universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

"Influence of glucose starvation on endocytosis and endosomal compartments in primary mouse hepatocyte sandwich culture"

verfasst von / submitted by

Helena Petrovic BSc

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2016 / Vienna 2016

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 066 834
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Molekulare Biologie
Betreut von / Supervisor:	ao. Univ.-Prof. Dipl.-Ing. Dr. Johannes Nimpf

TABLE OF CONTENTS

ABSTRACT 4

ZUSAMMENFASSUNG 5

ACKNOWLEDGEMENT 6

1 INTRODUCTION 7

1.1 Type 2 diabetes and correlated metabolic syndrome – a big threat to the humanity 7

1.2 Liver – a key metabolic organ 8

1.3 Endocytosis – the crucial process enabling hepatocellular polarity 10

1.4 RabGTPases and their effectors regulate endosome trafficking and fusion 11

1.5 Reciprocal connection between endocytosis and metabolism 13

1.5.1 Metabolically important kinases 15

1.6 Aim of the work 16

2 MATERIALS AND METHODS 17

2.1 Antibodies and reagents 17

2.2 Isolation of primary mouse hepatocytes 17

2.3 Primary hepatocyte sandwich cultures 20

2.4 Glucose starvation assay 21

2.4.1 Receptor internalization assay (LDL and EGF uptake assay) 23

2.4.2 Ice binding assay 23

2.4.3 Immunofluorescence 23

2.4.4 Immunofluorescence quantification by Fiji 24

2.5 SDS–PAGE, Western blots and quantification 25

3 RESULTS 28

3.1 Establishment of primary hepatocyte collagen sandwich system in new environment 28

3.2 Endosomal system is able to differentiate between different glucose availability 29

3.2.1 Internalization of signaling EGF cargo as a readout for endocytic transport 29

3.2.2 Internalization of nutritional LDL cargo as a readout for endocytic transport 32

3.3 Primary hepatocytes showed increased EGF and LDL ligand–receptor binding upon low glucose starvation 34

3.4 No alteration of intracellular compartments after glucose starvation 37

3.4.1 Glucose effect on endosomal compartments 37

3.4.2 No clear sign of glucose influence on other intracellular compartments 40

3.5 Kinases as a potential mediator between glucose metabolism and endocytosis 43

4 DISCUSSION 48

5 LITERATURE 54

6 LIST OF FIGURES 59

ABSTRACT

The endosomal system is a complex network of membrane compartments, important for communication between hepatocytes and extracellular environment. Hepatocytes, as highly polarized cells take up nutrients, hormones and metabolites by a process called endocytosis. Endocytosis is a key mechanism for maintaining glucose and lipid homeostasis in the liver. The molecular mechanism regulating the reciprocal relationship between endocytosis and metabolism are still poorly understood. In this study, we investigated the changes in endocytosis triggered by extracellular metabolic factors i.e. glucose stimulation. For this, the primary hepatocyte sandwich culture was stimulated with different glucose concentrations and the effects on endocytosis, endosomal compartments and molecular players were monitored using immunofluorescence and quantitative imaging as well as western blotting for protein analysis. We find that endocytic activity is enhanced in the presence of high glucose concentration and that there is less receptor–cargo binding on the cell surface under this condition. Furthermore, the enhanced recruitment of LAMP1 to the late endosomes under low glucose condition might suggest activation of autophagy. The kinases critical in metabolism, AMPK and Akt, showed activation after exposing the primary hepatocytes to different glucose concentrations. Together these results suggest that endosomal system is able to act accordingly to different glucose availability in the environment and to adapt its function corresponding to it.

ZUSAMMENFASSUNG

Das endosomale System stellt ein komplexes Netzwerk aus Membrankompartimenten, wichtig für die Kommunikation zwischen Hepatozyten und dem extrazellulären Extrazellularraum, dar. Hepatozyten nehmen über den Prozess der sogenannten Endozytose Nährstoffe, Hormone und Metabolite auf. Endozytose ist ein Schlüsselmechanismus in der Erhaltung der Glucose– und Lipidhomöostase der Leber. Weitgehend ungeklärt sind jedoch bis heute die zugrundeliegenden molekularen Mechanismen der Regulation des Zusammenspiels von Endozytose und Metabolismus. In dieser Studie haben wir Veränderungen der Endozytose in Zusammenhang mit extrazellulären metabolischen Faktoren als Trigger, wie z.B. Stimulation durch Glucose, untersucht. Hierfür wurde die primäre Hepatozyten–Sandwich–Kultur mit unterschiedlichen Glucosekonzentrationen behandelt und Auswirkungen auf die Endozytose, endosomale Kompartimente und molekulare Faktoren über Immunfluoreszenz, quantitative Imaging sowie western blotting für die Proteinanalyse ermittelt. Gezeigt hat sich, dass sich die endozytotische Aktivität in Anwesenheit hoher Glucosekonzentrationen verstärkt und Rezeptor–Liganden–Bindungen auf der Oberfläche abnehmen. Die erhöhte Rekrutierung von LAMP1 zu späten Endosomen bei geringen Glucosekonzentrationen legt eine Aktivierung von Autophagie–Prozessen nahe. Weiter fand sich bei den für den Metabolismus ausschlaggebenden Kinasen AMPK und Akt, nach Exposition der primären Hepatozyten zu unterschiedlichen Glucosekonzentrationen, eine Aktivierung. Gemeinsam lassen die Resultate den Schluss zu, dass das endosomale System fähig ist auf das unterschiedliche Glucoseangebot der Umgebung zu reagieren und seine Funktionen darauf anzupassen.

ACKNOWLEDGEMENT

I would first like to thank my thesis advisor Dr. Anja Zeigerer of the Helmholtz Diabetes Center (HDC) at Helmholtz Center Munich. The door to Dr. Zeigerer office was always open whenever I ran into a trouble spot or had a question about my research or writing. She consistently allowed this paper to be my own work, but steered me in the right the direction whenever she thought I needed it.

I would also like to thank the experts Prof. Dr. Johannes Nimpf at University of Vienna for validation of this thesis and Prof. Dr. Stephan Herzig of the Institute for Diabetes and Cancer (IDC) at Helmholtz Center Munich, who provided me an opportunity to join his team as intern and for their very valuable comments.

I thank my fellow lab mates for the stimulating discussions, advices and for all the fun we have had.

Finally, I must express my very profound gratitude to my parents, my sister and to my boyfriend for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them. Thank you.

Author

Helena Petrovic

1 INTRODUCTION

1.1 Type 2 diabetes and correlated metabolic syndrome – a big threat to the humanity

Diabetes mellitus is one of the 10 leading causes of death in the world according to the World Health Organization. The number of cases hit the 382 million mark and is on the rise all over the world, threating to become pandemic. The rates of obesity and diabetes are not only increasing in western societies, but even more in developing countries, where 80% of diabetes patients are living. It has been presumed that in 20 years´ time there will be 592 million people living with the disease (International Diabetes Federation, 2014) see Figure 1 for details. The showed data does not distinguish between type 1 and type 2 diabetes. It has been reported that in high–income countries approximately 87% to 91% of all people with diabetes are estimated to have type 2 diabetes (International Diabetes Federation (IDF), 2015).

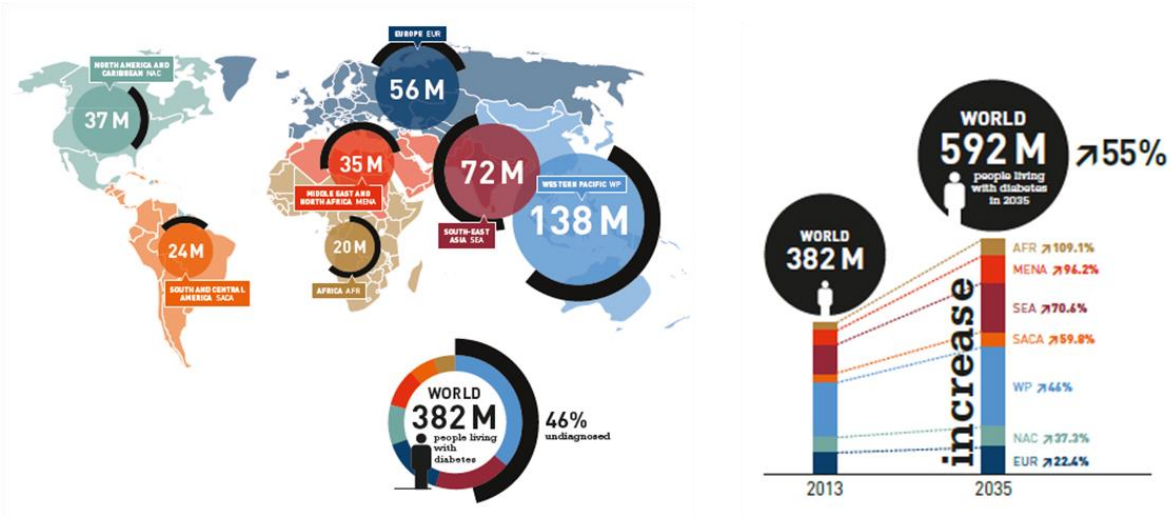


Figure 1 Diabetes is a huge and growing problem, and the costs to society are high and escalating. (See oben. Image and text from IDF Diabetes Atlas, 6th edition.)

Until now, effective treatments against type 2 diabetes and its associated diseases e.g. metabolic syndrome are still lacking. There is an overlap of symptoms and syndromes, which makes the disease systemic. Metabolic syndrome consists of many different diseases such as obesity, hypertension, dyslipidemia and heart failure, but its main characteristic is the development of

insulin resistance. Insulin resistance contributes to type 2 diabetes and the metabolic syndrome. In these diseases, the pancreas is producing insulin but the insulin target organs e.g. adipose tissue, muscle and liver stop responding to it. Over time, metabolic syndrome precipitate to more severe disease – type 2 diabetes. Insulin levels decrease and the blood glucose levels subsequently increase, since the insulin fails to suppress glucose production in liver. Frequent urination, excessive thirst, weight loss and blurred vision are only some of leading symptoms caused by this condition. More serious complications are coronary heart disease, stroke, blindness, renal failure and gangrene. Liver is not only responsible for endogenous glucose production, but it is crucial for lipid metabolism. Lipoprotein formation and internalization is especially enhanced in diabetic state, due to insulin´s function to stimulate protein and lipid production in the liver. Therefore, understanding the regulation of hepatic glucose production and lipogenesis is of essential meaning for potential anti–diabetic interventions.

1.2 Liver – a key metabolic organ

The liver comprises different cell types: parenchymal and non–parenchymal cells see Figure 2 for more information.

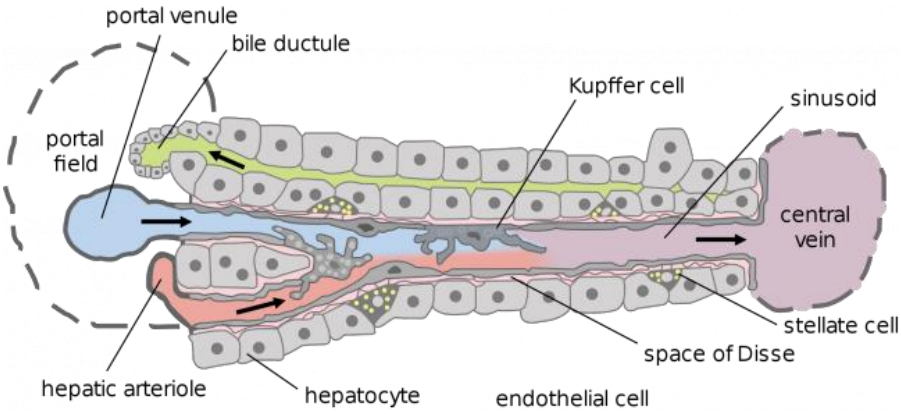


Figure 2 The liver comprises multiple cell types. The hepatic sinusoid is lined with the non–parenchymal cells: sinusoidal endothelial cells, Kupffer cells and hepatic stellate cells. Parenchymal cells commonly referred to as hepatocytes are organized as plates or sheets along hepatic sinusoids, thereby showing a remarkable heterogeneity. (Author of image: originally by Frevert U, Engelmann S, Zougbédé S, Stange J, Ng B, et al. Converted to SVG by Viacheslav Vtyurin who was hired to do so by User: Eug. Source of image: Intravital Observation of Plasmodium berghei Sporozoite Infection of the Liver, PLoS Biology.)

The prevalent cell type with 80% of the liver volume, are hepatocytes (parenchymal cells). Hepatocytes perform the liver's metabolic functions, including regulation of glucose and lipid metabolism, glycogen storage, plasma protein synthesis, detoxification and bile acid production.

Any perturbation of these functions can lead to diseases e.g. drug–induced liver diseases, cholestasis, type 2 diabetes and non–alcoholic fatty liver disease (NAFLD) (Zeigerer, A., Seifert, S., Marsico, M., Kalaidzidis, Y. & Zerial, manuscript under review in Exp. Cell Res.).

Hepatocytes are characterized by a polar plasma membrane, which is divided into two domains fulfilling different functions. Their apical membranes form a continuous network of bile canaliculus throughout the liver, while their basal membranes are in contact with the sinusoidal blood network. In comparison to normal polarized cells, which show a columnar polarity (Treyer & Müsch, 2013), hepatocytes form the apical membrane between neighboring cells. These apical membrane fuse and build a continuous network, called the bile canalicular network (Treyer & Müsch, 2013). The main role of the bile canaliculus is to collect bile secreted by hepatocytes see Figure 3.

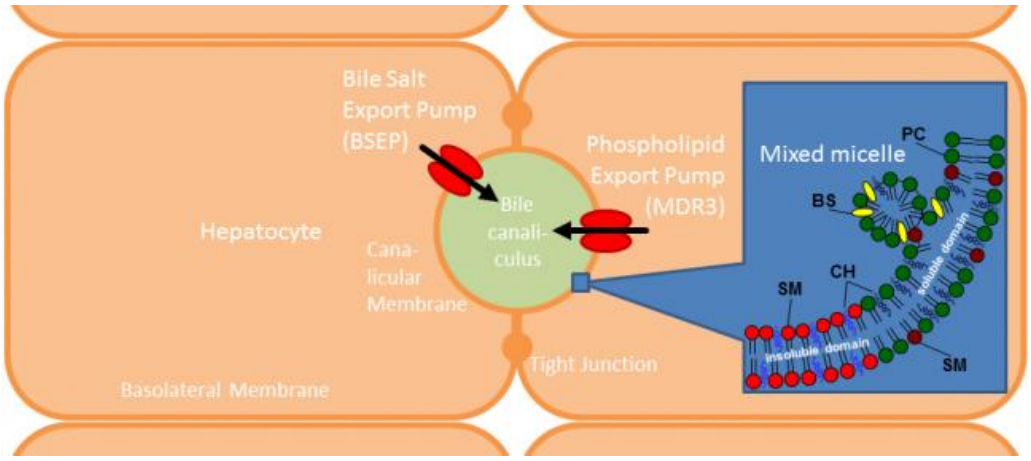


Figure 3 Liver cells secrete bile. The apical membrane facing the bile canaliculus contains specific proteins in charge of pumping bile salts into the bile canaliculus and proteins pumping lipids from the inner to the outer side of the membrane. BS: bile salt, CH: cholesterol, PC: phosphatidylcholine, SM: sphingomyelin (Author of image: Johannes Eckstein).

The tight junctions are responsible for forming the barrier between apical and basolateral membranes. The basolateral membrane contains proteins and lipids that are involved in exchange of metabolites and proteins with the blood.

1.3 Endocytosis – the crucial process enabling hepatocellular polarity

Hepatocellular polarity in the liver is of essential importance for the understanding of liver tissue structure and function. Hepatocytes are highly polarized cells. From the blood, hepatocytes take up nutrients, signaling molecules (e.g. hormones) and metabolites by a process called endocytosis. The internalized cargos are metabolized by hepatocytes and the products are released into the bile canalicular network by membrane trafficking pathways. The endosomal system is a complex network of membrane compartments, important for communication between hepatocytes and extracellular environment. Endosomal compartments can be seen as regulatory "hubs", which decide what kind of destiny the cargo will have e.g. being recycled back to the plasma membrane, retro transported to the Golgi, or further transported to late endosomes and degraded in lysosomes see Figure 4. For example, low–density lipoprotein (LDL) and epidermal growth factor (EGF) are internalized into clathrin–coated vesicles via process of clathrin–mediated endocytosis. After being internalized in the early endosome, cargo can be further processed along two different routes. It can be either recycled back to the cell surface via recycling endosomes, as in case of LDL receptor and EGF receptor or it can be degraded via late endosomes and lysosomes, as known for LDL and EGF (Jovic, Sharma, Rahajeng, & Caplan, 2010).

