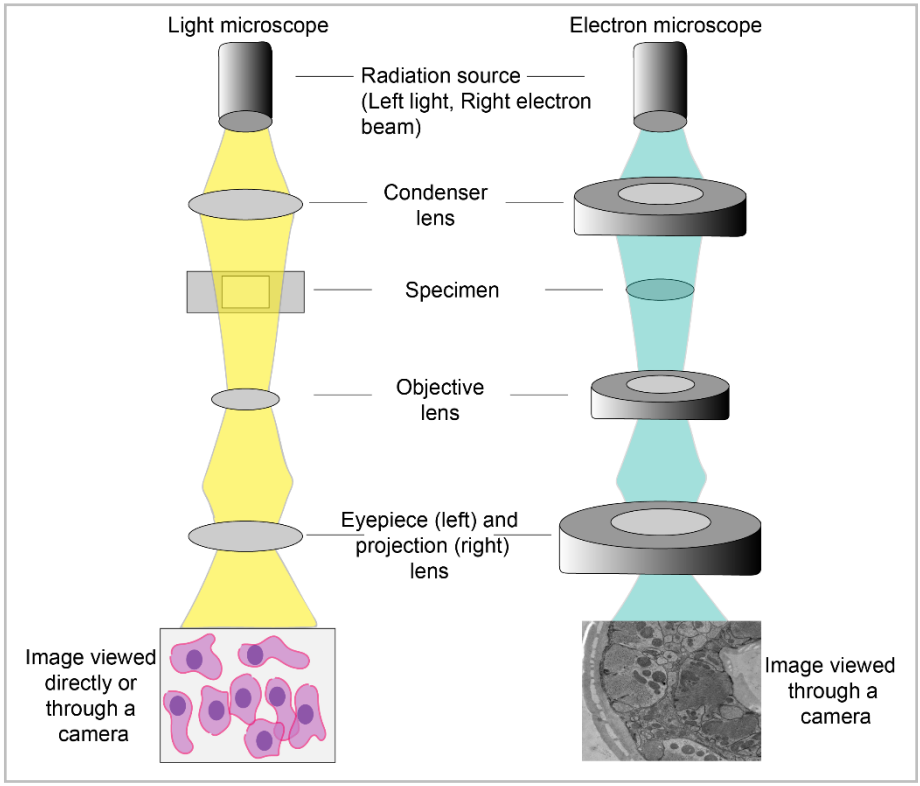

Figure 7. Comparison between a light and an electron microscope. Schematic representation of the structure of a light (left) and an electron (right) microscope. The light microscope uses a lamp as a radiation source whereas the electron microscope has a cathode from which electrons are extracted. Both microscopes use lenses to focus and orient the beam. A detector device is used for the acquisition of the image.

Room temperature electron microscopy

Depending on the model organism of interest and the scientific question to be addressed, the most suitable EM technique and sample preparation procedure must be selected. If the sample is of a small size (<0.5 micrometer thick) and the structure of interest is repeating, allowing for an improved signal to noise ratio due to image averaging, it is often preferred to use the cryo-EM approach. The sample is then plunged into liquid ethane for rapid freezing and imaged at

cryogenic temperature. Although this method provides the opportunity of visualizing a sample nearly at its native state, avoiding chemical fixatives, contrasting agents and dehydration of the sample, it comes with its limitations on sample size, low contrast, and lack of reusability as the sample can be imaged only once[92, 93]. Concurrently to the development of cryo-EM, other protocols have also been developed over the years that allow room temperature sample preparation and fixation, giving an almost limitless range of samples that can be visualized. There are two ways that samples can be prepared for room-temperature imaging. First, through chemical fixation followed by dehydration, plastic resin infiltration and polymerization. The second approach includes high-pressure freezing followed by freeze substitution where the sample is dehydrated, lightly stained, and infiltrated with resin at low temperatures with gradual elevation to room temperature after the polymerization of the plastic[94].

As an overview, chemical fixation refers to the use of different chemical cocktails such as glutaraldehyde, formaldehyde, which stabilizes ("fixes") the sample, osmium tetroxide, uranyl acetate and tannic acid that adds contrast to the sample in order to visualize it in the EM[95]. After the fixation/contrasting step, an epoxy or acrylic resin is used to embed the sample making it accessible for cutting ultrathin sections of about 20 to 100 nm, depending on the scientific question. Though this method has been well developed and does not require advanced equipment to perform, it has a few drawbacks. For example, it could notably alter the state of the biological sample, as many phospholipids are removed during the glutaraldehyde fixation step. Additionally, osmium staining is known to diffuse in an aqueous environment and many of the used fixatives (such as the osmium tetroxide) require an extended time to penetrate tissues, thus increasing the risk of deformation during the fixation process[96]. Finally, the dehydration step can lead to a significant shrinkage of the sample.

Such occurrences change the morphology and structure of the intracellular compartments, making it harder to draw fair conclusions on the appointed question[97].

To avoid such artifacts, one could use the high-pressure freezing approach for sample preparation purposes (Figure 8). During high-pressure freezing, the sample is frozen under liquid nitrogen temperatures at the same time as high-pressure is applied. The high pressure allows for an increase in the cooling rate of the sample, preventing the water molecules from forming a crystalline lattice but instead shape into an amorphous "vitreous" ice. Successful vitrification of a specimen depends on several factors such as the thickness of the sample, the use of cryoprotectants and the heat extraction flow at the surface of the specimen. Though this technique may seem complex and requires advanced equipment, it provides for a high-quality preservation of various samples closest to what one can observe through cryo-EM[98].

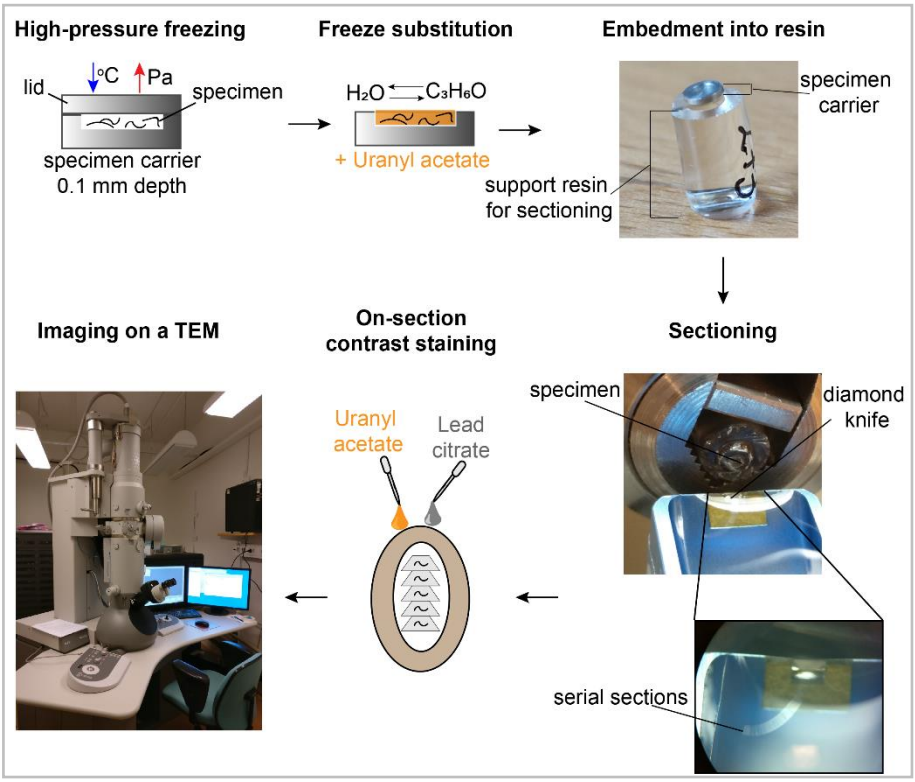

Figure 8. Flowchart of the sample preparation process for TEM imaging. Samples are high pressure frozen in liquid nitrogen followed by a freeze substitution process. Then, a resin of choice is used for sample embedding that will allow for the sectioning of the sample to take place. One last step of on-section staining is performed to enhance contrast before visualizing the samples under a TEM.