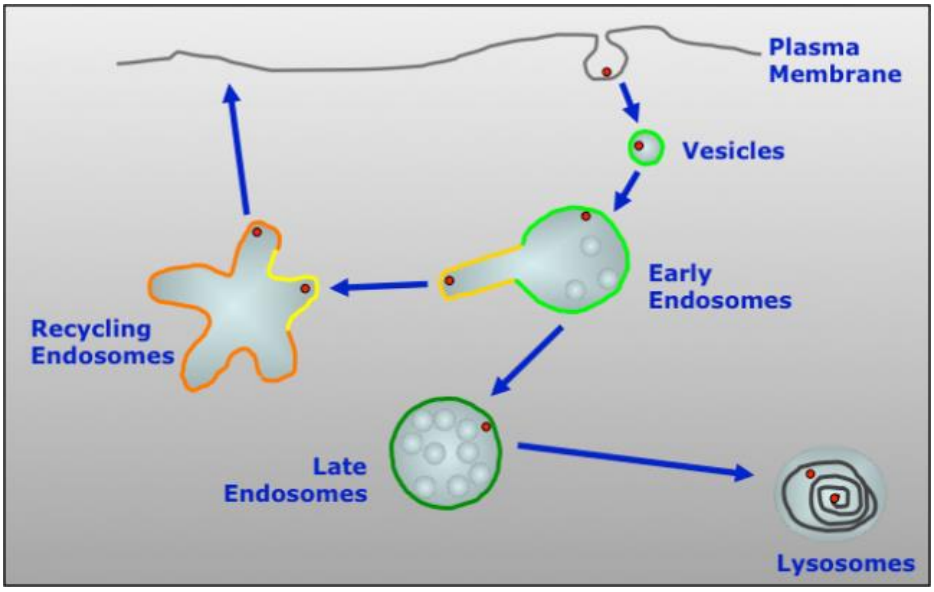


Figure 4 Endosomal transport in liver cells. Vesicles containing internalized cargo are transported to early endosomes for further sorting: The cargo is either recycled back to the cell surface through recycling endosomes or is alternatively transported via late endosomes to the lysosomes, to be degraded. (Authors of text and image: Anja Zeigerer and Marino Zerial.)

Endocytosis plays an important role for the correct sorting of cargos and junctional components to the membrane. This is achieved by involvement of specialized apical and basolateral endocytic and recycling membrane trafficking pathways (Zeigerer, A., Seifert, S., Marsico, M., Kalaidzidis, Y. & Zerial, manuscript under review in Exp. Cell Res.). Between endocytosis and polarity proteins coexist a tight and bidirectional relationship (Di Fiore & von Zastrow, 2014). Intact endocytosis is crucial for the maintenance of hepatocyte polarity (Zeigerer et al., 2012). It was shown that under perturbation of the endo–lysosomal system, apical proteins failed to be transported to the bile caniculi but misrouted to other subcellular compartments. The main regulator of this process, Rab5 seems to have a main role in polarized cargo sorting and in the maintenance of hepatocyte polarity necessary for liver function (Zeigerer et al., 2012).

1.4 RabGTPases and their effectors regulate endosome trafficking and fusion

Communication between hepatocytes and extracellular environment via endocytosis is of main importance for maintaining glucose and lipid homeostasis in the liver (Zeigerer et al., 2015). In order to absorb and release nutrients and receive and process signals, cells need endosomes. These organelles are specialized for sorting the cargo to the correct target. The structural and functional identity of intracellular compartments and specificity of their transport steps is ensured by Rab GTPases (Wandinger-Ness & Zerial, 2014) see Figure 5. The reversible GTP/GDP cycle of these small GTPases from Ras superfamily is in charge of assembling protein machineries on the intracellular membranes and by that affecting the membrane composition and fate of organelles. The cycle base on guanosine triphosphate (GTP) binding and hydrolysis to guanosine diphosphate (GDP). These reactions are regulated by guanine–nucleotide exchange factors (GEFs) and GTPase–activating proteins (GAPs). In their inactive state GTPases are bound to GDP in the cytosol. GEFs activate Rab proteins by stimulating the release of GDP to allow binding of GTP. In this GTP–bound conformation, GTPases dissociate from inhibitors and are “free” to bind effectors and to be stabilized on the membrane of the specific compartment (Wandinger-Ness & Zerial, 2014).

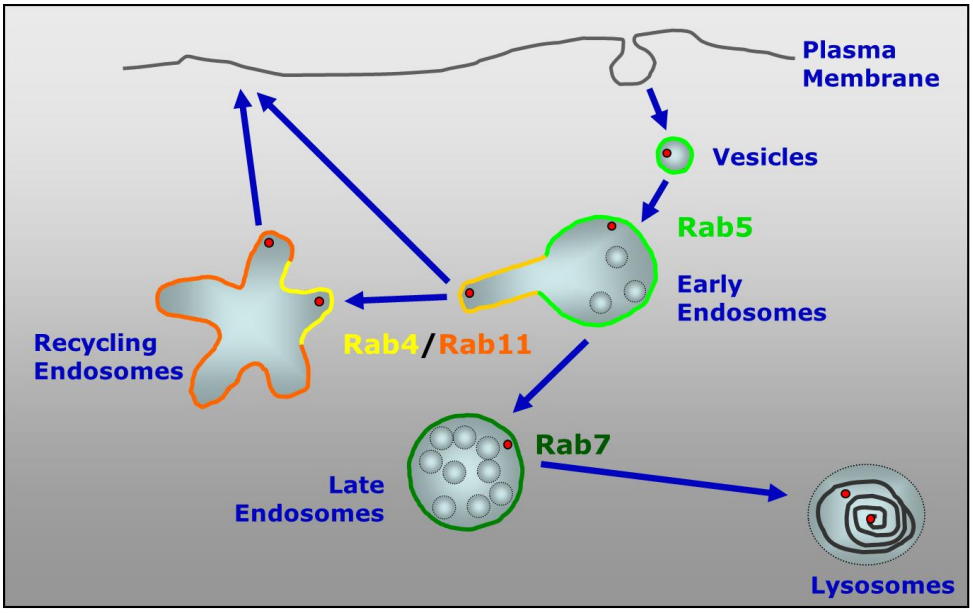


Figure 5 Rab proteins are organelle specific. For example, Rab5 is the GTPase which is found on the membrane of the early endosomes. Rab4/Rab11 are recycling endosome specific, whereas Rab7 is characteristic for late endosomes. (Authors of image: Anja Zeigerer and Marino Zerial.)

For example, Rab5 is the GTPase, which is found on the membrane of the early endosomes. Rab GTPases Rab5, Rab4, and Rab11 are characteristic for the early endocytic pathway, whereas Rab7 and Rab9 are present on the late endocytic pathway (Wandinger-Ness & Zerial, 2014). Rab4 and Rab11 control the trafficking along the recycling pathway, from early and recycling endosomes to the cell surface. Rab11 and belonging effectors can influence cell polarity, whereby causing cancer, gastrointestinal, and degenerative disease (Wandinger-Ness & Zerial, 2014).

Activated Rab proteins bind corresponding Rab effectors, whereby enabling the coordination of individual transport steps of the endocytic pathway. Accordingly, each endosome corresponds to a specific compartment and executes its specific function. It is known, that some Rab effectors act as tethering molecules for membranes compatible for fusion. Rab effectors cooperate with soluble NSF attachment protein receptors (SNAREs) in order to fuse with the corresponding membrane (Wandinger-Ness & Zerial, 2014). Rab5, the master regulator of early endosomes (Zeigerer et al., 2012), recruits an effector early endosome antigen 1 (EEA1), which further interacts with the early endosomal SNAREs and tethers endosomal membranes. EEA1 is downstream of clathrin–coated pits and is selectively recruited from the cytosol onto the membrane of early endosome. It ensures directionality to vesicular transport from the plasma

membrane to the early endosome (Wilson et al., 2000). One of the key effectors of Rab5, phosphatidylinositol-3-kinase (PI3K) Vps34/Vps15 complex catalyzes the phosphorylation of phosphatidylinositol to generate phosphatidylinositol-3-phosphate (PI3P) (Wandinger-Ness & Zerial, 2014). PI3P exists in higher amount on early endosomes and is the recognition motif for binding of FYVE–domain proteins e.g. EEA1 which enables regulation of early endocytic trafficking (Wandinger-Ness & Zerial, 2014). Activation of PI3K causes the accumulation of EEA1 in maturing early endosomes resulting in termination of Akt signaling (Zoncu et al., 2009). Dynamic interactions between Rab GTPases and their effectors on endosomal membranes are responsible for endosome biogenesis, cargo transport, and signaling.

1.5 Reciprocal connection between endocytosis and metabolism

Endocytosis, as a dynamical transport system between plasma membrane and intracellular compartments, affects intra–organelle communication. It has been shown, that Rab5 knockdown in mouse liver *in vivo* led to the loss of the entire degradative pathway i.e. early endosomes, late endosomes and lysosomes (Zeigerer et al., 2012) see Figure 6. The loss of Rab5 did not only have an impact on number of early endosomes. The changes in other cellular compartments such as late endosomes/multivesicular bodies and lysosomes, clathrin–coated pits and clathrin–coated vesicles (CCV), endoplasmic reticulum (ER), Rab11–positive structures were monitored. However, the expression of membrane tethering and fusion proteins e.g. EEA1 was unchanged. Appearance of lipid droplets and glycogen granules in hepatocytes refer to metabolic dysfunction (Zeigerer et al., 2012), meaning that endocytosis does not only regulate uptake of nutrients but also mediates signal transduction and metabolic activities (Antonescu, McGraw, & Klip, 2014; Sorkin & von Zastrow, 2009). As a consequence of Rab5 knockdown, perturbations in glucose and lipid metabolism were observed. The Rab5KD phenotype mimicked von Gierke's disease, which is characterized by a glucose-6-phosphatase (G6Pase) deficiency in liver, kidney and intestine. Furthermore, depletion of Rab5 led to hypoglycemia by inhibiting gluconeogenic gene expression. This study suggested that there is an unknown mechanism that links endocytosis with gluconeogenesis and therefore glucose metabolism (Zeigerer et al., 2015).

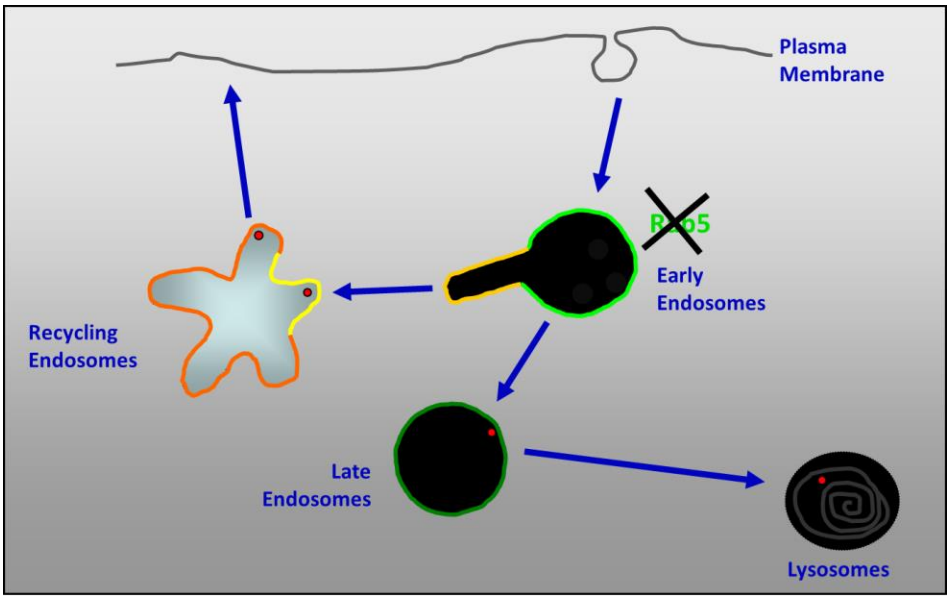


Figure 6 Rab5 is the master regulator of endosome biogenesis. One effect of the Rab 5 knockdown is the dramatic reduction in the number of early endosomes, late endosomes and lysosomes. (Authors of image: Anja Zeigerer and Marino Zerial.)

There are more evidences, showing that endocytosis is involved in metabolic regulation. It has been found out that Rab4KD in flies resulted in a decrease in glycogen levels, suggesting its involvement in growth of larvae and metamorphosis by regulating insulin and 20E pathways (Hou, Cai, Liu, Song, & Zhao, 2012). Furthermore, overexpression of Rab25 led to accumulation of glycogen in epithelial cancer cells upon nutrient stress (Cheng et al., 2012). In addition, depletion of the endosomal PI3K, Vps34 in mice *in vivo* caused hepatomegaly and hepatic steatosis, as well as a defect in glycogen storage (Jaber et al., 2012). Alternatively, many new studies suggest that metabolic activities can also regulate endocytosis. Accordingly, knockdown of metabolic genes in a genome–wide RNAi screen had a huge impact on endocytosis, inducing many defects (Collinet et al., 2010). Study in flies fed with high sugar showed an up–regulation of many endocytosis genes (Mattila et al., 2015). It has already been seen in yeast that glucose acts as both a metabolic substrate and a potent regulator of intracellular signaling cascades (Aoh, Graves, & Duncan, 2011). Cargos internalized by clathrin–mediated endocytosis are characterized by their specific sorting signals, which are recognized by adaptors located in clathrin coats (Traub & Bonifacino, 2013). Clathrin–dependent traffic at the trans–Golgi network and endosomes is transiently blocked upon glucose starvation (0% glucose) in yeast cells (Aoh et al., 2011). This blockage is due to disassociation of clathrin adaptors from membranes. The change

in the mechanism of adaptor recruitment may result in reduction of overall traffic rates. Clathrin adaptors at the trans–Golgi network and endosomes showed a changed localization upon glucose starvation and the protein kinase A (PKA) and AMP–activated protein kinase (AMPK) pathway seem to indirectly regulate this process through changes in energy metabolism. All this suggests that metabolism is controlled by endosomal traffic and any change of it may affect the signaling and proliferation of the cell by altering the protein composition on the cell membrane and endosomal compartments (Aoh et al., 2011).

1.5.1 Metabolically important kinases

Since the energy metabolism is required for adaptor localization and ATP production is affected by glucose starvation in yeast (Aoh et al., 2011), we focus in this work on AMPK and related kinases and how they are affected by glucose starvation in primary hepatocytes. AMPK is activated by cellular stress factors such as hypoxia, isochemia and glucose deprivation. In these cases, AMPK acts as a metabolic master switch by decreasing energy consumption and increasing energy production (Hardie, Ross, & Hawley, 2012). Lipid, glucose and protein homeostasis, and mitochondrial biogenesis, as well as some glycolytic, lipogenic and gluconeogenic genes are controlled by activity of AMPK (Fu, Wakabayashi, Ido, Lippincott-Schwartz, & Arias, 2010). Nevertheless, AMPK regulates canicular network formation and maintenance i.e. hepatocyte polarization. Another kinase of meaning, target of rapamycin (TOR) is responsible for cell growth and metabolism and is controlled by nutrients, growth factors, and energy status. Mammalian TOR complex 1 (mTOR1) phosphorylates different substrates e.g. ribosomal protein S6 kinase (S6K) that regulates protein synthesis (Hagiwara et al., 2012). Protein kinase B (Akt) is the kinase that is activated upon insulin binding and consequent PI3K activation (Hers, Vincent, & Tavars, 2011). Phosphorylation of Akt can cause alterations in glucose and lipid metabolism, since Akt stimulates the translocation of glucose transporters to the cell surface which leads to glucose uptake into the cell and increased glycogen synthesis and lipogenesis (Leto & Saltiel, 2012). Taken all together, the relationship between endocytosis and metabolism seems to be bi–directional. The molecular mechanisms behind this reciprocal regulation and connection between endocytosis and metabolism are still to be understood.

1.6 Aim of the work

Since the mechanisms ruling the interplay between endosomal trafficking and glucose and lipid metabolism are still unexplored, the aim of my work was to investigate the changes in endocytosis triggered by extracellular metabolic factors e.g. glucose stimulation. Therefore, we hypothesize that endosomal system is able to differentiate between different glucose availability.

In order to dissect the question how does endosomal system sense and respond to metabolic cues such as glucose, the primary hepatocytes were stimulated with different glucose concentrations and the effect on endocytosis, endosomal compartments and molecular players was monitored using immunofluorescence and quantitative imaging as well as western blotting for protein analysis.

2 MATERIALS AND METHODS

2.1 Antibodies and reagents

Primary antibodies

Rabbit anti EEA1 was developed in the Zerial lab. Other rabbit polyclonal antibodies were as follows: anti–AKT, phospho–AKT (Ser473), anti–AMPK, anti–Rab5, phospho–S6 RP from Cell Signaling Technology, phospho–AMPK (Thr172), anti–Rab11 from BD Bioscience, anti–G6PC and anti–CLTC from Atlas Antibodies, anti–Tom20 from Santa Cruz. Rat monoclonal anti–LAMP1 was purchased from BD Bioscience and mouse monoclonal anti–GAPDH from Sigma.

Secondary antibodies

HRP–secondary antibodies were anti–mouse–HRP from Thermo Fisher Scientific and anti–Rabbit–HRP from Sigma. Secondary antibodies labelled with Alexa fluorophores, Alexa–488–phalloidin, Dapi and EGF, biotinylated, complexed to Alexa Fluor–555 were purchased from Molecular Probes. LDL was purified from human serum und labelled as previously described (Rink, Ghigo, Kalaidzidis, & Zerial, 2005).

2.2 Isolation of primary mouse hepatocytes

Primary hepatocytes were isolated from C57BL/6NHsd mice (8 to 12 weeks old) via collagenase perfusion. The mouse was anesthetized by intraperitoneal injection of 50 µl Ketamine (100 mg/ml): Rompun (20 mg/ml) and fixed onto sterilized surface after being tranquillized. The abdominal cavity was opened and the liver was perfused through *venae cave* with prewarmed EGTA buffer, avoiding air bubbles. After successful cannula insertion, the hepatic portal vein was sequentially cut, in order to avoid harming hepatocytes by high pressure. The peristaltic flow rate was set on 6.8 ml/min, corresponding the mouse blood flow rate and the liver rinsed for approx. 15 minutes. The pressure of the perfusion should not be above a 20 cm water column.

EGTA buffer

- 124 ml Glucose (9 g/l)
- 20 ml KH buffer
- 20 ml HEPES buffer
- 30 ml amino acid solution
- 2 ml Glutamine solution (7 g/l)
- 0.8 ml EGTA solution

HEPES buffer

- 60 g/l HEPES
- pH adjusted with NaOH to 8.5

EGTA solution

- 47.5 g/l dissolved in H_2O + NaOH
- pH adjusted with HCl to 7.6

KH buffer

- 60 g/l NaCl
- 1.75 g/l KCl
- 1.6 g/l KH_2PO_4
- pH adjusted with NaOH to 7.4

Amino acid solution

- 0.27 g/l L–Alanine
- 0.14 g/l L–Aspartic acid
- 0.4 g/l Asparagine
- 0.27 g/l Citrulline
- 0.14 g/l L–Cysteine
- 1 g/l L–Histidine
- 1 g/l L–Glutamic acid
- 1 g/l L–Glycine
- 0,4 g/l L–Isoleucine
- 0.8 g/l – Leucine
- 1.3 g/l L–Lysine
- 0.55 g/l L–Methionine

- 0.65 g/l L–Ornithine
- 0.55 g/l L–Phenylalanine
- 0.55 g/l L–Proline
- 0.65 g/l L–Serine
- 1.35 g/l L–Threonine
- 0.65 g/l L–Tryptophan
- 0.55 g/l L–Tyrosine
- 0.8 g/l L–Valine
- Amino acids, which cannot be resolved at neutral pH, can be solubilized using 10 N NaOH. The pH can be adjusted afterwards with HCl to 7.6.

After adding 60 mg of fresh collagenase (≥ 125 collagen digestion unit/mg solid) to the collagenase buffer, the buffers were exchanged and the liver rinsed for following 7 – 10 min with corresponding buffer, until the collagen fibers in the liver lost its elasticity.

Collagenase buffer

- 155 ml Glucose–solution
- 25 ml KH–buffer
- 25 ml HEPES–buffer
- 38 ml amino acid solution
- 10 ml $CaCl_2$ (19 g/l $CaCl_2$*$2H_2O$)
- 2.5 ml Glutamine-solution
- Add the collagenase during the perfusion with EGTA to the collagenase buffer.

Afterwards, the liver was cut out and placed into a petri dish filled with approx. 15 ml suspension buffer, containing 400 mg of BSA.

Suspension buffer

- 124 ml Glucose
- 20 ml KH–buffer
- 20 ml HEPES–buffer (pH adjusted to 7.6)
- 30 ml amino acid solution
- 2 ml Glutamine solution
- 0.8 ml $MgSO_4$–solution (24.6 g/l $MgSO_4*7H_2O$)
- 1.6 ml $CaCl_2$–solution
- 400 mg BSA

The hepatocytes were extracted under the sterile hood by gently holding the liver with pincers and by repeatedly soaking it in the suspension buffer until the hepatocytes were dissociated. This suspension was passed through a wire net (pore size of 100 μm) and centrifuged for 5 min at 50x g and at 4°C. The pellet was washed twice in 50 ml of suspension buffer. Finally, the pellet was resuspended in 10 ml suspension buffer by gently inverting the tube. Furthermore, the cell number and vitality was determinated. For this hepatocytes were diluted 1:1 in a trypan blue solution (4 g/l) and the number of vital hepatocytes was calculated using the Neubauer counting chamber. In order to get the final concentration of living hepatocytes in 10 ml, the average of the cell number counted in squares was multiplied with the dilution factor 2 and further with the volume factor $10*10^4$. Specifically, 100 µl of resuspended cell suspension was pipetted with cut–off tip into 400 µl of suspension buffer and from there 25 µl of suspension was mixed with 25 µl Trypan blue solution. This was applied to the counting chamber and the number of dead and live cells were counted for all four squares. The following equation for concentration was used: number of live cells x 10000 x dilution factor = number of cells/ml. The common isolation results in total of 30 to 70 Mill cells. The vitality rate should not be less than 75%. For plating approx. 80000 cells/cm^2 were used, which means 200000 cells per 24 well (one well of a 24 well plate has 1.89 cm^2).

2.3 Primary hepatocyte sandwich cultures

The cells were seeded onto collagen layer (approx. 0.9 mg/ml) in 24 well plates (Thermo Fisher Scientific), with or without autoclaved coverslips. For that, 10 mg Rat tail collagen I (Sigma–Aldrich) dissolved in 10 ml of sterile 0.2% (v/v) acetic acid over night at 4°C was used. Furthermore, 10x DMEM (Biozol) was added to the final concentration of 1x and adjusted to pH 7.4 with 1M NaOH using pH–strips. The solution has to be kept on ice all the time, in order to

prevent timely polymerizing. For bottom layer, 100 µl of collagen solution was spread in the well with the pipette tip. It was left for approx. 45 min with open lid to polymerize in the incubator (37°C, 5% CO_2, humidified atmosphere). Afterwards, the cell culture medium was added in order to equilibrate the collagen.

Culture medium

- William's E Medium with 2.24 g/l $NaHCO_3$ (PAN Biotech)
- 10% FCS
- 5% penicillin/streptomycin (Thermo Fisher Scientific)
- 5% L–Glutamine
- 100 nM dexamethasone (Sigma–Aldrich)

The media was removed by gently aspirating before plating the cells. The required volume of cells was transferred to a falcon containing culture medium in order to gain the final concentration of 200000 cells/ml. Approximately 1 ml of cell solution was added drop wise to each well. The cells were incubated for 1 – 3 hours, then washed once with phosphate–buffered saline (PBS), and incubated for further 5 – 3 hours. After 6 hours, the cells were washed once again with PBS to remove dead and unattached cells and the top collagen layer was applied. The collagen solution was diluted in 0.2 % acetic acid in order to achieve a lower concentration (0.6 mg/ml). After the pH was adjusted, 160 µl of collagen solution was applied to the cells and incubated up to 1 hour until the formation of the sandwich. The cells were covered with 0.5 ml culture medium and left in incubator to recover overnight. Media was exchanged every day and experiment performed at day 3 to 5 post–isolation.

2.4 Glucose starvation assay

The primary hepatocyte cell culture was first starved for 2 hours in starvation medium and maintained at 37°C in an atmosphere with 5% CO_2.

Starvation medium

- phenol–free, FCS–free 1xDMEM medium (Gibco)
- 25 mg/l pyruvate
- 5% penicillin/streptomycin
- 1 mM (180.2 mg/l) glucose

Afterwards, the cells were stimulated for the next 2 hours with corresponding low / intermediate / high glucose medium. High glucose medium contains 4.5 g/l glucose, which mimics diabetic condition. Intermediate glucose medium contains 2 g/l glucose and simulates feeding condition, whereas low glucose medium represents fasting condition in the given context.

Low glucose starvation medium

- phenol–free, FCS–free 1xDMEM medium
- 25 mg/l pyruvate
- 5% pen/strep
- 5 mM (0.9 g/l) glucose

Intermediate glucose starvation medium

- phenol–free, FCS–free 1xDMEM medium
- 25 mg/l pyruvate
- 5% pen/strep
- 11 mM (2 g/l) glucose

High glucose starvation medium

- phenol–free, FCS–free 1xDMEM medium
- 25 mg/l pyruvate
- 5% pen/strep
- 25 mM (4.5 g/l) glucose

Furthermore, experiments for endocytosis, imaging and signaling were conducted as described below.

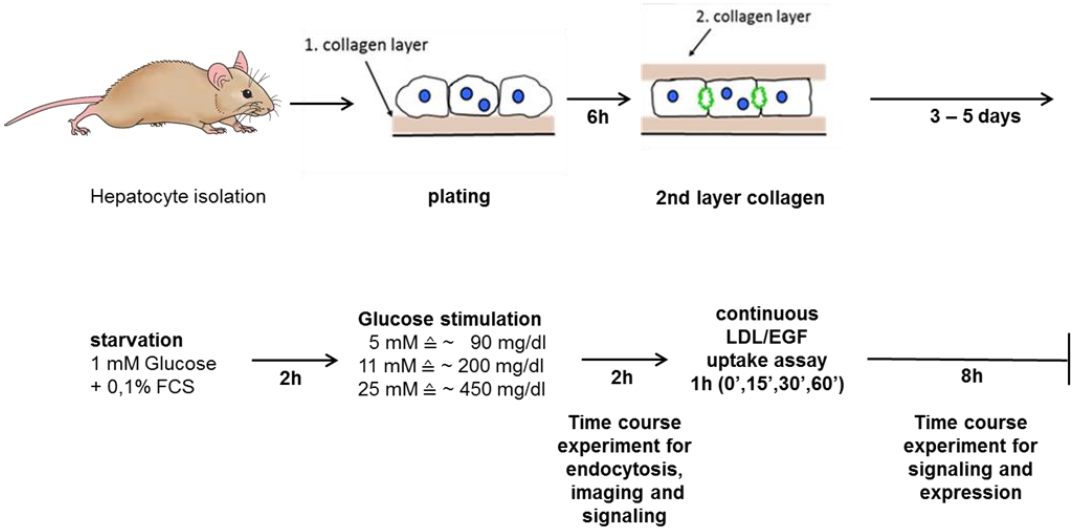


Figure 7 Experimental setup for glucose stimulation assays (see 2.4).

2.4.1 Receptor internalization assay (LDL and EGF uptake assay)

The glucose starvation assay can be followed by LDL or EGF continuous uptake for various time points in corresponding glucose starvation medium followed by a cold wash with PBS and fixation in 4% paraformaldehyde for 30 min. Two kinds of fluorescently lipid–core–labeled LDLs were used, Dil and DiD LDL (2.5 µg/ml) and fluorescently labeled EGF, alexa555–EGF and alexa647–EGF (400 ng/ml).

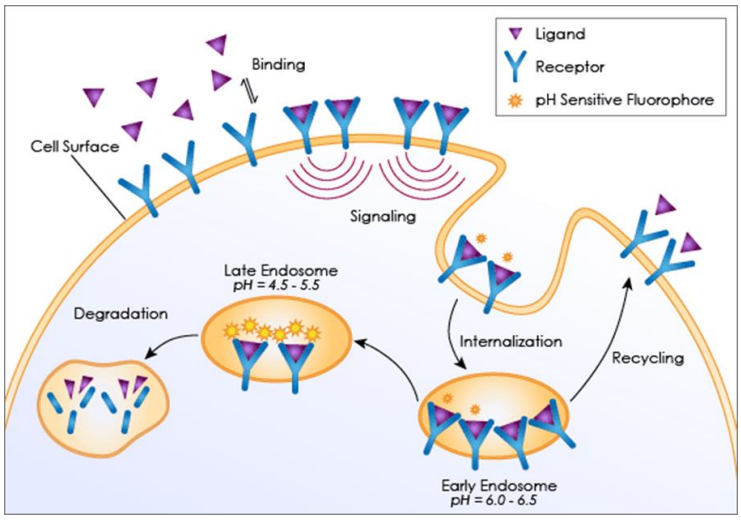


Figure 8 Receptor internalization assay. Receptor Internalization Assay measures the absorption of membrane receptors into the cell via endocytosis. The event is activated by the binding of ligand to surface receptor that signals the formation of plasma membrane-formed inward vesicles to enclose the target receptors (shown right). After the vesicles are formed and internalized, they are redirected to fuse with early endosomes (pH 6.0-6.5) that can recycle the receptors back to the plasma membrane, or they can be degraded via late endosomes and lysosomes (pH 4.5-5.5). (Source: Nexcelom Bioscience)

2.4.2 Ice binding assay

For quantification of LDL or EGF receptor surface levels, primary hepatocytes were starved and exposed to different glucose concentrations for 2 hours, prior to incubation on ice with fluorescently labeled EGF and LDL for 2 hours followed by a cold wash with PBS and fixation in 4% paraformaldehyde.

2.4.3 Immunofluorescence

After the corresponding assay, primary hepatocytes in collagen sandwich were washed with PBS and fixed in 4% paraformaldehyde at room temperature for 30 min, washed twice with PBS, permeabilized for one hour with 0.1% Triton X-100, washed and blocked in 10% horse serum for

1 hour. Holes were aspirated in collagen in order to allow better antibodies penetration, and primary antibodies were added to the cells to incubate overnight at room temperature. On the next day, the cells were washed for a day in TNT wash buffer (300 mM NaCl, 0.1% Tween, 10 nM Tris/HCl) with often exchanges of buffer, incubated with secondary antibodies for 6 hours at 37°C and washed again extensively overnight at 4°C. Afterwards, cells were stained with DAPI for nuclei and with phalloidin for actin, washed with distillated water and mounted onto glass slides using 0.1 g/ml Mowiol (Calbiochem).

Images were taken with a Laser Scanning Confocal Microscope (Olympus Fluoview 1000, IDC, Munich) equipped with an Olympus UPIanSApo 60x 1.35 Oil immersion objective at a resolution of approx. 100 µm/pixel and 600 nm step size.

2.4.4 Immunofluorescence quantification by Fiji

The average fluorescent intensity of approx. 20 – 30 cells per condition were measured by quantitative image analysis using Fiji software in order to determine the level of cargo internalization or binding upon different time points. Each individual cell was manually selected in phalloidin or dapi channel using free hand selection tool and further transferred to the EGF or LDL or other specific marker channel, which fluorescence intensity was calculated using ROI (region of interest) manager tool see Figure 9. Background fluorescence was determined from cells that were not incubated with first antibody or fluorescently labeled cargo.