After the cryo-fixation step has been performed, further dehydration, resin infiltration and polymerization are needed under low temperatures of about -90 to -50 °C. This could be performed either in a self-made setup of a Styrofoam box filled with dry ice or in designated machines that allow for a more automated and regulated process. A freeze substitution step at -90 °C allows water molecules to be replaced by an acetone solution and different reagents, such as uranyl acetate, may also be added for additional contrast. Afterwards, the temperature is increased to -50 °C and the resin infiltration

begins by exposing the sample to an increasing concentration of the chosen plastic. Depending on the type of resin, samples can then polymerize either in a 60 °C oven or at -50 °C under a UV lamp followed by room temperature UV polymerization.

The samples are now ready for trimming and sectioning into ultra-thin sections (20 nm to 100 nm) or thicker sections (150 nm to 350 nm) if tomography is to be performed. Sectioning is important especially for thick samples as there is a limitation in the ability of the electrons to penetrate a biological sample. A last step of contrast stain is performed on the acquired sections of the sample with the use of uranyl acetate and lead citrate before the sample is ready for imaging under a TEM (Figure 8)[99].

Room temperature electron tomography

A basic TEM process as described above allows for the detailed visualization of the intracellular compartments from any biological sample. The acquired images though represent only a two-dimensional projection section of the sample. If one would like to gain a better z-resolution and a 3D depiction of the sample, then electron tomography (ET) can be used[100]. The sample preparation process is very similar to that of a normal image acquisition protocol with the difference being in the sectioning of the sample and the imaging procedure. For ET, the specimen should be cut into serial thick sections of about 150 to 300 nm in order to provide more volume, depending on the voltage power of the electron microscope that will be used. The limitation here lies again in the fact that lower voltage microscopes would not provide electrons with sufficient energy to penetrate thicker samples. After sectioning and contrast staining of the sample, colloidal gold fiducial markers are added to both sides of the sections. These will later be of help for a proper orientation of the tomographic tilt series. Finally, the microscope is set to

acquire many different images of the specimen in determined tilt angles, usually ±60 degrees. Once all images are taken, they are aligned using one of the many available software (e.g., IMOD) to reconstruct the 3D volume of the specimen using weighted back-projection (Figure 9)[101, 102].

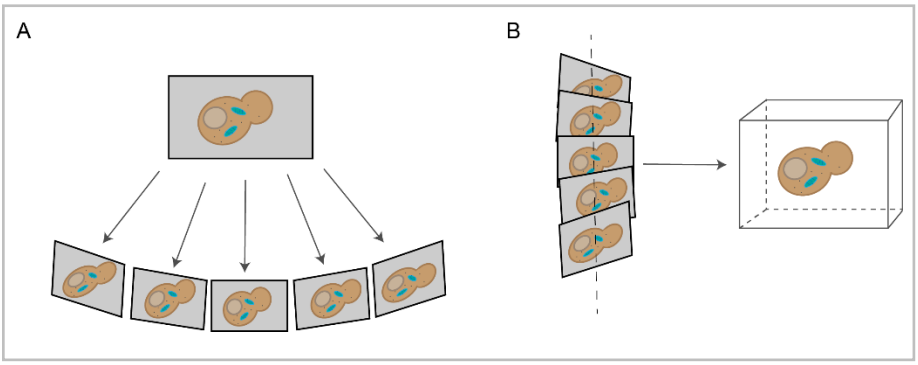

Figure 9. Schematic representation of the tomography reconstruction process. (A) Several micrographs of the specimen are acquired at different tilt series between -60° and 60°. (B) The acquired tilt series is then loaded to the IMOD software where a 3D reconstruction of the specimen is generated.

The development of ET has made it possible to reclaim the lost information of a 2D projection and enable the study of pleomorphic biological structures (such as organelles and cells), both in their cellular context and in an isolated state[103]. However, just like any other scientific technique, this method also comes with its own set of artifacts. As mentioned above, during the tilt series, the specimen is rotated at specific angles which normally range between -60° and 60°. At the higher tilts though, the specimen is not visible due to the edges of the sample holder covering part of the sample. Thus, some information is lost during acquisition, a phenomenon often referred to as the "missing wedge". This limitation can be partially avoided if a second round of tilt series is also acquired after rotation of the sample at about 90° (dual axis tomography). This

approach can reduce the "missing wedge" to a "missing cone" and provides higher 3D information[104, 105].

Finally, besides the reconstruction process, the IMOD software package comes with many more functionalities related to EM and ET. One feature that facilitates a better visualization of the 3D volume is the reconstruction of a 3D model of the acquired tomogram. Different structures or cellular compartments can be modelled and presented all together into a 3D model, creating an analysis tool that provides additional information on volumes, areas and distances as well as a more general 3D organization of the structures residing in the acquired tomogram. It then becomes possible to compare different conditions and evaluate changes in the shape and size of specimens, which would have been challenging or even impossible in a 2D projection setup. Finally, besides the improved resolution (especially in z), it also gives the added benefit of a 3D volume that can be sectioned and viewed in any possible direction.

Immunoelectron microscopy

An additional way to strengthen our comprehension of the relationship between structure and function is through a technique named immunoelectron microscopy (immuno-EM). This technique combines the high spatial resolution of EM with the ability to unravel the location and distribution of proteins at the subcellular level, banishing the belief that EM is a pure morphological approach[106]. One could say that immuno-EM is the equivalent of immunofluorescence for the electron microscopy field. As the name implies, immuno-EM involves the antibody detection of a protein of interest. The primary antibody is then detected using a secondary antibody conjugated to gold particle that is visible by an electron microscope (Figure 10, A). Due to the high atomic number of the gold particle, when the electron beam hits the

gold, the high-density atom reflets the electrons generating a dark spot on the electron micrograph (Figure 10, B). This process can be done either before the embedding stage of the sample preparation or directly on ultrathin sections[107, 108].

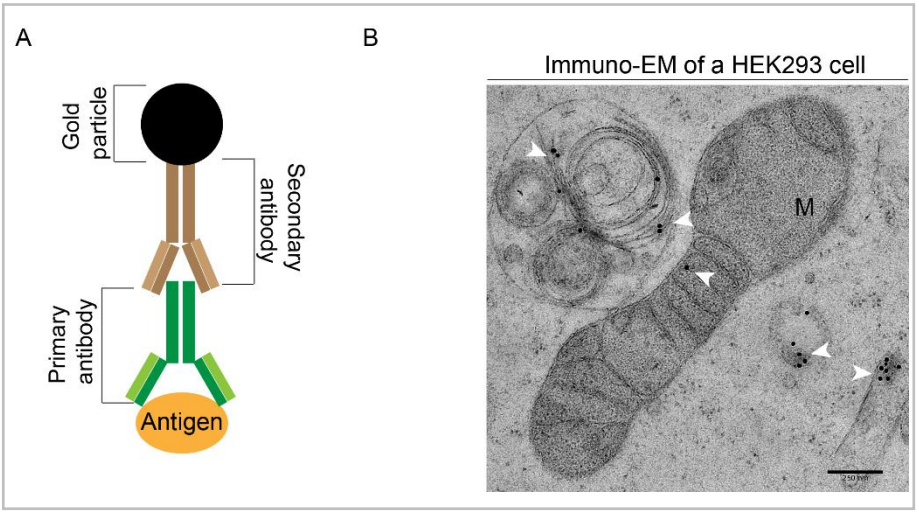

Figure 10. Example of an immuno-EM experiment. (A) A graphical representation of the antibody sandwich. The primary antibody will bind to the specific antigen and afterwards a gold-labelled secondary antibody will detect the presence of the primary. The length of the sandwich may vary depending on if the whole antibody or just a fraction of it is used, but would approximately be 30 nm long when using whole IgGs. (B) An example of a HEK293 cell electron micrograph after on-section immuno-EM. White arrowheads indicate the presence of gold particles that were found either clustered together or as individuals. Abbreviations: HEK293; human embryonic kidney 293 cells, M; mitochondria. Scale bar: 250 nm.

Though the theory behind this technique seems quite achievable, many parameters should be adjusted for the success of the experiment. Firstly, the fixation, embedding and polymerization approach during sample preparation are of great importance for the maintenance of the antigens of interest. Many fixatives and conditions denature antigens, interfering with the antibody's

affinity to them. Specific protocols have been developed where harsh fixatives and high temperatures are avoided in order to overcome such limitations. A second limitation, mostly related to the on-section labeling process, is the low number of accessible antigens. As the staining is performed on one side of a thin section, and the antibodies cannot penetrate the plastic, only the properly oriented antigens on the surface area are available to form bonds with the antibodies. Adjusting the appropriate antibody concentration, the use of control samples without the primary antibody and quantifications of gold per area are essential to evaluate the amount of specific labeling that occurs[109, 110].