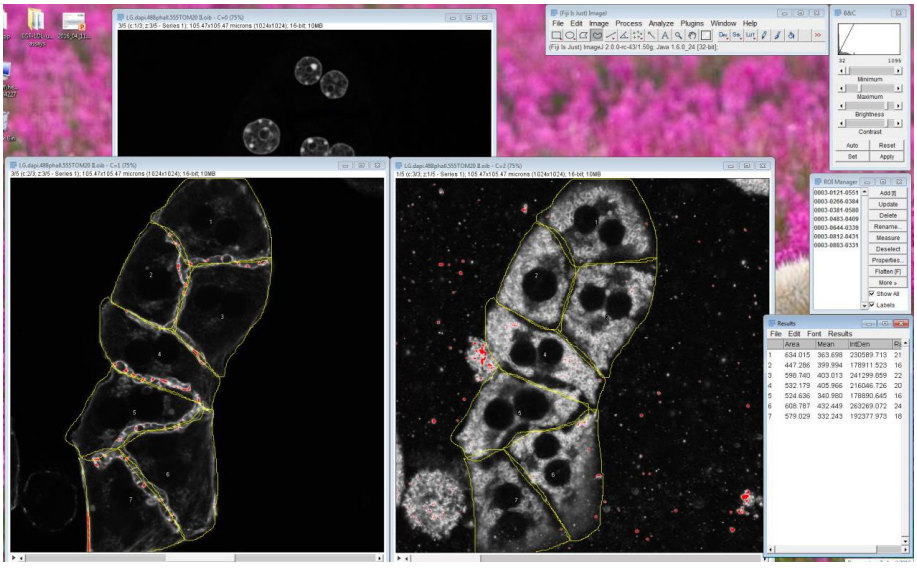


Figure 9 Cell based assay quantification using Fiji software (see 2.4.4 for explanation)**.**

2.5 SDS–PAGE, Western blots and quantification

Western blot lysates were prepared from primary hepatocytes sandwich culture. For this purpose, cells were washed once with PBS and incubated in 100 µl of SDS lysis buffer for 5 min on ice.

SDS–Lysis buffer

- 20 mM Tris pH 7.5
- 150 mm NaCl
- 1 mM EDTA
- 1 mM EGTA
- 1% SDS
- 1% NP40 or 1% Na–deoxycholate
- CLAAAP (1:1000) and Phosphatase inhibitors (1:100)

Approx. 3 – 4 wells were pooled together and the lysate pipetted until resolved. Afterwards, the lysates were incubated for 30 min at 4°C on a rotor and sonicated for 3 min at 37°C. Next, the lysates were span for 5 min at 10000 rpm and the pellet was discarded. Protein concentration was determinated with Bio-Rad Protein Assay kit. The proteins were resuspended in 5x SDS Laemmli buffer and incubated at 95°C for 5 min before the samples were applied to the gels. Approx. 50 µg of total protein per lane were run on SDS-PAGE in SDS running buffer at 100 V constant.

10 % resolving gel for Tris–glycine SDS–polyacrylamide gel electrophoresis

- H_2O 1.9 ml
- 30% acrylamide mix 1.7 ml
- 1.5 M Tris (pH 8.8) 1.3 ml
- 10% SDS 0.05 ml
- 10% ammonium persulfate 0.05 ml
- TEMED 0.002 ml

5% stacking gel for Tris–glycine SDS–polyacrylamide gel electrophoresis

• H_2O	1.4 ml
• 30% acrylamide mix	0.33 ml
• 1.0 M Tris (pH 6.8)	0.25 ml
• 10% SDS	0.02 ml
• 10% ammonium persulfate	0.02 ml
• TEMED	0.002 ml

SDS running buffer (10x)

- 720 g Glycine
- 150 g Tris base
- 50 g SDS
- 5 l H_2O

5x SDS Laemmli buffer

- 250 mM Tris–HCl pH 6.8
- 10% SDS
- 30 % Glycerol
- 5 % 2 - Mercaptoethanol
- 0.02 % Bromophenol blue

The samples were electrophoresed in a polyacrylamide gel and then transferred to nitrocellulose membranes at 90 V constant for 3 hours at 4°C using wet blotting system. In order to check the success of protein transfer, the membranes were stained using Ponceau staining, scanned and distained afterwards.

Blotting buffer (10x)

- 558.3 g Glycine
- 121.6 g Tris base
- 5 l H_2O

TNT buffer (1x)

- 10 mM Tris HCl pH 8.0
- 300 mM NaCl
- 0.1% Tween 20
- 1 l H_2O

TBST buffer (10x) pH 7.6

- 24 g Tris HCl
- 5.6 g Tris base
- 88 g NaCl
- 600 ml H_2O
- 0.1% Tween 20

The membranes were blocked in 5% BSA–TNT in case of phosphorylated proteins (pAMPK, pAkt and pS6 RP), otherwise in 5% skimmed milk–TBST for 30 – 45 min. Following, blots were incubated with a 1:500 dilution of anti–pAMPK, 1:1000 dilution of anti–pAkt and 1:10000 dilution of anti–GAPDH first antibodies overnight at 4°C on shaker. The next day, membranes were intensively washed with corresponding buffer and incubated with HRP–secondary antibodies accordingly in milk or BSA for 45 min at room temperature on shaker. pAMPK, pAkt and pS6 RP

were incubated with a 1:6000 dilution of horseradish peroxidase–conjugated anti–rabbit antibody in BSA–TNT. To detect the total level of AMPK and Akt proteins, the blots were stripped for 30 min at RT, and then washed so long until 2–Mercaptoethanol was completely washed out.

Stripping buffer

- 1.042 m 3M Tris HCl pH 6.8
- 10 ml 10% SDS
- 573 µl 2 - Mercaptoethanol
- ad 50 ml H_2O

The blots were incubated with a 1:1000 dilution of anti–AMPK and 1:2000 dilution of anti–Akt in milk–TBST overnight at 4°C. On the following day, the blots were washed with TBST and incubated with a 1:6000 dilution of HRP–conjugated anti–rabbit antibody in milk–TBST for 45 min at RT on shaker. GAPDH part of blots was incubated with a 1:4000 HRP–conjugated anti–mouse antibody. After few washing steps, membranes were developed with ECL (1:1) using ChemiDoc Imaging System. Protein bands were quantified with Fiji as described in the method outlined at http://lukemiller.org/index.php/2010/11/-analyzing-gels-and-western-blots-with-image-j/.

3 RESULTS

3.1 Establishment of primary hepatocyte collagen sandwich system in new environment

The availability of a reproducible and physiologically relevant cell culture system to study the connection between endocytosis and metabolism in the liver is of crucial interest for this study. As demonstrated by Zeigerer A. at al., the primary hepatocyte collagen sandwich system recapitulates key structural and functional properties of the hepatocytes in the liver and therefore, can be used as a model system to study liver cell biology. This know–how was transferred from Dresden to Munich, when setting up the IDC. Initially we came across some difficulties in culturing primary hepatocytes e.g. stressed cells, de–differentiated cells, apoptotic cells due to different environmental conditions in Munich. Nevertheless, some improvements on primary hepatocyte culture were necessary to obtain a reliable cellular system for this study. We have switched from BioWhittaker William's E medium for cell culture (Lonza) that did not contain sodium bicarbonate to the William's E medium (PAN biotech) with 2.24 g/l sodium bicarbonate. The addition of sodium bicarbonate is necessary for the cells growing under CO_2 condition, since it enables high buffer capacity in the cell culture medium and keeps the pH–value in the physiological area. Furthermore, the collagenase concentration in collagenase buffer was reduced from 90 mg per ca. 255 ml collagenase buffer to 60 mg, since the recent purchased collagenase has a higher activity. After plating the cells, the washing step was performed after only 1 – 2 hours of attachment, as distinct to the original protocol. We also changed the time of cell attachment, 6 hours in total instead of 3 hours, which showed better results. After washing step, the cells were left to recover in 200 µl instead of 1000 µl cell culture medium, and were further on grown in 500 µl of cell culture medium. By this way, the oxygen restriction e.g. hypoxia was reduced. The improvements resulted in better cell confluency and less cell debris i.e. apoptosis see Figure 10. After optimizing all these condition, the primary hepatocyte culture seems to be more reliable, so it can be used as a consistent tool for conducting further experiments.

a b

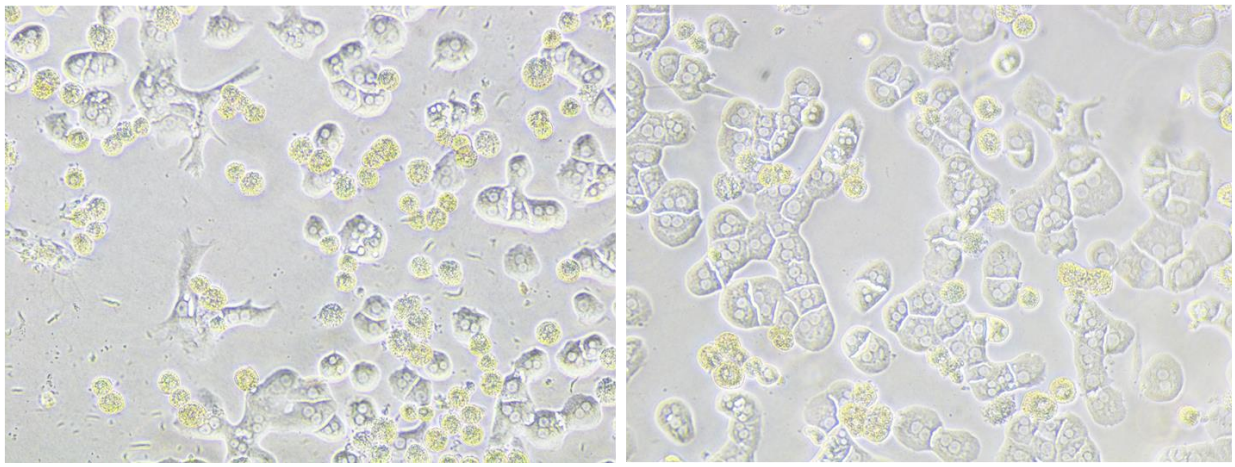


Figure 10 Optimization of primary hepatocyte collagen sandwich culture. a) Primary hepatocytes before optimization of conditions. **b)** Primary hepatocytes after optimization of conditions. Images were acquired using Nikon Eclipse TS 100, IWD 20X/0.4.

3.2 Endosomal system is able to differentiate between different glucose availability

In order to find out how the endosomal system responds to different metabolic cues and if this could be a way to sense the external metabolic conditions, we investigated the effect of different glucose concentrations on endosomal transport process by measuring the endocytic uptake kinetics of hepatocytes. Two established cargos, LDL as a nutritional cargo and EGF as a signaling cargo, were used as readout for endocytic transport. After they have been internalized from the hepatocyte plasma membrane into clathrin–coated vesicles their further transport, from early endosomes to degradation in lysosomes, can be nicely followed by using fluorescent microscopy.

3.2.1 Internalization of signaling EGF cargo as a readout for endocytic transport

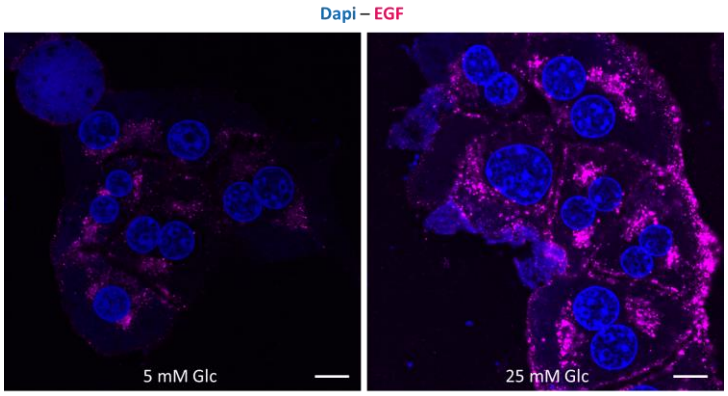
The EGF or LDL receptor uptake can be used to measure the rate of endocytosis, since the influx of internalized receptors equals the efflux of degraded one. When the system is fully occupied the plateau is reached and degradation decelerates. In order to estimate the rate of speed of EGF or LDL receptors absorption into the cell via endocytosis, we performed the receptor internalization

assay. The assay is based on the following principle. The receptors on the surface of the cell membrane are bound by a labeled cargo, which are then enclosed into plasma membrane–formed inward vesicles. After the vesicles separate from the membrane they fuse further with early endosomes. Due to the acidic pH, the cargo dissociates from the receptor and is degraded via late endosomes and lysosomes, while the receptor is recycled. The fluorescence signal is detected and accordingly quantified. The experiment was conducted when the hepatocytes reached polarized state in the collagen sandwich, after day 3. Hepatocytes were starved in 1 mM glucose medium without serum for 2 hours in order to bring them on the basal metabolic rate, and additional 3 hours upon addition of different glucose containing media (high: 25 mM, intermediate: 11 mM and low: 5 mM). In a final hour the continuous EGF uptake assay was conducted. The optimal concentration of EGF was estimated to be 400 ng/ml. For this purpose, fluorescently labeled cargos were added at different time points i.e. 60, 30, 15 and 0 min, washed with cold PBS and fixed. Accordingly, images were acquired using Olympus confocal scanning microscope. The fluorescent intensities of approx. 20 cells per condition were measured by quantitative image analysis using Fiji in order to determine the level of cargo internalization upon different time points see Figure 11.

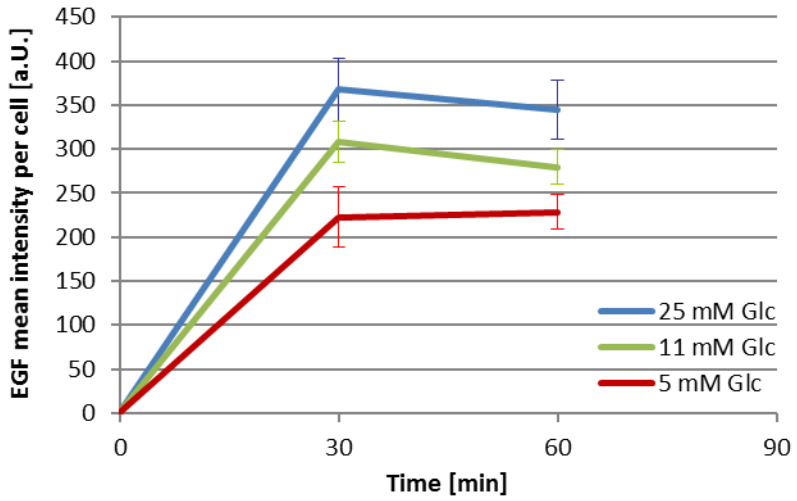
After 30 min time point, the fluorescent intensity begins to decrease i.e. reaches a plateau even though the uptake is still ongoing. By this point the endosomal system is fully occupied by cargo and the degradation pathway is involved. Accordingly, the saturation intensity of a laser has been reached. In order to compare different experiments, the results of all trials were collected and normalized to curve maxima see Figure 11 section c and Figure 12 section c. In the case of not saturated curve the mean intensity value of each time point was normalized to 60 min time point and when saturated each value was normalized to the average value of high glucose time points.

Faster internalization of EGF was observed in high glucose stimulated hepatocytes, meaning enhanced endocytosis under high glucose conditions.

a


Dapi – EGF
5 mM Glc
25 mM Glc

b


EGF mean intensity per cell [a.U.]
450
400
350
300
250
200
150
100
50
0
0
30
60
90
Time [min]
25 mM Glc
11 mM Glc
5 mM Glc

c

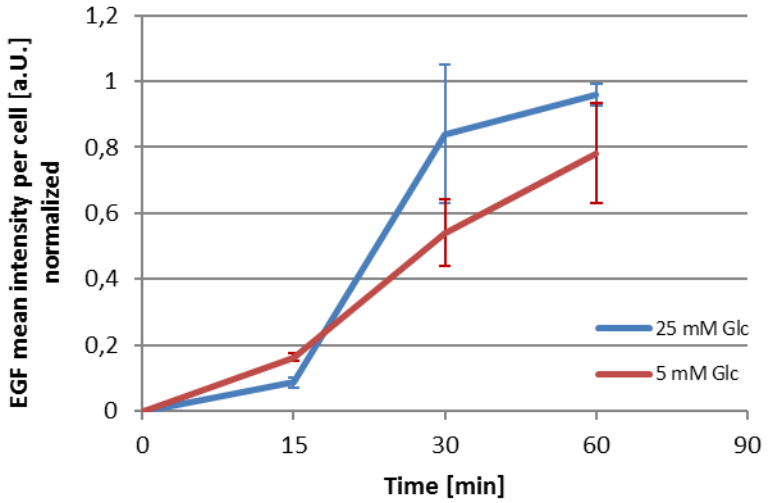


Figure 11 EGF uptake upon high (25mM) and low (5mM) glucose stimulated primary hepatocytes. **a)** Representative confocal images of primary hepatocytes incubated with EGF for 30 min. Scale bar=10 µm. **b)** Quantification of this experiment with Fiji. (mean ± s.e.m). **c)** Summary of three independent internalization experiments normalized to curve maxima. (mean ± s.e.m).

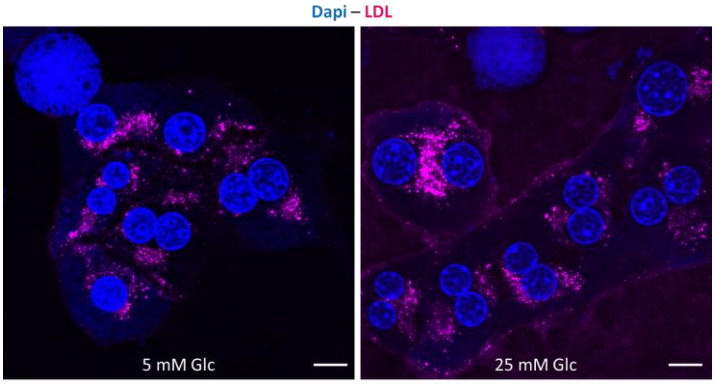
3.2.2 Internalization of nutritional LDL cargo as a readout for endocytic transport

The experimental setup is explained in 3.2.1 section. Internalization of LDL into glucose starved hepatocytes was followed over a one–hour time course.

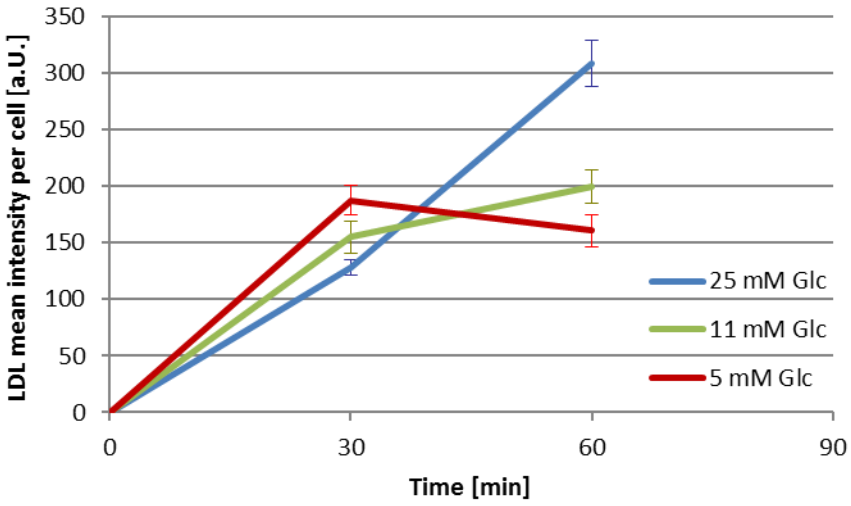
High glucose exposure resulted in enhanced internalization of LDL see Figure 12.

These results suggest that the endosomal system can sense between different extracellular glucose concentrations by actively adapting the internalization of nutritional and signaling cargos e.g. LDL and EGF.

a


Dapi – LDL
5 mM Glc
25 mM Glc

b


LDL mean intensity per cell [a.U.]
350
300
250
200
150
100
50
0
0
30
60
90
Time [min]
25 mM Glc
11 mM Glc
5 mM Glc

c

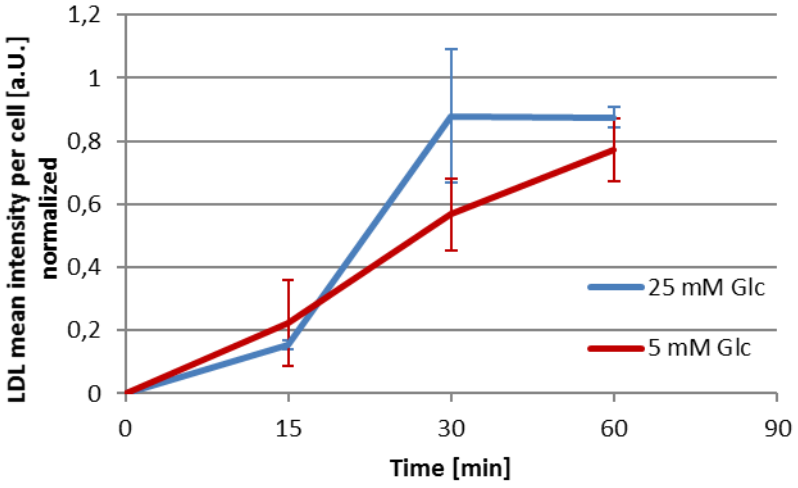
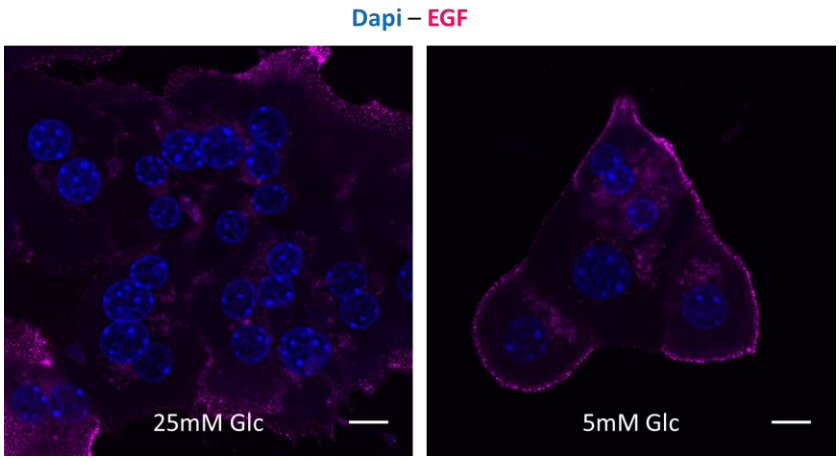


Figure 12 LDL uptake upon high (25mM) and low (5mM) glucose stimulated primary hepatocytes. a) Representative confocal images of primary hepatocytes incubated with LDL for 30 min. Scale bar=10 µm. **b)** Quantification of this experiment with Fiji. (mean ± s.e.m). **c)** Summary of three independent internalization experiments normalized to curve maxima. (mean ± s.e.m).

3.3 Primary hepatocytes showed increased EGF and LDL ligand–receptor binding upon low glucose starvation

Glucose starvation leads to a decrease in receptor–mediated endocytosis. The decrease in endocytosis upon low glucose stimulation could be due to a lower abundance of EGF and LDL receptors on the cell surface. Therefore, we measured the amount of EGF and LDL receptor on the cell surface of primary hepatocytes by incubating the cells with LDL and EGF on ice for 2 hours in same high or low glucose medium in which they were stimulated beforehand. By “freezing” the process of any further endocytosis after glucose stimulation, we were able to monitor the cargo binding to the receptor. The fluorescent image analysis revealed that the hepatocytes exposed to high glucose condition had less receptors on the membrane surface, ruling out that the decrease of endocytosis is due to a reduction of receptor amounts on the plasma membrane under low glucose condition see Figure 13 and Figure 14.

a



b

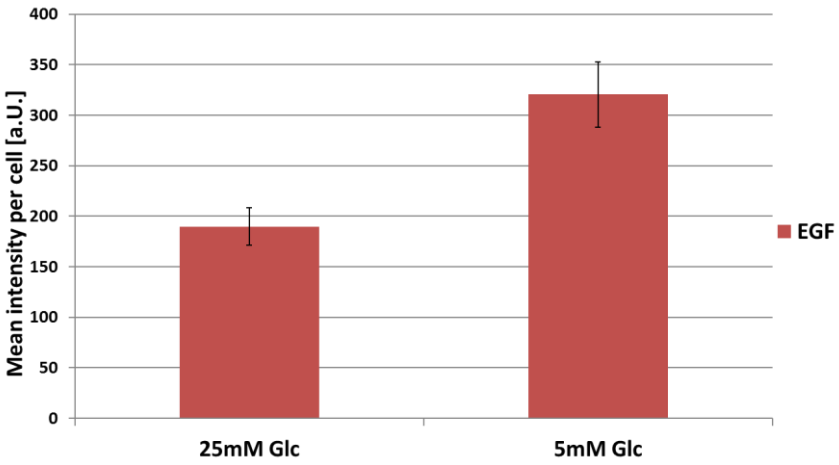
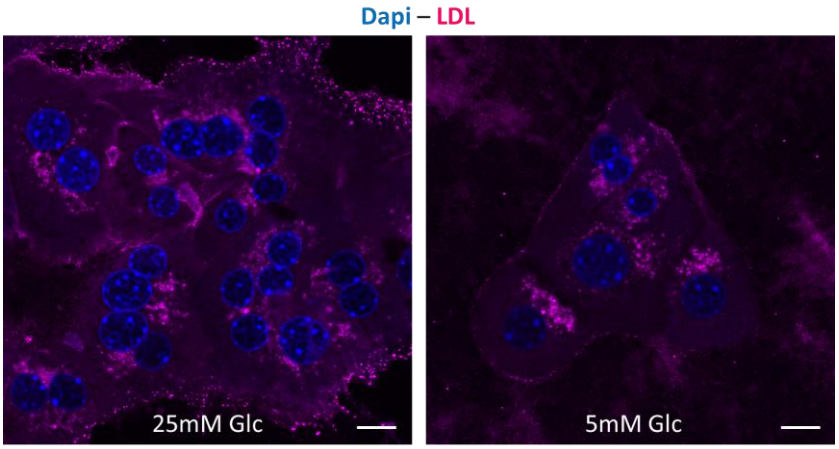


Figure 13 EGF–receptor binding on the surface of primary hepatocytes. a) Representative confocal images of middle confocal section. Scale bar, 10 µm. **b)** Quantification of EGF per high (25 mM) and low (5 mM) glucose condition (mean ± s.e.m.).

a



b

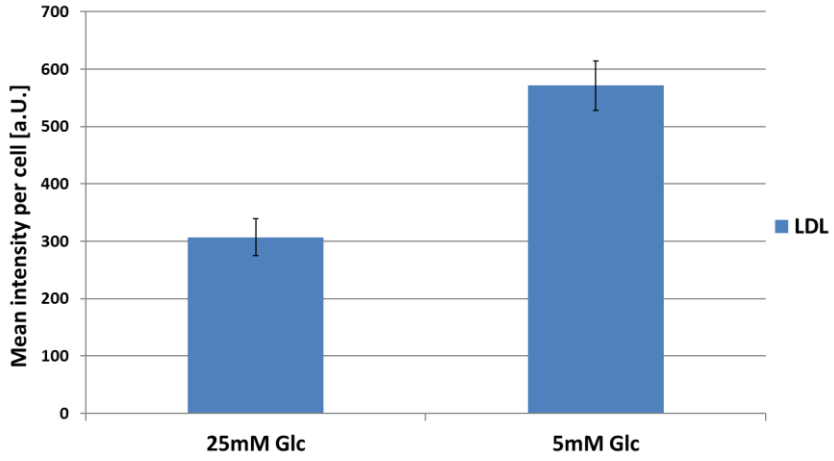


Figure 14 LDL–receptor binding on the surface of primary hepatocytes. a) Representative confocal images of middle confocal section. Scale bar, 10 µm. **b)** Quantification of LDL per high (25 mM) and low (5 mM) glucose condition (mean ± s.e.m.).

3.4 No alteration of intracellular compartments after glucose starvation

Since glucose starvation led to a reduction in endocytic transport, we next examined whether the integrity of intracellular endosomal compartments was also affected. For this, specific well–established membrane markers were stained and the mean fluorescent intensity per cell was measured.

3.4.1 Glucose effect on endosomal compartments

The effect on the distribution of endosomal compartments of glucose–starved hepatocytes was monitored, since the alterations in Rab effector recruitment to endosomal membranes should lead to changes in endosomal transport. The endosomal compartments were stained using established markers, such as EEA1 for early endosomes and LAMP1 for late endosomes and lysosomes. Using image analysis (Fiji see 2.4.4 for more information), we measured total intensity of specific marker after treating hepatocytes with high and low glucose concentration.

Thus far two experiments have been conducted to investigate the effect of different glucose concentrations on EEA1 intensities see Figure 15, which gave inconsistent results see Figure 16. Therefore, we conclude that the decrease in endosomal transport under low glucose stimulation are most likely independent of EEA1 function.

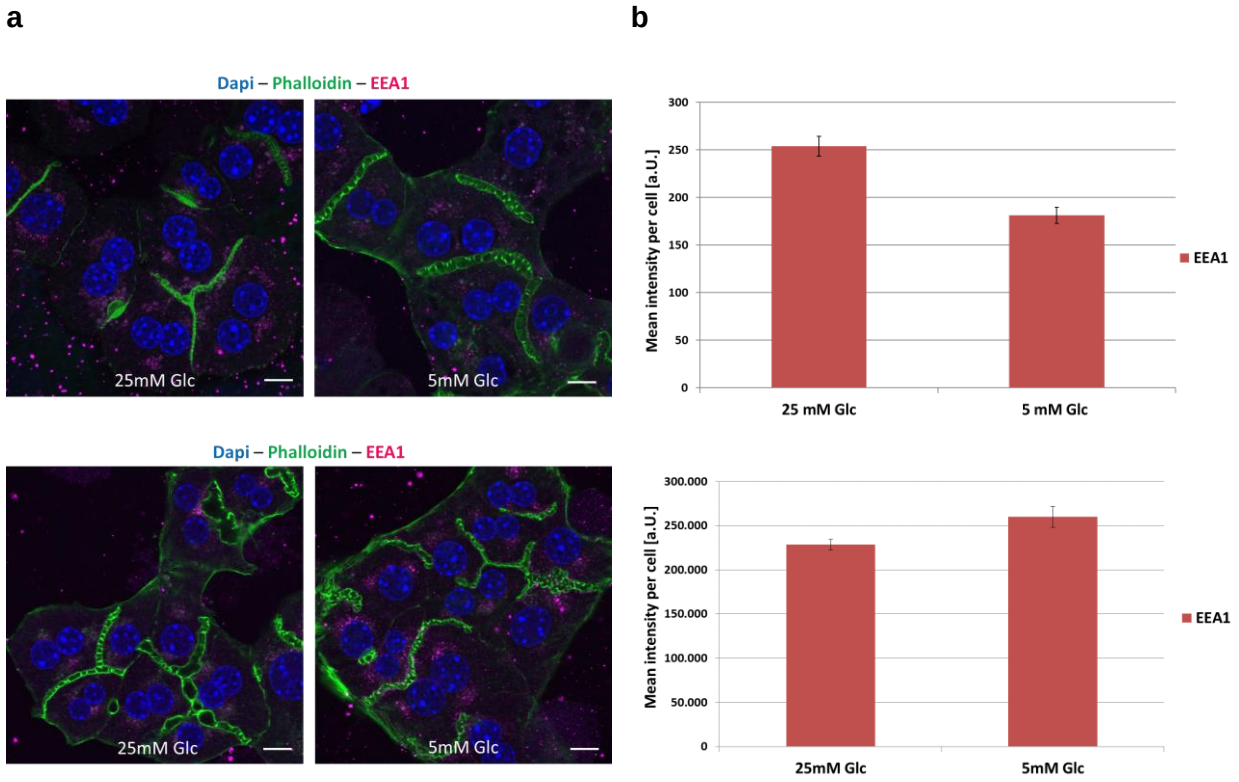


Figure 15 Distribution of early endosomes specifically stained with anti–EEA1 antibody in hepatocytes starved with low and high glucose concentration. a) Representative confocal microscopy images of primary hepatocytes stained with anti-EEA1, phalloidin and DAPI. Scale bar, 10 µm. **b)** Quantification of mean fluorescent intensity of EEA1 vesicles in low and high glucose stimulated hepatocytes (mean ± s.e.m.). Two separate experiments are shown.

Based on the so far performed experiments there is no significant change in EEA1 recruitment to the early endosomes upon starvation with low or high glucose concentrations. However, due to experimental inconsistencies these experiments need to be repeated to obtain convincing conclusions.

The high to low glucose fluorescent ratio of primary hepatocytes stained specifically for LAMP1, showed it enhanced traffic to the late endosomes in low glucose condition, suggesting the activation of the degradation pathway see Figure 16 and Figure 17.

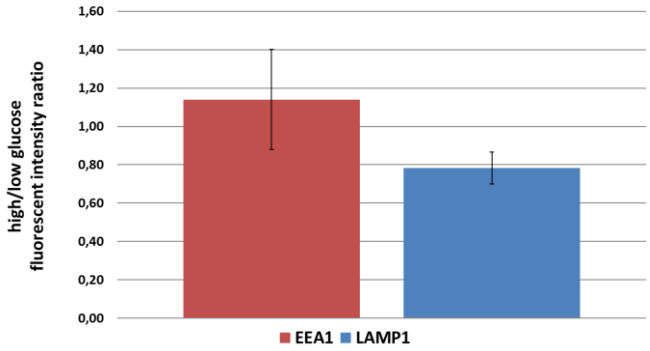


Figure 16 Quantification of ratio between high and low glucose stimulated hepatocytes specifically stained with anti–EEA1 and anti–LAMP1 antibodies (mean ± s.e.m.).