The existence of different sized gold particles can also provide the opportunity of labeling two proteins within the same sample. Such an approach is necessary when questions of co-localization and interaction between more than one protein of interest are addressed[111]. Immuno-EM has been proven to be a powerful tool for locating proteins at an ultrastructural level.

RESULTS AND DISCUSSION

This thesis is divided into two separate subjects which share the common element of membrane morphology and diversity. The first part focuses on the formation of distinct nuclear membrane buds which have been previously observed but are understudied in a variety of model organisms. We thus try to investigate this phenomenon and connect it to potential cellular pathways in an effort to better understand and describe its functionality. The second part aims more to elucidate membrane homeostasis and the involved pathways. We highlight the important role of membrane regulators and how the absence of such mechanisms can affect morphologically and functionally normal cellular processes. Most of the work is performed with the help of advanced TEM techniques as described in previous sections.

PAPER I: Nuclear envelope budding is a response to cellular stress.

Aim

This project was based on previous observations in the lab of NEBs that were forming between the inner and outer nuclear envelope. A thorough literature search revealed such events to have been previously noted but not properly examined for their prevalence and functionality. As NPCs have so far been the only known gateway for molecules to go through the nuclear envelope, the presence of NEBs could prove to be an unnoticed alternative pathway for such transactions. This project focuses on the quantification and detailed structural description of NEB events and the investigation of its cellular role.

Results

NEB events have a higher frequency during cellular stress.

For the examination of the NEB events, the budding yeast *Saccharomyces cerevisiae* was initially used as a model organism. Five stressors of various nature were introduced to *S. cerevisiae* cultures and electron micrographs of nuclear cross-sections were acquired randomly for the quantification of the number of observed NEB events. The different stress conditions included constant mild heat stress (38°C), exposure to hydrogen peroxide, sodium arsenite, azetidine-2-carboxylic acid (AZC) (Figure 11), and inhibition of the proteasome. All five conditions significantly increased the frequency of NEB events in comparison to the untreated cells, which had a frequency of NEB events at about 2% of the sections.

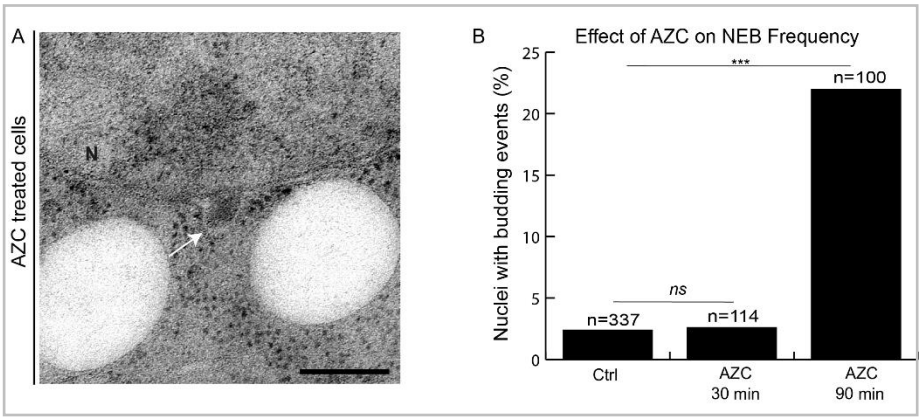

Figure 11. NEB in AZC treated cells. (A) Electron micrograph depicting an example of a NEB event in cells treated with AZC. White arrow points at the formed bud. (B) Quantification of the frequency of NEB events found in the examined cell sections per condition. The frequency of NEB events was significantly increased after exposure of 90 minutes to the drug. N; nucleus. ***p≤0.001. Scale bar: 200 nm.

These results strongly indicated that NEB events are a necessary pathway that is needed when the cells are under stress. The AZC drug is a proline analog which can be incorporated into proteins, changing their natural conformation resulting in an increased number of misfolded proteins. Though all the above stressors can also cause the formation of misfolded proteins among other things, the fact that both the AZC treatment (Figure 11) and the proteasome inhibition triggered the highest frequency of NEB events (between 15% and 22%), pointed towards a link between protein quality control and formation of NEBs, with the potential hypothesis that aggregated proteins could perhaps be a part of the transported cargo.

The cargo of NEBs contains ubiquitin and Hsp-104.

Most proteins that become misfolded or aggregated will soon be detected and marked for degradation, as they can pose a hazard for the survival of the cell. This process involves tagging of the protein with a poly-ubiquitin chain, a signaling molecule used to activate and guide the proteasome system to initiate degradation. If the previous hypothesis on the nature of the cargo being associated to aggregated proteins is accurate, then ubiquitin is most likely to be bound to the transported cargo. To test this connection, immuno-EM was performed in heat shocked yeast cells for the detection of ubiquitin. Indeed, these results revealed the presence of ubiquitin molecules inside the NEB events.

Besides the ubiquitination step that signals for degradation, other molecules are also involved in the protein quality control process such as the Hsp-104 molecule. Hsp-104 is a well-characterized chaperone that localizes to aggregated proteins and acts as a disaggregase, supporting the degradation of ubiquitinated proteins[112]. Following our previous findings, we performed an additional immuno-EM experiment on heat shocked cells for the potential

detection of Hsp-104. These findings were consistent with the proposed hypothesis as NEB events appeared to be highly labeled for Hsp-104 in comparison to the appropriate negative control (in this setup lipid droplets were used as a negative control).

NEB structures are not related to misassembled NPCs.

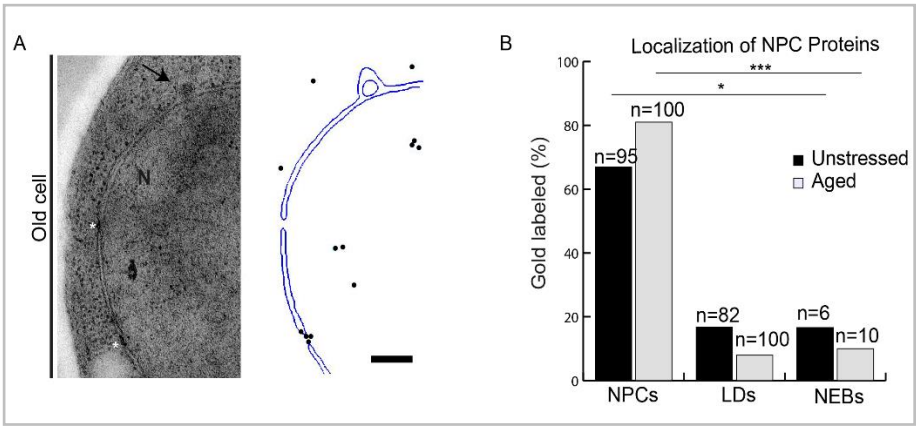

Figure 12. Immunoelectron microscopy of NPC components. (A) Electron micrograph and its corresponding model of an old yeast cell depicting a NEB event (black arrow) and two NPCs (white asterisks), as well as the gold particles bound to NPC components. (B) Quantification of the localization of NPC proteins in intreated and old yeast cells. Most NPCs were highly labeled in contrast to the lipid droplets (LDs) and NEBs. N; nucleus. *p≤0.05, ***p≤0.001. Scale bar: 200 nm.

Previous publications have highlighted a particular structure that appeared to be the result of a defective assembly of NPCs, creating a protruding structure formed by both nuclear envelope membranes[67]. These protrusions often resembled the NEB events that were observed in the *S. cerevisiae* cells. In order to clarify if NEB events and the misassembled NPCs are one and the same thing, we performed an immuno-EM experiment for an antibody which is able to recognize four different NPC components (Nup62, Nup153, Nup214, and Nup358). The results revealed that NEB events were labeled with NPC

components as often as the assigned negative control (lipid droplets) proposing that these occurrences are independent of the misassembled NPCs (Figure12). Although we could not confidently assume that none of the observed NEB events were confused with the NPC related herniations, there is an obvious distinction between the two phenomena.

NEBs are evolutionarily conserved and part of a normal cellular function.