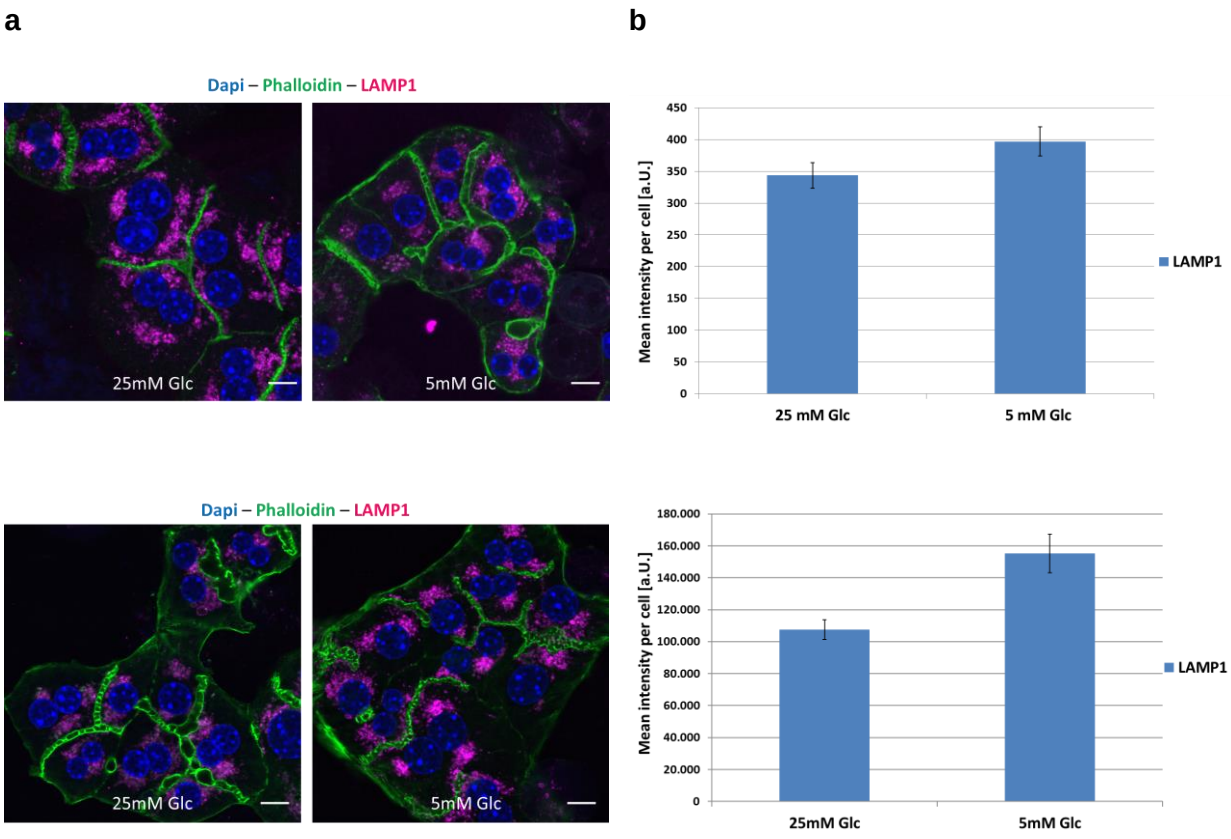
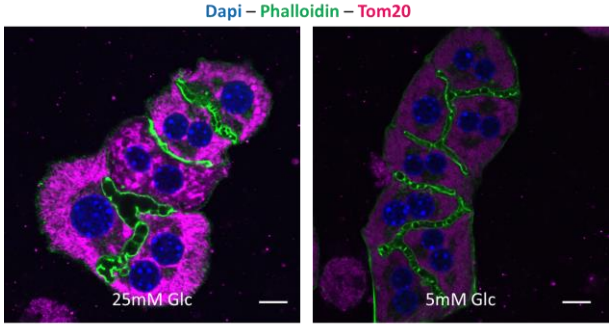


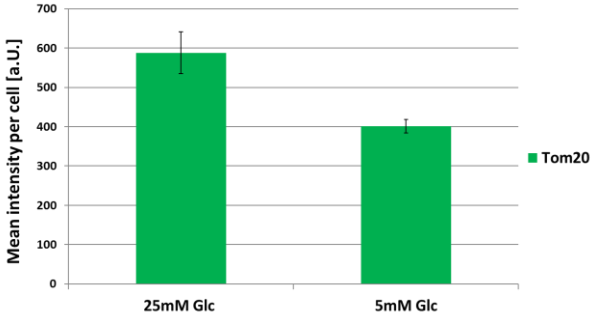
Figure 17 Distribution of late endosomes and lysosomes specifically stained with anti–LAMP1 antibody in hepatocytes starved with low and high glucose concentration. a) Representative confocal microscopy images of primary hepatocytes stained with anti-LAMP1, phalloidin and DAPI. Scale bar, 10 µm. **b)** Quantification of mean fluorescent intensity of LAMP1 vesicles in low and high glucose stimulated hepatocytes (mean ± s.e.m.). Two separate experiments are shown.

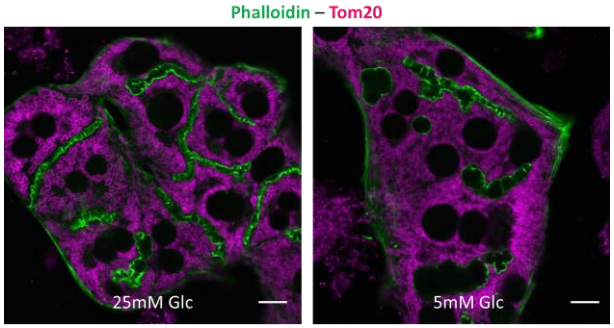
3.4.2 No clear sign of glucose influence on other intracellular compartments

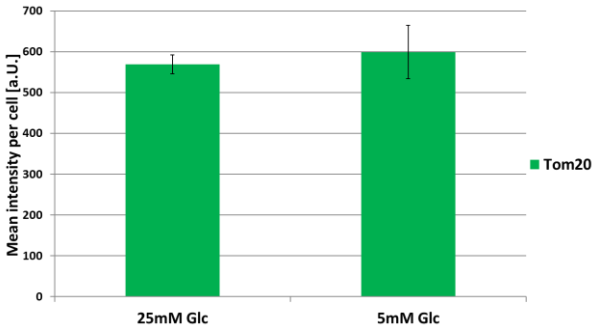
It has been suggested that the depletion of endosomal compartments upon Rab5 knockdown has an effect on other cellular organelles, e.g. Mitochondria. Proteomics of Rab5KD livers reveal alterations in mitochondrial metabolism e.g. down–regulated proteins in citrate cycle, fatty acid metabolism, butanoate metabolism, different amino acids metabolism etc. (Zeigerer et al., 2015). Changes in appearance of organelles, such as enlarged and engulfed mitochondria as well as swelled endoplasmic reticulum (ER) support the indication of metabolic perturbations (Zeigerer et al., 2012). Therefore, we want to observe how other intracellular membranes, e.g. Golgi, ER and Mitochondria, react upon glucose stimulation and investigate the potential interconnection between these different compartments. Therefore, some preliminary images for organelles were acquired and quantified see Figure 18. In order to stain for different organelles well–established markers were used. Mitochondrial import receptor subunit Tom20 was used to visualize the mitochondrial outer membrane. Two separate experiments were done with different outcomes. The results were normalized to high glucose condition in order to get high to low glucose fluorescent ratio. No significant change in Tom20 intensity was observed. Furthermore, glucose-6-phosphatase (G6Pase), an abundant enzyme found on the lumen side of the ER membrane was monitored and showed increased intensity in hepatocytes stimulated by low glucose. To visualize clathrin–coated vesicles (CCVs) that mediate the vesicular transport of cargo by connecting the trans–Golgi network, endosomes, lysosomes and the cell membrane (Uniprot), we stained for a clathrin heavy chain (CLTC). There was no difference in intensity levels of CLTC under low and high glucose conditions. The Rab5 antibody was used additionally to the above mentioned EEA1 antibody as an early endosome marker. The Rab11 antibody was used as a recycling endosome marker. Neither did Rab5 or Rab11 show clear changes in low or high glucose stimulated hepatocytes. In each case, the staining needs to be repeated, since this was an initial experiment.

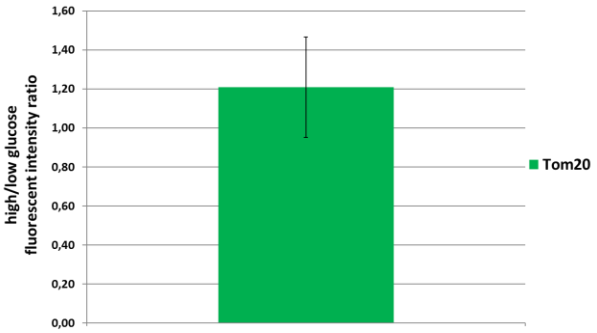
a


Dapi – Phalloidin – Tom20
25mM Glc
5mM Glc

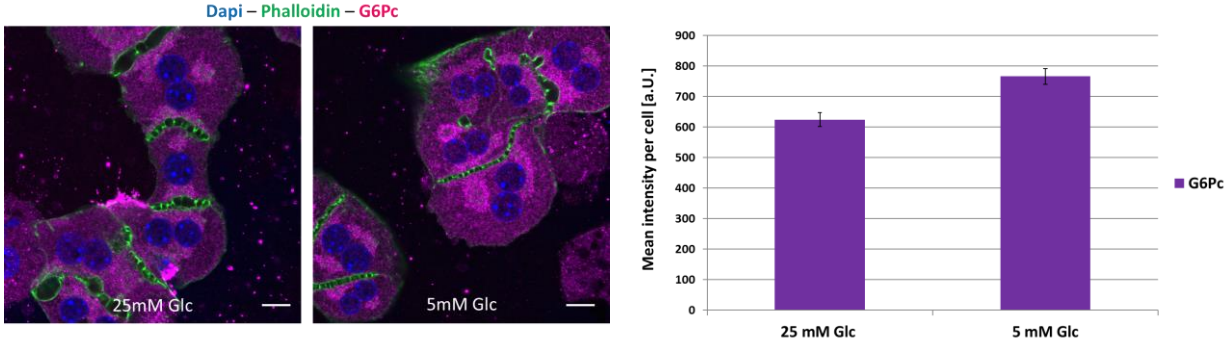

Mean intensity per cell [a.U.]
700
600
500
400
300
200
100
0
25mM Glc
5mM Glc
Tom20


Phalloidin – Tom20
25mM Glc
5mM Glc

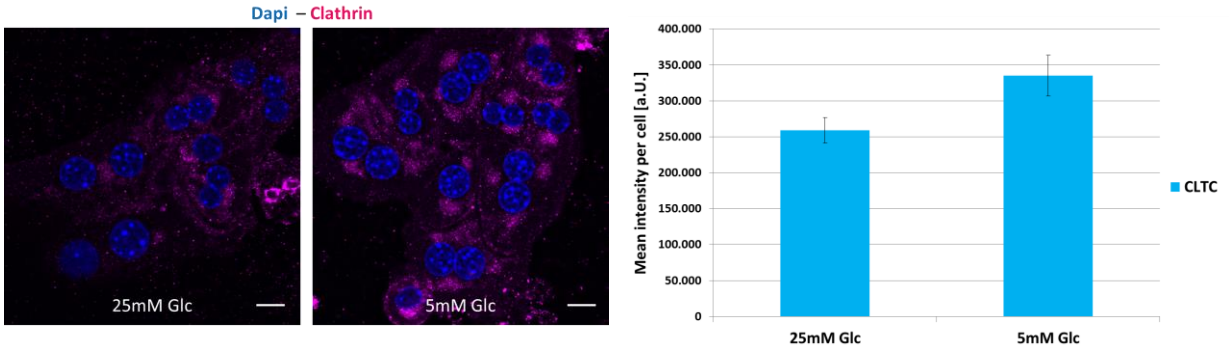

Mean intensity per cell [a.U.]
700
600
500
400
300
200
100
0
25mM Glc
5mM Glc
Tom20


high/low glucose fluorescent intensity ratio
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
Tom20

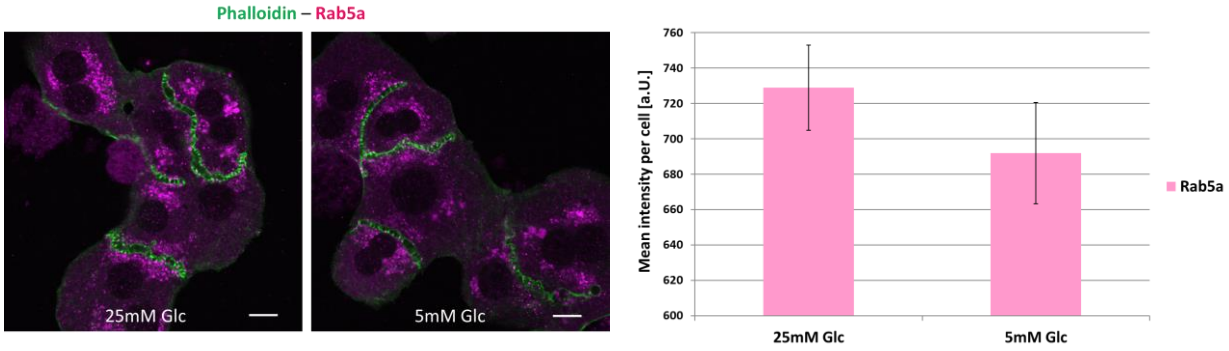
b


Dapi – Phalloidin – G6Pc
25mM Glc
5mM Glc
Mean intensity per cell [a.U.]
900
800
700
600
500
400
300
200
100
0
25 mM Glc
5 mM Glc
G6Pc

c


Dapi – Clathrin
25mM Glc
5mM Glc
Mean intensity per cell [a.U.]
400.000
350.000
300.000
250.000
200.000
150.000
100.000
50.000
0
25mM Glc
5mM Glc
CLTC

d


Phalloidin – Rab5a
25mM Glc
5mM Glc
Mean intensity per cell [a.U.]
760
740
720
700
680
660
640
620
600
25mM Glc
5mM Glc
Rab5a

e

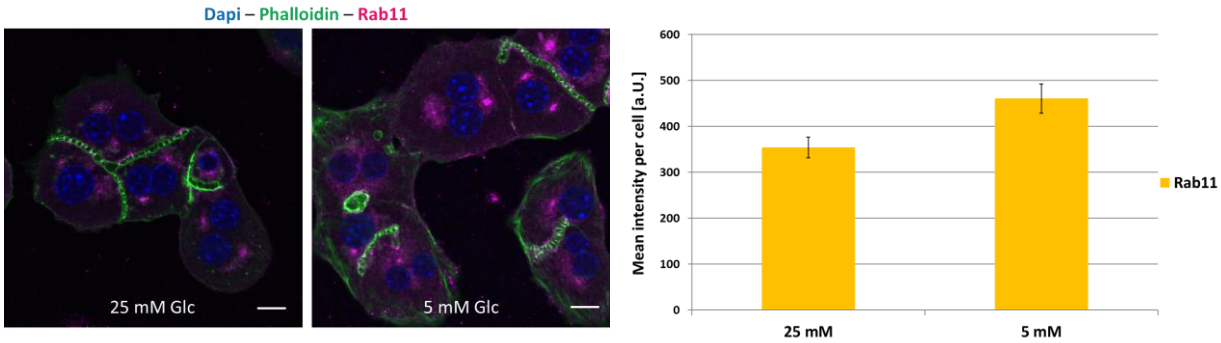


Figure 18 Staining with different markers of primary hepatocytes upon low and high glucose stimulation. a) Representative confocal image of primary hepatocytes stained with anti-Tom20, DAPI and phalloidin; quantification of mean intensity and high to low glucose fluorescent ratio. Two separate experiments are shown. Scale bar, 10 µm (mean ± s.e.m.). **b**) Representative confocal image of primary hepatocytes stained with anti-G6Pc, DAPI and phalloidin and quantification thereof. **c)** Representative confocal image of primary hepatocytes stained with anti-CLTC plus DAPI and quantification thereof. **d)** Representative confocal image of primary hepatocytes stained with anti-Rab5a, and phalloidin and quantification thereof. **e)** Representative confocal image of primary hepatocytes stained with anti-Rab11, DAPI and phalloidin and quantification thereof.

3.5 Kinases as a potential mediator between glucose metabolism and endocytosis

The molecular mechanism regulating glucose effect on endocytosis is not known. Therefore, we address the question which signaling pathways are altered after exposing primary hepatocytes to different glucose concentrations. In order to identify the potential activation of kinases important for energy metabolism or possible alterations in their signaling pathways, we chose 3 candidates i.e. AMPK, Akt, mTOR (pS6 Ribosomal Protein) to take a closer look at, by performing western blot analysis and quantifying phospho/total ratio.

For this, multiple repetitions were conducted, and higher amount of protein was loaded on the gel as well as different developing time points were tested in order to get stronger band intensity after blot development. Quantification was done in Fiji and results were normalized to values of samples stimulated with low glucose concentration.

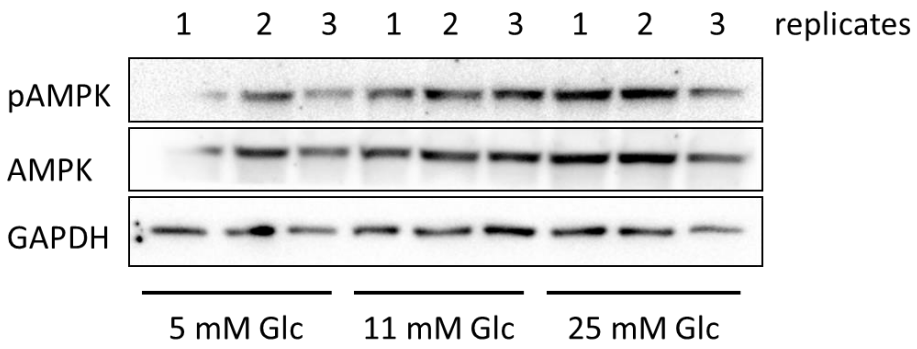
The last trial was chosen as a representative experiment so far, since it showed a tendency towards changed AMPK and Akt phospho/total ratio see Figure 19 A and B and Figure 20 A and B. AMPK protein levels were slightly increased after 2 hours of high glucose stimulation. This

gives us a hint that AMPK might be involved in process of regulation of endocytosis upon glucose stimulation. Akt protein levels were significantly elevated under low glucose condition. pS6 RP levels were not influenced by glucose see Figure 21.

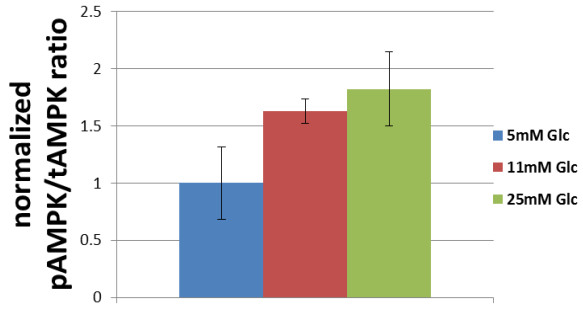
Neither AMPK, Akt nor pS6RP showed significant change in phosphorylation level when the separate experiments were summarized and normalized see Figure 19 C, Figure 20 C and Figure 21 C. Nevertheless, because of fluctuations this experiment needs to be repeated.

Taken together, our data shows that endosomal transport is sensitive to alterations in glucose exposures and slows down internalization under glucose starvation conditions. However, the molecular mechanism responsible for these alterations still needs to be elucidated.

A



B



C

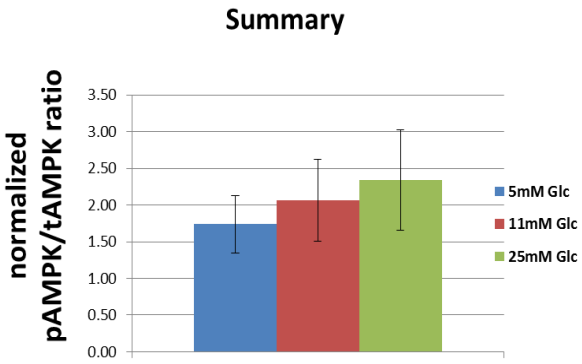
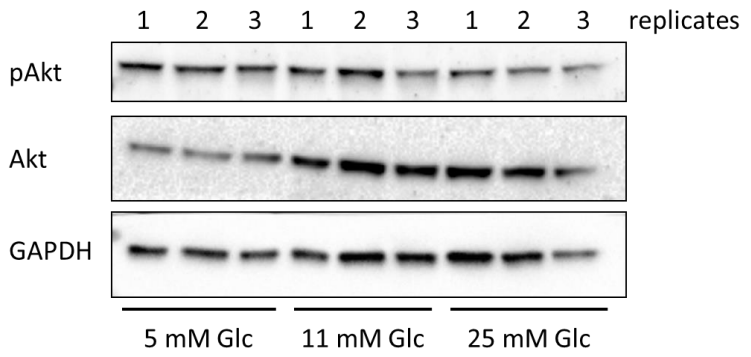
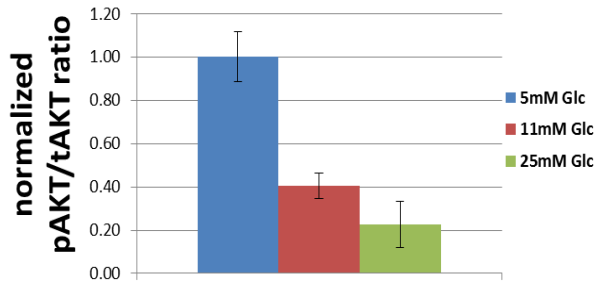


Figure 19 pAMPK upon stimulation of primary hepatocytes with high, intermediate and low glucose concentration. A) Representative western blot of pAMPK and AMPK **B)** Quantification of the representative western blot experiment (mean ± s.e.m.). **C)** Quantification of the summarized western blot experiments (mean ± s.e.m.).

A



B



C

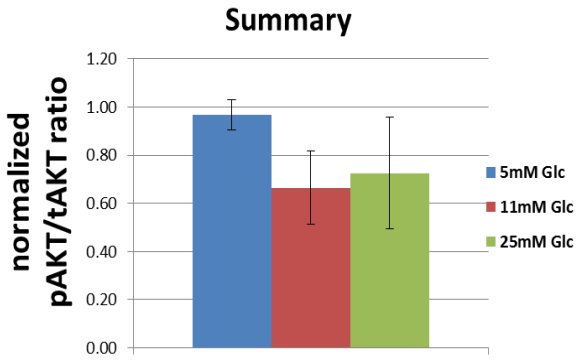
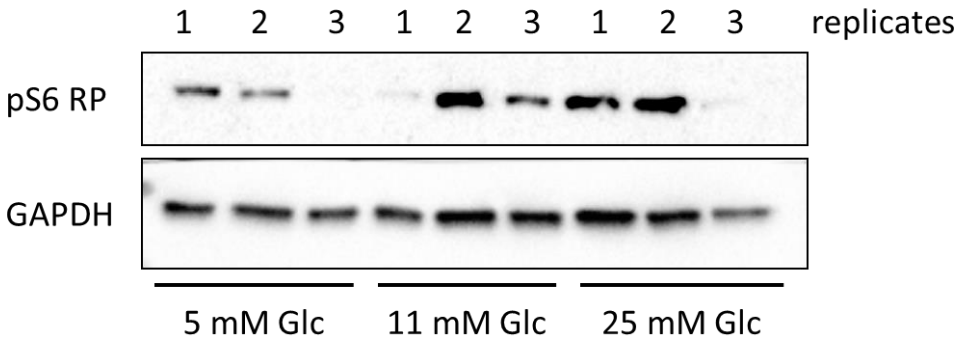
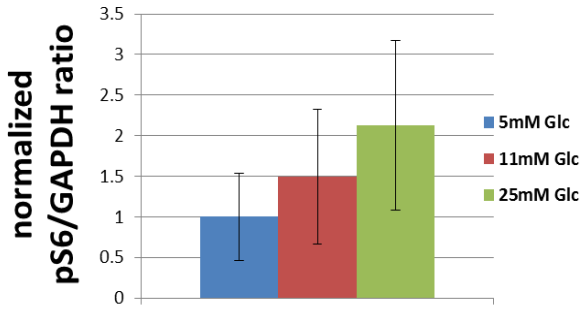


Figure 20 pAkt upon stimulation of primary hepatocytes with high, intermediate and low glucose concentration. A) Representative western blot of pAkt and Akt **B)** Quantification of the representative western blot experiment (mean ± s.e.m.). **C)** Quantification of the summarized western blot experiments (mean ± s.e.m.).

A



B



C

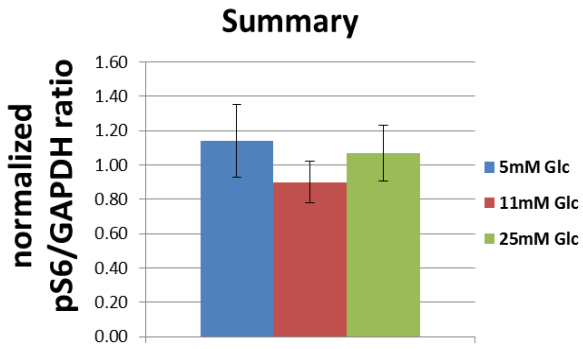


Figure 21 pS6 RP upon stimulation of primary hepatocytes with high, intermediate and low glucose concentration. A) Representative western blot of pS6. **B)** Quantification of the representative western blot experiment (mean ± s.e.m.). **C)** Quantification of the summarized western blot experiments (mean ± s.e.m.).

4 DISCUSSION

In order to further elucidate and stress the importance of the reciprocal relationship of hepatic metabolism and endocytosis for the intact function of hepatocytes we exposed primary hepatocytes to the different concentrations of glucose. There are more evidences suggesting that the uptake of nutrients through endocytosis and metabolism are finely modulated processes that depend from each other see 1.5. Recent data in yeast showed that glucose starvation influences endosomal traffic (Aoh et al., 2011) and that endocytosis is required for survival in glucose starvation (Lang et al., 2014). However, until now this concept has not been shown for higher and more complex eukaryotic cells. Therefore, the aim of my work was to answer the question if the highly metabolic cells such as hepatocytes respond to different glucose stimulation by adapting their endosomal transport system. Hepatocytes were treated by different glucose concentrations under starving conditions. Here, it was found that the endosomal system differentiates between high and low glucose concentrations by adapting the kinetics of cargo internalization. Thus, this work underlines once more the interconnection between metabolism and endocytosis.

The internalization of LDL and EGF showed increased kinetics when primary hepatocytes were stimulated with high glucose concentration. EGF and LDL cargos were used as a general readout for endocytosis, in order to track two different pathways after internalization of cargo e.g. LDL for degradation pathway and EGF for recycling pathway. For more physiological and pathophysiological approach, the cells should be exposed for a longer time to glucose. In that case, a potential toxic effect of glucose on gene regulation and protein expression may be observed. It is shown that endocytosis is energy dependent process (Schmid & Carter, 1990). Since we monitored enhanced endocytosis during oversupply of glucose, it gave us a hint that more glucose may lead to higher ATP consumtion. The stimulatory effect of glucose on inducing uptake is cargo independent as both EGF and LDL are changed, suggesting a general influence on endocytosis.

To answer the question, what do endosomes in fact sense, glucose per se or glucose flux i.e. change in energy, one can do 2–deoxy–d–glucose (2–DG) uptake assay. 2–DG is a glucose molecule which is characterized by its inability to undergo further glycolysis, since it is metabolized to 2–D G–6–phosphate (2-DG6P). It accumulates in the cells and is directly proportional to glucose uptake by cells (Yamamoto et al., 2011). Further on, it will be interesting to observe the general response on influence of glucose availability in cell culture. The question arises, if we block endocytosis by knockdown does it change the amount of energy that is being produced?

Does the information for endosomal behavior come from exogenous or endogenous factors? To find the further answers, the glycolysis stress assay can be used to detect changes in O_2 and H+ concentrations. By Seahorse XF Analyzer, the cellular metabolism i.e. the two major energy producing pathways, glycolysis and oxidative phosphorylation, can be measured. The rate of oxygen consumption is the direct readout for mitochondrial respiration, whereas the rate of extracellular acidification is proportional to glycolysis (XF Glycolysis Stress Test Kit User Manual, Seahorse Bioscience).

While conducting an experiment, we came across some difficulties. Some points in time e.g. 60 min time point often showed variability or did not reached saturated state. This can be due to the stressed cells that are not metabolizing very well or the cargo e.g. LDL, which is a big protein and does not penetrate well through the second collagen layer. Anyway, by pooling the results of multiple trials and normalizing it to curve maxima we came to the consistent result in both cases of LDL and EGF uptake, which is enhanced endocytosis upon stimulation by high glucose.

The reduction of endocytosis under glucose starvation could be induced by a lower expression of cargo receptors on the cell surface. However, we found the opposite. Low glucose stimulation resulted in a higher abundance of surface receptors, suggesting that 1) internalization is strongly reduced leading to a higher number of receptors on the plasma membrane, or 2) recycling of the EGF and LDL receptors must be strongly increased. However, the time course data of internalization reveal a slower kinetic also in the later stages of the curve, which is dominated by degradation and recycling. Therefore, we favor the first explanation. But further experiments directly measuring degradation and recycling are necessary to address this question.

After being internalized, cargo and receptor mostly go separate ways. In the pathway promoting degradation, cargo proteins are subsequently degraded in late endosomes and lysosomes. However, receptors preferentially take the recycling pathway. To observe this pathway separately, one can do recycling assay in which only LDL or EGF receptor is labeled and chased. The result can be compared with the one from uptake and ice assay, in order to be sure from which pathway the effect of changed endosomal kinetics comes from. Recycling assay can be performed by occupying the receptors with labeled cargo first and then washing them out from surface. Afterwards, the cells are incubated with unlabeled cargo under different time points. The loss in fluorescence intensity represents efflux of the different reporters to the cell surface. Also the degradation assay can be performed by following the labeled cargo through the time course (e.g.

90 min). The labeled EGF or LDL is detectable until degradation and is observed with vanishing fluorescence.

The cells can be pretreated with different glucose concentration and incubated with a saturating amount of EGF receptor antibody against extracellular domains for various periods of time in order to measure the internalization rate constant. Nonetheless, in ice binding and uptake assay experiments, the cells were incubated with well–defined saturating concentration of ligand, so that the amount of fluorescent cargos correspond directly the number of receptors they bind. The receptor cargo bond is highly specific, since all receptor epitopes are occupied.

With all these experiments we can see at which step of endocytosis (early, late, degradation) or recycling there is more cargo intensity and at which step comes to change. If we know, which step of endocytosis, under given different glucose concentrations is enhanced then we can address the affected pathway for further research.

The main advantage of a fluorescence–based endocytosis uptake assay is that the cargo uptake can be specifically measured per single cell in contrast to radioactive methods of analysis. Since our 3D cell culture system consist of both pericentral and periportal hepatocytes, this allows individual approach and comparison of each cell. Primary hepatocytes do not divide and are more physiological cells than the cell line. Therefore, they are well suitable for manipulation and observation of specific effects e.g. knockdown. The cell based uptake assay tells us more specific if the process of endocytosis is affected or not. More advanced steady state uptake can be reached by adding more time points to the internalization time course. The image acquisition can be automated using the automatic microscope and kinetics derived from the resulting curve. Apart from this fact, non–radioactive assays are not hazardous to human health, don't produce radioactive waste, don't require special laboratory conditions and are less expensive than radioreceptor assays, which on the other hand are fast, easy to use and reproducible (De Jong, Uges, Franke, & Bischoff, 2005).

Furthermore, one should perform western blot analysis for LDL and EGF receptors in order to see if the protein expression levels change upon different glucose stimulation. Different amount of bounded receptor cargos can be consequence of how fast endocytosis occurs or based on regulation of receptor expression. Anyhow, we premised that the total level of LDLR and EGFR does not change after 2 hours exposure to glucose, since the turnover half–life of both receptors lasts longer than 2 hours. In the case of LDLR half–life, it takes approx. 12 hours and for EGFR

between 8 and 24 hours to degrade, depending on the cell line (Casciola, van der Westhuyzen, Gevers, & Coetzee, 1988; Sorkin & Duex, 2010).

It has been reported that during glucose starvation (0% glucose) in the yeast, plasma membrane proteins are delivered to the vacuole (lysosome in mouse) through reduced recycling at the endosomes. The recycling seems to be strongly inhibited by glucose starvation while endocytosis is less affected (Lang et al., 2014). Still, this needs to be shown in more complex organisms.