Besides the fact that cellular stressors are capable of increasing the frequency of NEB events, it is of great importance to highlight the fact that undisturbed yeast cultures also formed these nuclear buds though in a lower frequency. The existence of such events even in normal growing control cells is an indication that the NEB pathway is a natural occurrence that serves a purpose even in the absence of stressors.

Though the budding yeast was selected as a model organism due to its conveniences in growing, maintaining, and genetically modifying, previous publications had already proven the existence of NEB events in different organisms. Thus, it seemed natural to investigate other model organisms for the formation of NEBs, investigating if these events are evolutionarily conserved. For this purpose, images of nuclei from the yeast *Schizosaccharomyces pombe* (*S. pombe*), the protist *Trypanosoma brucei* (*T. brucei*), the round worm *Caenorhabditis elegans* (*C. elegans*) and the Human Mast Cell Line 1 (HMC-1) were also acquired and examined for the presence of NEBs. The results showed that all the selected organisms produced NEB events though exhibiting differences in their morphology and frequency.

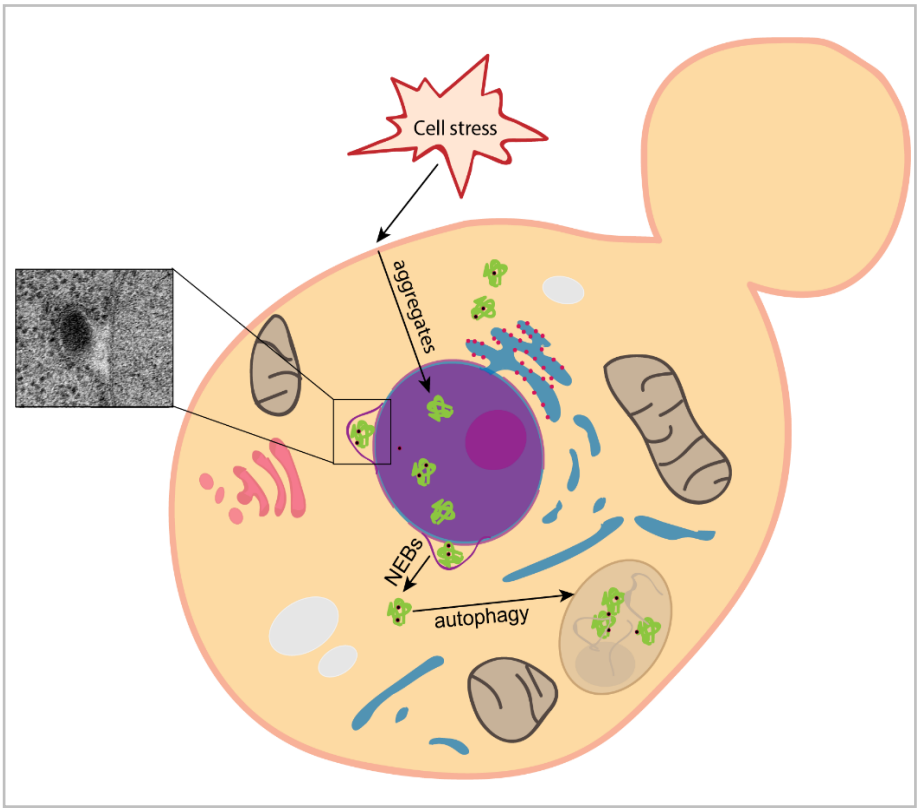

Figure 13. Graphical representation of the NEB pathway. NEB events are strongly associated to the amount of protein aggregates in the cell and the functionality of the proteasome system. The cargo most likely includes ubiquitinated protein aggregates which are targeted for degradation.

Discussion

In this study, we investigated the previously noted formation of buddings on the nuclear envelope membranes. We used five different stressors to assign possible functionalities on such events and our results lead to the conclusion that NEB is strongly related to the proteasome pathway and the formation of protein aggregates. The two conditions that yielded the highest frequencies of NEBs were the AZC treatment and the inhibition of the proteasome

degradation system. AZC has a very similar structure to proline, though it has four instead of five carbon atoms in its ring. This similarity is what makes it possible for AZC to incorporate into polypeptides and the difference in carbon atoms is what creates the conformational changes that cause proteins to misfold[113]. Most misfolded proteins tend to create aggregates which become a target for ubiquitination and degradation via the proteasome pathway[114]. The fact that accumulation of protein aggregates, and the inability of the proteasome pathway to function significantly, both trigger the formation of NEB events strongly suggests that the transported cargo could be related to the degradation pathway.

Further immuno-EM experiments strengthen this hypothesis as both ubiquitin and the protein aggregation related chaperone Hsp-104 were found inside the budding events. A previous publication revealed that the cargo of NEB events in the fruit fly *Drosophila melanogaster*, consisted of megaRNPs. Our observations in combination with previous investigations suggest that NEB creates a gateway for molecules to exit or enter the nuclear envelope barrier and that the transported cargo can vary in nature[115].

Finally, we have additionally proven the fact that NEBs are evolutionarily conserved among many different organisms and that such events occur as a natural phenomenon even in the absence of any external stressors.

Own contributions to Paper I

Preparation of samples for electron microscopy: I prepared *C. elegans* as well as many of the yeast samples included in this study for electron microscopy through high-pressure freezing and freeze substitution. I have sectioned, contrast stained and imaged samples for the examination of the presence of NEB events.

NEB quantification: I have imaged and quantified micrographs for most of the included experiments for the presence of NEB events. I have categorized the events based on morphology and measured the size among the examined organisms.

Quantification and plotting of immuno-EM experiments: I have quantified and plotted the results from most of the immuno-EM experiments that were performed in this study. Also, I have conducted some of the immuno-EM experiments myself.

3D model reconstruction: I have modeled NEB events from existing tomographic data using the IMOD software package.

Manuscript preparation: I have written the first draft of this manuscript and prepared all related materials from figures to supplemental material and supporting videos.

PAPER II: Palmitic acid-induced membrane deformations in AdipoR2 depleted cells.

Aim

This project was based on previous findings on the effects of PA on cellular membranes and lipid homeostasis. In particular, several studies showed that PA provided exogenously in the media of cultivated cells can cause an increase in membrane rigidity and that this can be countered by activation of AdipoR2 that promotes fatty acid desaturation and incorporation of unsaturated fatty acids (UFAs) into phospholipids. The goal of this study was to provide details of the PA-induced membrane defects using primarily electron microscopy. Control cells and cells lacking the AdipoR2 receptor were treated in various ways that would challenge membrane homeostasis, and electron microscopy images were obtained that allowed quantification of any obvious membrane defect. This study therefore focused on the description of such deformations and their possible implications in the survival of the cell.

Results

Exogenous PA induces obvious deformations on an intracellular level.

As an initial experiment, control cells and AdipoR2 siRNA-treated cells were grown either on basal conditions or in the presence of PA. Samples were then prepared for electron microscopy and examined for potential alterations of their membranous compartments. This experimental setup revealed that exogenous PA causes defects in the morphology of three distinct compartments: cytoplasmic membranes of the ER, mitochondria, and the nuclear envelope intramembrane space (Figure 14). In the absence of a functional AdipoR2 receptor, all deformations were more frequent and more severe when compared with the control cells.

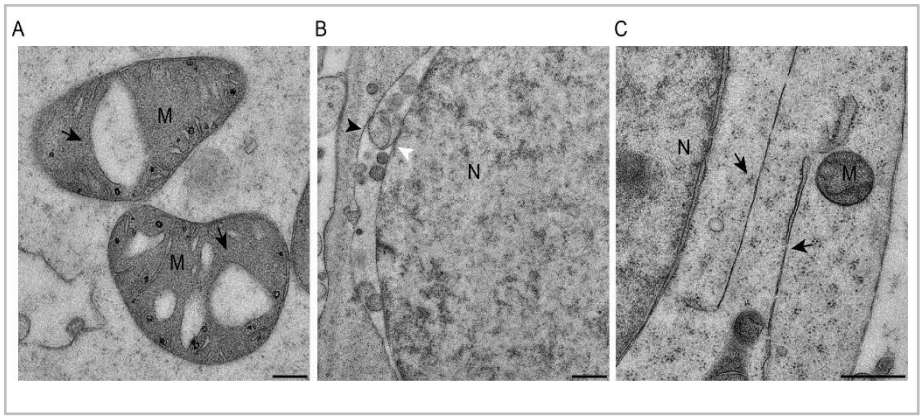

Figure 14. PA-induced cellular deformations in non-targeted siRNA HEK293 cells. (A) Electron micrograph illustrating the deformed mitochondrial cristae (black arrows) induced by treatment of cells with exogenous PA. (B) Electron micrograph depicting the presence of NEBs between the nuclear membranes (black arrowhead at ONM, white arrowhead at INM) in PA treated cells. (C) Electron micrograph showing the closely apposed cytoplasmic membranes (black arrows) in the presence of PA. M; mitochondria, N; nucleus. Scale bars: A, B; 500 nm, C; 250 nm.

More specifically, in PA-treated cells the cytoplasmic membranes formed closely apposed structures resembling straight lines. These lines could be either long and continuous through much of the cytoplasm or appear as many small lines clustered together in specific areas of the cell. There were also occasions where these lines would have a swollen area with a blebbing of unknown origin located inside the wider part of the membrane. Mitochondria with a disturbed and swollen cristae were also noted in the PA-treated samples. Though such deformed mitochondria were also present in some of the control cells, their frequency was increased in cells lacking AdipoR2, especially upon PA treatment. Finally, nuclear envelope blebbings similar to the NEBs of the previous paper were also present in high frequency and severity in the AdipoR2 siRNA cells challenged with PA.

OA can alleviate PA-induced deformations.

Previous studies on the rigidifying effects of saturated fatty acids such as PA have shown that co-treatment of cells with a monounsaturated fatty acid (OA) can restore the balance in membrane lipid composition and hence prevent SFA-induced membrane rigidification. To investigate if OA could also alleviate the intracellular effects of PA, we co-treated cells with both PA and OA and examined them for the presence of membrane deformations. In agreement with the previous findings, OA was able to decrease the frequency of the closely apposed membranes, the deformed mitochondria, and the nuclear envelope blebbings. OA alone did not induce any obvious cellular defects.

Other cell lines are similarly affected by PA.

All the aforementioned experiments were conducted on HEK293 cells and, when desired, AdipoR2 was silenced with the use of the siRNA technique. To investigate if different cell lines would be equally affected by such treatments and if the siRNA process itself was influencing in any way the observed deformations, a nearly haploid human (HAP1) AdipoR2 knock out cell line was used, thus excluding the need for an siRNA silencing of the receptor. We found that HAP1 cells produced similar phenotypes in the presence of PA and that these could also be rescued with addition of OA. The most significant difference between the cell lines was the frequency of the nuclear envelope blebbings: the HAP1 cells did not form many blebbings in any of the treated groups, indicating that different cell lines may use alternative ways to deal with membrane homeostasis and lipid imbalances.

PINK-1 was frequently localized in the closely apposed membranes.

PINK1 is a protein commonly used as an indicator of mitophagy, a process where defective mitochondria are targeted for degradation. Under normal conditions, PINK1 is synthesized in the ER and transported towards the mitochondria. If there are no defects, then PINK1 enters the mitochondria where it is degraded. However, PINK1 is unable to pass through the outer mitochondrial membrane of defective mitochondria and so accumulates on their perimeter. This accumulation triggers the production and recruitment of PARKIN protein which binds to PINK1 and targets the defective mitochondria for degradation[116, 117].

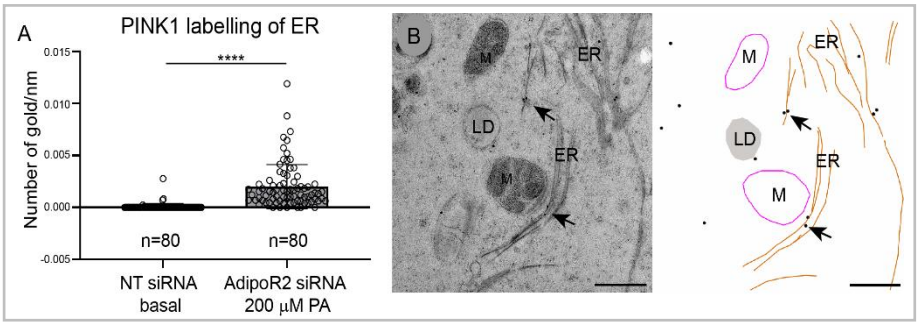

Figure 15. PINK1 labeling of ER membranes in HEK293 cells. (A) Quantification of the PINK1 labeling on the ER between non-targeted siRNA cells on basal condition and AdipoR2 siRNA cells on PA. (B) Representative electron micrograph and the corresponding model depicting the localization of gold particles in the closely apposed ER membranes (black arrows). LD; lipid droplets, ER; endoplasmic reticulum, M; mitochondria. ****p≤0.0001. Scale bars: 500 nm.

In an attempt to investigate if the observed mitochondria with abnormal cristae are also a target for mitophagy, we performed immuno-EM for the PINK1 protein both in control cells and AdipoR2 siRNA cells under PA. Overall, AdipoR2 siRNA cells on PA had a significantly higher labeling frequency in their mitochondria, independent of their morphology, compared to the control

group. When the mitochondria from the AdipoR2 group were separated based on morphology to deformed and non-deformed and compared for frequency of labeling, the localization of PINK1 did not seem to differ between these two groups. To our surprise, PINK1 seemed to be highly enriched in the closely apposed ER membranes, suggesting that PINK1 trafficking or processing is impaired in these deformed membranes (Figure 15).

Discussion

PA can cause many defects on a cellular level. When introduced exogenously in a culture, it disrupts the lipid balance in the membranes and thus disturbs membrane homeostasis: cellular membranes become more rigid, mitochondrial respiration is partially impaired, accumulation of ROS is more profound and apoptotic pathways are often activated in accordance with the severeness. Though all these have been previously observed with the use of molecular standard techniques and fluorescence microscopy, little to nothing is known on an ultrastructural level on the PA-induced phenotypes. Our study has proven that PA influences the morphology of several cellular compartments such as ER, mitochondria, and the nuclear envelope.

Some of the observed membrane phenotypes were dose-dependent and most of them were decreased in frequency and severity in the presence of the monounsaturated OA. Two different cell lines were used yielding similar results though with differences in the intensity and frequency of the deformations. These variations may be an indicator that each cell line reacts in a different way to PA-induced stress and promotes specific pathways to restore any produced defects.

Focusing especially on the cytoplasmic membranes, such as the closely apposed ER membrane phenotype, it is quite interesting to explore how cells

maintain their basic functionality even when much of their cytoplasmic membranes are severely deformed. From the PINK-1 experimental results, it becomes apparent that such a peculiar morphology almost resembling straight lines could potentially affect normal cellular processes such as the translocation of ER-synthesized proteins to their final destination. It is possible that the observed PINK1 trafficking defect reflects a more generalized defect in the trafficking of ER-produced proteins across tightly apposed membranes, begging the question of how the cells maintain functionality in spite of this membrane defect. One possibility is of course that the closely apposed membranes amount to a mechanism to sequester rigid, non-functional membranes while protecting the rest of the cell. This study therefore provides a good basis for a detailed exploration of pathways that provide robustness against structural membrane defects.

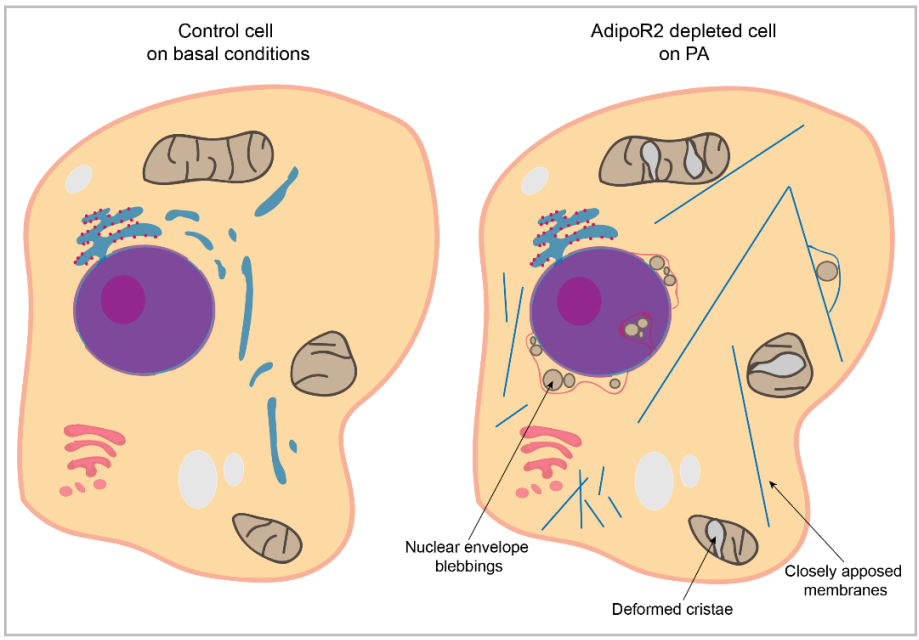

Figure 16. Schematic representation of the PA-induced cellular deformations. Illustration of a control cell grown under basal conditions (left cell) in comparison to

an AdipoR2-deficient cell growing on PA (right cell). In the AdipoR2-deficient cell, three distinct PA-induced membrane deformations are observed (closely apposed membranes, mitochondria with deformed cristae and nuclear envelope blebbings).