Since the uptake kinetics of endocytosis changed upon glucose stimulation, we monitored if the intensity of endosomal compartments correspondingly adapts. The fluorescence intensity of EEA1– and LAMP1–positive structures was observed, in order to determine the gain or loss of early and late endosomes. The enhanced recruitment of LAMP1 to the late endosomes in low glucose condition points towards activation of degradation pathway. Energy depletion e.g. lack of glucose induces autophagy, which is characterized by lysosome and autophagosome membrane fusion, where no longer needed cargo or organelle is degraded (reviewed in Singh & Cuervo, 2011). In this process, the vesicle clustering is enhanced in order to conserve the energy and to adapt the cargo homeostasis to the new condition. Therefore, the enhanced recruitment of LAMP1 under glucose starved conditions could reflect an activation of the autophagy pathway upon starvation. In the case of EEA1 Rab5–effector, we could not see differential recruitment to endosomes upon different glucose stimulation within a two–hour time frame. Other interesting phenomena to look at in the future would be the number of endosomes after exposure to glucose, since it does not necessary correlate with the endosomal trafficking kinetics. Different parameters, such as number of organelles, intensity, cargo uptake and content etc. can be measured by Motion Tracking software (Collinet et al., 2010; Zeigerer et al., 2012).

Certainly, the internalization of plasma membrane proteins is affected by glucose starvation. The proteins endocytosed from the plasma membrane may be forwarded to intracellular compartments instead of being recycled back to the membrane (Lang et al., 2014). As this can lead to further changes, we monitored markers for different organelles such as Rab5 for early endosomes, Rab11 for recycling endosomes, Clathrin heavy chain for clathrin–coated vesicles, Tom20 for Mitochondria after exposure to different glucose concentrations but no significant change in intensity was noticed.

As the loss of endosomes in the mouse liver upon Rab5 silencing caused glucose–6–phosphatase deficiency and “swollen” ER (Zeigerer et al., 2012), we observed the levels of ER

marker G6Pase. G6Pase is the rate limiting enzyme of gluconeogenesis and glycogenolysis and therefore plays a key role in the homeostatic regulation of blood glucose levels. We observed the higher level of G6Pase protein in hepatocytes stimulated with low glucose concentration, which points towards altered glucose metabolism after stimulation by different glucose concentrations. Even though the influence of glucose on different organelles showed only a minor change in intensity of markers, the tendency is still immanent, so the experiment needs to be repeated.

In order to address the questions, which are the mechanisms that are ruling the changes in endocytosis upon glucose stimulation and which conserved metabolic kinases are activated or changed through these conditions, we targeted AMPK, Akt and mTOR kinases as a potential regulating pathways. We observed an increase in Akt and AMPK protein levels upon low or high glucose stimulation correspondingly. This can be of potential meaning since the AMPK is a crucial cellular energy sensor which controls metabolic energy balance at the whole–body level (Hardie et al., 2012). AMPK is activated when intracellular ATP levels lower and acts by inhibiting anabolic pathways meanwhile promoting catabolic pathways (Mihaylova & Shaw, 2011). Stresses as low nutrients or prolonged exercise can activate AMPK, which then regulates growth and reprogramming metabolism as well as cellular processes such as autophagy and cell polarity (Mihaylova & Shaw, 2011). It was expected to see the activation of AMPK rather in low glucose condition, but AMPK levels were slightly increased in high glucose condition. Still, this suggests that glucose stimulation may alter kinase modification.

On the other hand, the serine/threonine kinase Akt is a main player in cell signaling downstream of growth factors, cytokines, and other cellular stimuli and perturbed activation can lead to diverse diseases e.g. type 2 diabetes and cancer (Manning & Cantley, 2007). In cancer cells, increased Akt pathway signaling correlates with increased rates of glucose metabolism (Simons Andrean L, Orcutt Kevin P, Madsen Joshua M, Scarbrough Peter M, 2012). The oxidative stress that arises from the inhibition of Akt signaling inhibits glycolysis and increases hydroperoxide production, which can potentially have cancer–killing properties (Simons Andrean L, Orcutt Kevin P, Madsen Joshua M, Scarbrough Peter M, 2012). Anyhow, our findings are only tendencies that have to be confirmed by phosphoproteomics analysis.

In this study, we improved the setup of primary hepatocytes sandwich culture, since the reliable cell culture is important for success of experiments. Furthermore, it has been suggested that endosomal transport reacts upon glucose stimulation by slowing down the system and the internalization of receptors under low glucose condition. We monitored the enhanced recruitment

of late endosomal marker LAMP1 under low glucose conditions that may refer to activated autophagy pathway. AMPK and Akt kinases showed tendency to be activated under high or low glucose condition accordingly, and by that possible ability to sense different glucose concentrations. As previously discussed, the endosomal system is important for sensing the environmental cues and its role in regulating the cell metabolism according to its energy needs has a great potential for finding the therapies for treatments of metabolic diseases such as hepatic insulin resistance.

5 LITERATURE

Antonescu, C. N., McGraw, T. E., & Klip, A. (2014). Reciprocal regulation of endocytosis and metabolism. *Cold Spring Harbor Perspectives in Biology*, *6*(7). https://doi.org/10.1101/cshperspect.a016964

Aoh, Q. L., Graves, L. M., & Duncan, M. C. (2011). Glucose regulates clathrin adaptors at the trans-Golgi network and endosomes. *Molecular Biology of the Cell*, *22*(19), 3671–3683. https://doi.org/10.1091/mbc.E11-04-0309

Casciola, L. a, van der Westhuyzen, D. R., Gevers, W., & Coetzee, G. a. (1988). Low density lipoprotein receptor degradation is influenced by a mediator protein(s) with a rapid turnover rate, but is unaffected by receptor up- or down-regulation. *Journal of Lipid Research*, *29*(11), 1481–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3241124

Cheng, K. W., Agarwal, R., Mitra, S., Lee, J. S., Carey, M., Gray, J. W., & Mills, G. B. (2012). Rab25 increases cellular ATP and glycogen stores protecting cancer cells from bioenergetic stress. *EMBO Molecular Medicine*, *4*(2), 125–141. https://doi.org/10.1002/emmm.201100193

Collinet, C., Stöter, M., Bradshaw, C. R., Samusik, N., Rink, J. C., Kenski, D., … Zerial, M. (2010). Systems survey of endocytosis by multiparametric image analysis. *Nature*, *464*(7286), 243–9. https://doi.org/10.1038/nature08779

De Jong, L. A. A., Uges, D. R. A., Franke, J. P., & Bischoff, R. (2005). Receptor-ligand binding assays: Technologies and applications. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*. https://doi.org/10.1016/j.jchromb.2005.10.002

Di Fiore, P. P., & von Zastrow, M. (2014). Endocytosis, signaling, and beyond. *Cold Spring Harbor Perspectives in Biology*, *6*(8). https://doi.org/10.1101/cshperspect.a016865

Fu, D., Wakabayashi, Y., Ido, Y., Lippincott-Schwartz, J., & Arias, I. M. (2010). Regulation of bile canalicular network formation and maintenance by AMP-activated protein kinase and LKB1. *Journal of Cell Science*, *123*, 3294–3302. https://doi.org/10.1242/jcs.068098

Hagiwara, A., Cornu, M., Cybulski, N., Polak, P., Betz, C., Trapani, F., … Hall, M. N. (2012). Hepatic mTORC2 activates glycolysis and lipogenesis through Akt, glucokinase, and SREBP1c. *Cell Metabolism*, *15*(5), 725–738. https://doi.org/10.1016/j.cmet.2012.03.015

Hardie, D. G., Ross, F. a., & Hawley, S. a. (2012). AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nature Reviews Molecular Cell Biology*, *13*(4), 251–262. https://doi.org/10.1038/nrm3311

Hers, I., Vincent, E. E., & Tavar??, J. M. (2011). Akt signalling in health and disease. *Cellular Signalling*. https://doi.org/10.1016/j.cellsig.2011.05.004

Hou, L., Cai, M. J., Liu, W., Song, Q., & Zhao, X. F. (2012). Small GTPase Rab4b participates in the gene transcription of 20-hydroxyecdysone and insulin pathways to regulate glycogen level and metamorphosis. *Developmental Biology*, *371*(1), 13–22. https://doi.org/10.1016/j.ydbio.2012.06.015

International Diabetes Federation. (2014). IDF Diabetes Atlas Sixth edition. *Idf.org*, 1–2. https://doi.org/2-930229-80-2

International Diabetes Federation (IDF). (2015). IDF Diabetes Atlas 7th edition. *Idf.org*. https://doi.org/10.1289/image.ehp.v119.i03

Jaber, N., Dou, Z., Chen, J. S., Catanzaro, J., Jiang, Y. P., Ballou, L. M., … Zong, W. X. (2012). Class III PI3K Vps34 plays an essential role in autophagy and in heart and liver function. *Proc Natl Acad Sci U S A*, *109*(6), 2003–2008. https://doi.org/1112848109 [pii] 10.1073/pnas.1112848109 [doi]

Jovic, M., Sharma, M., Rahajeng, J., & Caplan, S. (2010). The early endosome: A busy sorting station for proteins at the crossroads. *Histology and Histopathology*. https://doi.org/10.1016/j.bbi.2008.05.010

Lang, M. J., Martinez-Marquez, J. Y., Prosser, D. C., Ganser, L. R., Buelto, D., Wendland, B., & Duncan, M. C. (2014). Glucose starvation inhibits autophagy via vacuolar hydrolysis and induces plasma membrane internalization by down-regulating recycling. *Journal of Biological Chemistry*, *289*(24), 16736–16747. https://doi.org/10.1074/jbc.M113.525782

Leto, D., & Saltiel, A. R. (2012). Regulation of glucose transport by insulin: traffic control of GLUT4. *Nature Reviews. Molecular Cell Biology*, *13*(6), 383–96. https://doi.org/10.1038/nrm3351

Manning, B. D., & Cantley, L. C. (2007). AKT/PKB Signaling: Navigating Downstream. *Cell*. https://doi.org/10.1016/j.cell.2007.06.009

Mattila, J., Havula, E., Suominen, E., Teesalu, M., Surakka, I., Hynynen, R., … Hietakangas, V. (2015). Mondo-Mlx Mediates Organismal Sugar Sensing through the Gli-Similar Transcription Factor Sugarbabe. *Cell Reports*, *13*(2), 350–364. https://doi.org/10.1016/j.celrep.2015.08.081

Mihaylova, M. M., & Shaw, R. J. (2011). The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. *Nature Cell Biology*, *13*(9), 1016–23. https://doi.org/10.1038/ncb2329

Rink, J., Ghigo, E., Kalaidzidis, Y., & Zerial, M. (2005). Rab conversion as a mechanism of progression from early to late endosomes. *Cell*, *122*(5), 735–749. https://doi.org/10.1016/j.cell.2005.06.043

Schmid, S. L., & Carter, L. L. (1990). ATP is required for receptor-mediated endocytosis in intact cells. *Journal of Cell Biology*, *111*(6 PART 1), 2307–2318. https://doi.org/10.1083/jcb.111.6.2307

Simons Andrean L, Orcutt Kevin P, Madsen Joshua M, Scarbrough Peter M, S. D. R. (2012). The role of Akt pathway signaling in glucose metabolism and metabolic oxidative stress. *Oxidative Stress in Cancer Biology and Therapy*, 21–46. https://doi.org/10.1007/978-1-61779-397-4

Singh, R., & Cuervo, A. M. (2011). Autophagy in the cellular energetic balance. *Cell Metabolism*. https://doi.org/10.1016/j.cmet.2011.04.004

Sorkin, A., & Duex, J. E. (2010). Quantitative analysis of endocytosis and turnover of epidermal growth factor (EGF) and EGF receptor. *Current Protocols in Cell Biology / Editorial Board, Juan S. Bonifacino ... [et Al.]*, *Chapter 15*, Unit 15.14. https://doi.org/10.1002/0471143030.cb1514s46

Sorkin, A., & von Zastrow, M. (2009). Endocytosis and signalling: intertwining molecular networks. *Nature Reviews. Molecular Cell Biology*, *10*(9), 609–22. https://doi.org/10.1038/nrm2748

Traub, L. M., & Bonifacino, J. S. (2013). Cargo recognition in clathrin-mediated endocytosis. *Cold Spring Harbor Perspectives in Biology*, *5*(11). https://doi.org/10.1101/cshperspect.a016790

Treyer, A., & M??sch, A. (2013). Hepatocyte polarity. *Comprehensive Physiology*, *3*(1), 243–287. https://doi.org/10.1002/cphy.c120009

Wandinger-Ness, A., & Zerial, M. (2014). Rab Proteins and the Compartmentalization of the Endosomal system. *Cold Spring Harbor Perspectives in Biology*, *6*(11), 1–25. https://doi.org/10.1101/cshperspect.a016162

Wilson, J. M., de Hoop, M., Zorzi, N., Toh, B. H., Dotti, C. G., & Parton, R. G. (2000). EEA1, a tethering protein of the early sorting endosome, shows a polarized distribution in hippocampal neurons, epithelial cells, and fibroblasts. *Molecular Biology of the Cell*, *11*(8), 2657–71.

Yamamoto, N., Ueda, M., Sato, T., Kawasaki, K., Sawada, K., Kawabata, K., & Ashida, H. (2011). Measurement of glucose uptake in cultured cells. *Current Protocols in Pharmacology*, (SUPPL. 55). https://doi.org/10.1002/0471141755.ph1214s55

Zeigerer, A., Seifert, S., Marsico, M., Kalaidzidis, Y. & Zerial, M. (n.d.). Functional properties of hepatocytes in vitro are correlated with cell polarity maintenance. *Manusript under Review.*

Zeigerer, A., Bogorad, R. L., Sharma, K., Gilleron, J., Seifert, S., Sales, S., … Zerial, M. (2015). Regulation of Liver Metabolism by the Endosomal GTPase Rab5. *Cell Reports*, *11*(6), 884–892. https://doi.org/10.1016/j.celrep.2015.04.018

Zeigerer, A., Gilleron, J., Bogorad, R. L., Marsico, G., Nonaka, H., Seifert, S., … Zerial, M. (2012). Rab5 is necessary for the biogenesis of the endolysosomal system in vivo. *Nature*, *485*(7399), 465–70. https://doi.org/10.1038/nature11133

Zoncu, R., Perera, R. M., Balkin, D. M., Pirruccello, M., Toomre, D., & De Camilli, P. (2009). A Phosphoinositide Switch Controls the Maturation and Signaling Properties of APPL Endosomes. *Cell*, *136*(6), 1110–1121. https://doi.org/10.1016/j.cell.2009.01.032

6 LIST OF FIGURES

Figure 1 Diabetes is a huge and growing problem, and the costs to society are high and escalating. 7

Figure 2 The liver comprises multiple cell types. 8

Figure 3 Liver cells secrete bile. 9

Figure 4 Endosomal transport in liver cells. 10

Figure 5 Rab proteins are organelle specific. 12

Figure 6 Rab5 is the master regulator of endosome biogenesis. 14

Figure 7 Experimental setup for glucose stimulation assays. 22

Figure 8 Receptor internalization assay. 23

Figure 9 Cell based assay quantification using Fiji software. 24

Figure 10 Optimization of primary hepatocyte collagen sandwich culture. 29

Figure 11 EGF uptake upon high (25mM) and low (5mM) glucose stimulated primary hepatocytes. 32

Figure 12 LDL uptake upon high (25mM) and low (5mM) glucose stimulated primary hepatocytes. 34

Figure 13 EGF–receptor binding on the surface of primary hepatocytes. 35

Figure 14 LDL–receptor binding on the surface of primary hepatocytes. 36

Figure 15 Distribution of early endosomes specifically stained with anti–EEA1 antibody in hepatocytes starved with low and high glucose concentration. 38

Figure 16 Quantification of ratio between high and low glucose stimulated hepatocytes specifically stained with anti–EEA1 and anti–LAMP1 antibodies. 39

Figure 17 Distribution of late endosomes and lysosomes specifically stained with anti–LAMP1 antibody in hepatocytes starved with low and high glucose concentration. 39

Figure 18 Staining with different markers of primary hepatocytes upon low and high glucose stimulation. 43

Figure 19 pAMPK upon stimulation of primary hepatocytes with high, intermediate and low glucose concentration.. 45

Figure 20 pAkt upon stimulation of primary hepatocytes with high, intermediate and low glucose concentration. 46

Figure 21 pS6 RP upon stimulation of primary hepatocytes with high, intermediate and low glucose concentration..... 47