Own contributions to Paper II

Preparation of samples for electron microscopy: I have high-pressured frozen, and freeze substituted already treated cultures of HEK293 and HAP1 cells and prepared them for electron microscopy.

Immuno-EM for PINK-1: I have performed immuno-EM experiments on HEK293 cells for the detection of the PINK-1 mitophagy marker.

Quantification of phenotypes: I have acquired and quantified all the needed micrographs for the performed experiments. I have plotted and run statistical tests for all different treated groups and examined phenotypes.

Tomographic reconstruction: I have performed 3D acquisition and reconstruction of a HEK293 control and AdipoR2 siRNA treated sample for the investigation and comparison of a healthy and deformed mitochondrion.

Manuscript preparation: I have written the first draft of this manuscript and prepared all related materials from figures to supplemental material and supporting videos.

PAPER III: Sphingosine 1-Phosphate Mediates Adiponectin Receptor Signaling Essential for Lipid Homeostasis and Embryogenesis.

Aim

Previous studies have revealed that double knockout (DKO) mice for the membrane homeostasis regulators AdipoR1 and AdipoR2 are embryonic lethal[118]. The maintenance of at least one of these receptors is enough for the production of viable progenies, but the precise reasons for the double knockout's inability to develop were still unknown prior to our study. The AdipoRs have been described for their role in regulating lipid homeostasis through a signaling pathway which activates the production of fatty acids desaturases. At the molecular level, both AdipoR1 and AdipoR2 share the ability to catalyze the hydrolysis of ceramides to produce sphingosine and free fatty acids. The resulting sphingosine can then undergo a phosphorylation step which then produces the signaling molecule S1P.

The aim of this study was to examine if the observed embryonic lethality is related to a failure in membrane homeostasis, possibly caused by an inefficient S1P signaling.

Results

AdipoR1/2 DKO lethality connects to higher amount of SFAs

To examine what are the main physiological and biological differences between DKO mice and wild type mice, DKO embryos (E12.5) were collected and analyzed for histology, lipidomics, and proteomics. The histological results did not show any apparent difference suggesting that morphological defects probably occur at a later stage. The findings from the lipidomics and proteomics analysis, however, revealed a clear difference in the amount of

SFAs (mostly PA) that were incorporated into the phospholipids of the DKOs and the wild type mice. Apparently, most PCs and PEs of the DKO embryos had a higher amount of PA and a noticeably lower amount of any mono- or polyunsaturated fatty acid. Proteomics further supported these observations as pathways related to fatty acid metabolism were upregulated in DKO embryos whereas the "SNARE interactions in vesicular transport" pathway was downregulated. These results suggest a connection between the embryonic lethality of the DKOs and potential membrane malfunctions that occur due to the high amount of SFAs in the phospholipids.

DKO mouse embryonic fibroblasts (MEFs) share similar defects

To begin elucidating the underlying mechanism with which AdipoR1/2 acts as a lipid regulator, MEFs from WT, AdipoR1-KO, AdipoR2-KO and DKO embryos were used as a more flexible experimental model and studied for potential membrane abnormalities. AdipoR1-KO, AdipoR2-KO and DKO MEFs showed similar defects as the DKO embryos in terms of higher amount of PA in PCs and PEs and a decreased amount of OA. Addition of PA could worsen these defects whereas co-treatment with OA alleviated them. All defects and the saturation level of phospholipids were more severe in the DKO in comparison to the AdipoR2-KO and gradually even less severe in the AdipoR1-KO MEFs.

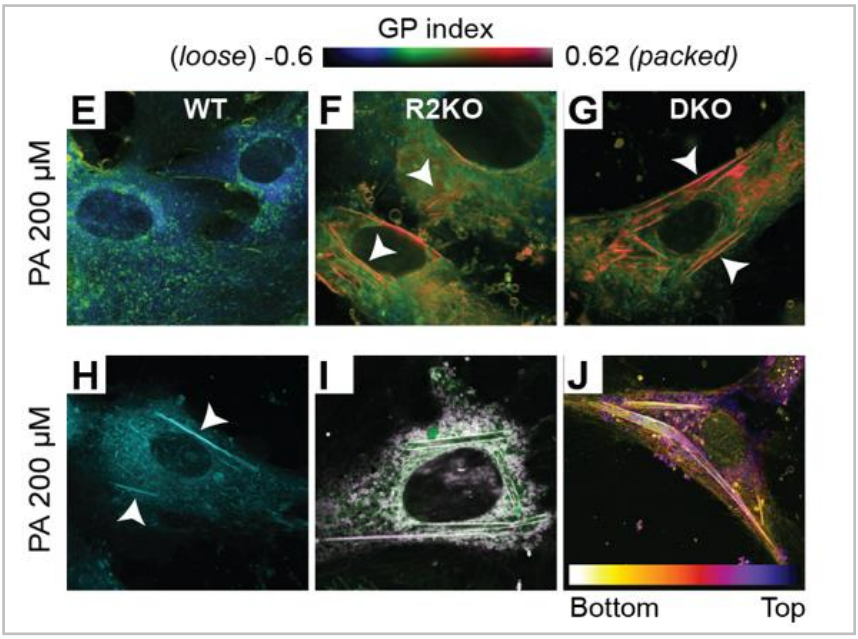

Figure 17. Confocal microscopy of MEFs. Representative pseudocolor images of wild type, AdipoR2 KO and DKO MEFs treated with exogenous PA showing the GP index (membrane rigidity indicator) derived from a Laurdan dye experiment (top images). Blue color indicates a loosely packed membrane whereas green and red indicate higher rigidity. White arrows point at high rigidity areas. Confocal images of AdipoR2 KO MEFs on PA stained with a lipid dye and an ER marker (bottom images). White arrows point at unique PA-induced thin solid-like structures in the cytoplasm.

Further Laurdan dye staining confirmed the excessive membrane packing in the AdipoR2-KO and the DKO MEFs that were treated with exogenous PA. Confocal and electron microscopy revealed unique membrane morphologies and deformations. Under the confocal microscope, thin solid-like structures appeared in the PA-treated DKO and AdipoR2-KO MEFs that were later confirmed to represent ER membranes (Figure 17). Electron micrographs presented peculiar spiral membranes in the cytoplasm as well as closely apposed membranes often representing ER. Nuclear envelope budding was also more frequent in the DKO MEFs that were challenged with PA (Figure

18). Overall, these results proved that DKO MEFs are a proper model to recreate and examine DKO embryonal membrane defects.

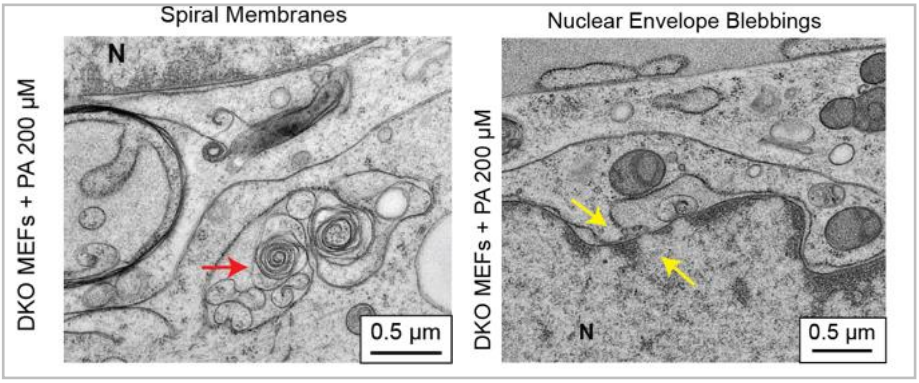

Figure 18. PA-induced ER spirals and NEBs in DKO MEFs. Representative electron micrographs showing the formation of spiral ER structures (left image, red arrow) and NEBs (right image, yellow arrows) in DKO MEFs treated with exogenous PA.

S1P can restore membrane defects in DKO cells

To test if the formation of the S1P signaling molecule through the ceramidase activity of the AdipoR1/2 is important for initiating membrane homeostasis, thus implying that the S1P is the downstream signaling molecule in the AdipoR1/2 pathway, PA-challenged DKO MEFs were grown in the presence or absence of exogenous S1P. In general, DKO MEFs appear to have an increased number of ceramides but low levels of S1P and sphingosine molecules, suggesting that the presence of the receptors are necessary for the hydrolysis of ceramides and the eventual formation of S1P. All cells grown in the presence of S1P had a significantly decreased amount of PA and a higher amount of OA and MUFAs in phospholipids, suggesting that S1P was able to initiate the fatty acid desaturation process and maintain the balance in the lipid composition. The tight membrane packing phenotype was restored after

addition of S1P as well as the SFA-induced ER stress. These results were consistent in different cell types and organisms, such as in the HEK293 cells and the round worm *C. elegans*.

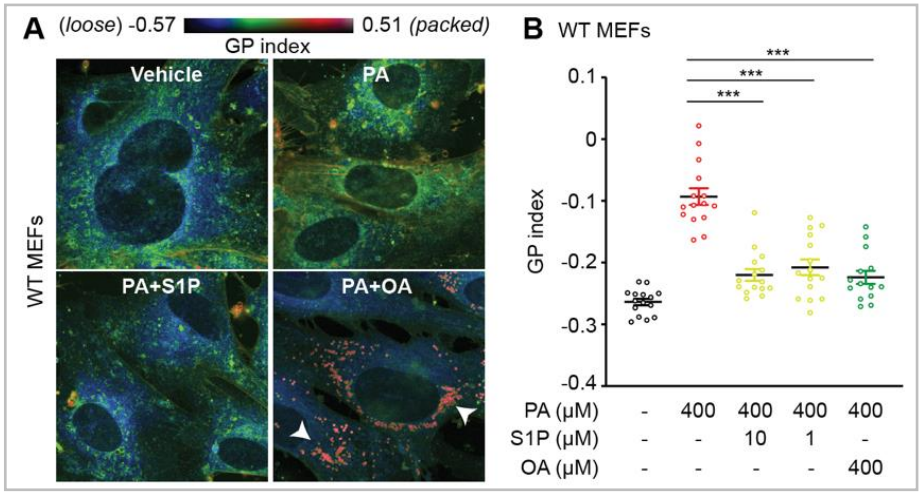

Figure 19. S1P and OA rescue of the PA-induced membrane rigidity in MEFs. Pseudocolor images and average GP index from several images of WT MEFs treated with vehicle, PA ±S1P or ±OA and stained with Laurdan. White arrows indicate the highly packed lipid droplets induced by PA+OA.

To further evaluate the implication of S1P in the membrane homeostasis process, wild type cells of various cell types (MEFs, HEK293, Jurkat E6-1 and INS-1E) were challenged with high concentrations (400 μM) of PA in the presence or absence of external S1P. Once more, the additional S1P was able to eliminate all PA-induced membrane deformations and help the cells to cope in such high SFA concentrations. S1P improved the membrane fluidity and promoted desaturation of the SFAs, increasing the levels of unsaturated fatty acids in the membrane phospholipids (Figure 19).

Overexpression of the AdipoR1/2 can also yield similar results in protecting WT cells from the overwhelming amounts of PA. Their ceramidase activity

strongly depends on the Zn^{+2} ion which resides in the catalytic domain and is coordinated by three His residues. To examine if this effect is connected to their ceramidase activity that creates the S1P molecules, wild type or mutated forms of AdipoR2 with one or all three of their His residues altered were overexpressed in HEK293 cells and examined for their membrane health. These results showed that the mutated AdipoR2 lost its ability to protect the cells from the excessive amounts of PA treatment. These effects were also confirmed in the *C. elegans* model through overexpression of their homolog receptor PAQR-2.

To examine one more step in the process of converting ceramidases to S1P, the two identified sphingosine kinases, Sphk1 and Sphk2, in HEK293 cells were knocked down and the cells were assessed for their membrane health. Interestingly, single silencing of each kinase did not influence the rigidity level, but double knockdown of the kinases yields an increase in lipid packing. Similar effects were also noted in the *C. elegans* model after silencing of the single known sphingosine kinase. The *sphk-1* mutant showed similar but less severe phenotypes suggesting that a second kinase may also exist but is not yet identified. Altogether, it has been made clear that the ceramidase activity of the AdipoRs and the presence of the S1P signaling molecule are necessary for the restoration and maintenance of membrane homeostasis.

S1PR3-SREBP1 and PPARγ are the downstream molecules in the S1P pathway

As the involvement of the S1P signaling molecule in the AdipoR1/2-driven membrane homeostasis has been confirmed, we aimed to further investigate the molecules that work downstream in the S1P pathway. There are five different S1P membrane receptors (S1PR1-5) so to clarify which receptor(s) is responsible for the regulation of membrane homeostasis, different assays were

performed. Examination of the expression levels of the receptors, the use of agonists/antagonists as well as silencing of the receptors in three different cell types (MEFs, HEK293, U-2 OS cells, and *C. elegans*) all pointed to the result that the S1PR3 is the main regulator downstream of the S1P pathway. Similar assays demonstrated the importance of the sterol regulatory element-binding protein-1 (SREBP1) in the restoration of membrane homeostasis as HEK293 cells lacking AdipoR2 or SREBP1/2 showed a similar RNAseq profile. The levels of unprocessed SREBP1 were also influenced by the presence of PA or the absence of AdipoR2, strengthening the conclusion that adipoR2-derived S1P acts via S1PR3 and SREBP1 to enhance the activity of fatty acid desaturation and restore membrane fluidity.

Following a similar hypothesis as before, we wanted to investigate the involvement of PPARγ, a known intracellular target of S1P, as well as the retinoid X receptor (RXR), a transcription factor known to be involved with the PPARγ activity. The use of specific antagonists or complete silencing at a gene level, both revealed the connection between PPARγ/RXRα and the AdipoR2/S1P pathway. Addition of S1P could not rescue the PPARγ/RXRα silencing phenotype, proving that these molecules function downstream of AdipoR2 and S1P.

SCD as a final regulator in the AdipoR2/S1P pathway

A previous RNAseq experiment in AdipoR2-KO HEK293 cells had shown a significant downregulation of SCD, a gene responsible for SFA to MUFA conversion and regulation of membrane homeostasis. To clarify if SCD is involved in the AdipoR2/S1P pathway, the expression levels both in protein and mRNA level were investigated. SCD levels were significantly lower in DKO MEFs as well as in cells lacking other important molecules of the S1P pathway such as sphk-1 or PPARγ. These results were confirmed in MEFs,

HEK293 cells and in *C. elegans*. Further lipidomic analysis verified our previous findings as the loss of AdipoR2 resulted in an excess of SFA which could be partially corrected after addition of S1P whereas loss of S1PR3, SREBP1, PPARγ and SCD resulted in a similar increase in SFA levels, but it could not be rescued by an addition of S1P (downstream of S1P).

Discussion

AdipoR1/2 were previously investigated under the scope of the potential antidiabetic effects of these proteins and characterized as adiponectin-dependent receptors. An increasing number of studies have now shifted the focus towards an evolutionarily conserved function of these receptors involving the detection and restoration of lipid imbalances and membrane homeostasis. In this study, these properties of AdipoR1/2 have been confirmed and the pathway has been examined for other signaling molecules involved in the process. Lack of AdipoR1/2 is associated with an increased amount of SFA in the membrane phospholipids at the expense of PUFA, significant changes in the membrane properties such as levels of rigidity and implications in the development and survival of the cells. The addition of MUFAs or other downstream molecules in the pathway can partially restore these defects. Mice lacking both receptors (DKO) are embryonic lethal, although the exact causes of such lethality have not been fully understood.

Our results have now shown that AdipoR1/2 acts as ceramidase that breaks ceramides into sphingosine and a free fatty acid. The released sphingosine can then become phosphorylated via a sphingosine kinase (sphk-1 or sphk-2) and form the signaling molecule sphingosine 1-phosphate, S1P. Downstream on the pathway, S1PR3, SREBP1, PPARγ, and RXRα are identified as molecules that contribute to the activation and recruiting of fatty acid desaturases such as SCD for the conversion of the SFAs into MUFAs and restoration of membrane

homeostasis (Figure 20). All these observations have been made in several cell types and organisms such as mice, *C. elegans*, MEFs, HEK293, Jurkat, U-2 OS cells, revealing an evolutionarily conserved mechanism of the AdipoR1/2. The observed lethality in the DKO mice, most likely caused by the excess amount of SFAs in the membranes, demonstrates the importance of the AdipoR1/2-mediated membrane homeostasis in a mammalian physiological environment.

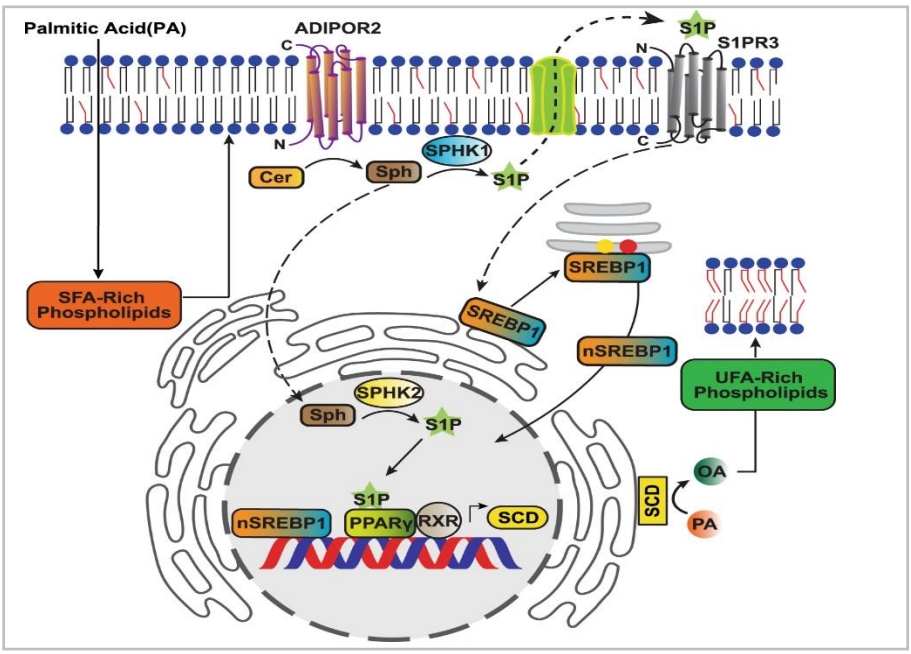

Figure 20. Schematic representation of the AdipoR2/S1P pathway. Excessive amount of SFAs into the phospholipids activates the AdipoR2 receptor to produce the S1P signaling molecule. S1P is then responsible for activation of S1PR3, production of SREBP1, PPARγ and eventual increase in the expression levels of SCD. The desaturase can then convert SFAs into MUFAs and restore membrane homeostasis. The image is taken from Paper III.

Own contributions to Paper III

Preparation of samples for electron microscopy: I have high-pressured frozen, and freeze substituted already treated cultures of WT and DKO MEFs and prepared them for electron microscopy.

Quantifications of phenotypes of interest: Quantified and plotted the frequency of the phenotypes of interest between WT and DKO MEFs as well as ER-mitochondrial contact site points and formation of lipid droplets.

Writing of the manuscript: Contributed to the writing and revision of the manuscript for the parts that I was involved with.

FUTURE PERSPECTIVES

NEB project

We have now shown that an alternative pathway exists that allows nucleocytoplasmic communication in healthy and stressed cells beyond the NPC pathway. Although electron microscopy has given a detailed insight on the shape and structure of the observed budding events, this method comes with its own limitations as information on directionality (heading inwards or outwards) and the fate of the bud cannot be obtained with the use of TEM. To overcome these obstacles, developing and using specific fluorescent markers for the formed buds would allow the live observation of these events and give us the opportunity of answering the previous questions. A better insight into the exact frequency of NEBs could also be accomplished as through TEM we only observe a section of the cell and to capture the real frequency, an extremely high number of images must be examined.

To date, we have examined five different stress conditions and presented a correlation between protein quality control and the frequency of NEB events. Further conditions, however, could also be investigated for the triggering of NEBs such as in cancer cells or other disease related models. It is very likely that NEB is not only involved in a single process, but could facilitate many cellular pathways. As these events appear to be evolutionarily conserved among several species, it would be of great interest to investigate more in detail if the functionality of NEB is similar or if it changes depending on the organism in question.

AdipoR2 project

We have currently proven that AdipoR2 has a ceramidase functionality that catalyzes the hydrolysis of ceramides into sphingosine and free fatty acids and

regulates membrane homeostasis. The regulation of membrane fluidity through AdipoR2 has been shown to act independently of their proposed ligand, adiponectin, though previous studies have shown that adiponectin may be able to enhance the AdipoR2-mediated ceramidase activity. Thus, it would be of great interest to further investigate if adiponectin really acts through AdipoR1/2 or if there is a separate pathway involved.

In paper II, we have investigated the observable morphological differences induced by addition of PA in wild type and AdipoR2 depleted cells. These observations gave rise to interesting phenotypes such as the formation of the closely apposed membranes that resembled straight lines. When we investigated the PINK-1 protein as a marker for mitophagy, we surprisingly saw that PINK-1 was significantly localized in the closely apposed ER in comparison to a healthy-looking ER. It would be interesting to evaluate if more proteins are trapped in the closely apposed ER due to its peculiar morphology that could reflect a rigid packing of the membrane phospholipids. In such a case, formation of closely apposed membranes could hinder several cellular pathways and may be a cause for activation of apoptosis. In the same paper, there was also a high number of NEB like events in the AdipoR2 depleted cells treated with PA. If these events are similar to the ones observed in paper I and if they share a similar functionality is to be further examined.

S1P project

In this project we thoroughly investigated the downstream pathway of the AdipoR1/2 as well as possible reasons for the embryonic lethality of double AdipoR1/2 mutant mice. While examining MEF cells under the electron microscope, unique spiral structures of the cytoplasmic membranes were observed. This phenotype so far appeared only in these primary cells, making it interesting to further investigate possible PA-induced structural deviations

in a variety of cell models. Similar to the closely apposed membranes of paper II, these spirals could potentially influence cellular functions and perhaps contact sites between different compartments.

Although mice experiments can be laborious and time-consuming, further investigation of the observed embryonic lethality may be of interest. The E12.5 embryos did not show any difference in their histological results but perhaps examination of different embryonic stages could give a better understanding of the physiological changes in the double mutants.

ACKNOWLEDGMENTS

I would like to express my deepest gratitude to my supervisor, **Richard Neutze**, for giving me the opportunity to be part of your lab and science. Even though I did not have any previous experience in this particular field, I was given the opportunity to learn and develop over the past six years and I am truly grateful for that. Thank you for always being there even in your most busy times, caring for both my science and my well-being in the working environment.

I am also extremely grateful for my two co-supervisors, **Marc** and **Johanna**, for introducing me to two completely different scientific fields. Marc, thank you for always being such an inspiration with your excitement about your science and the willingness to hear our thoughts, questions, and ideas. You are always efficient and fast in giving replies and feedback, supervising as much as needed but also giving lots of freedom for exploring our own ideas and experiments. Your obvious love for science is contagious and motivational! Johanna, thank you for introducing me to the vast world of EM! It has been such a beautiful and unique experience to see biology under an electron microscope! Thank you for always being compassionate and understanding of personal situations, especially after having two little kids that made VABing a regular part of my life.

To my examiners, **Sebastian** and **Gisela**, thank you for being there to check my process over the years and make sure everything was planned well and going smoothly!

From Höög's lab: I would first like to thank my colleague **Davide** who was actually the one to introduce me for this PhD position! Thank you so much for helping me start my PhD journey. Thank you for always being you, the nicest

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