Regulation of Nicotinamide N-methyltransferase Expression in Adipocytes

Regulation der Nicotinamide N-methyltransferase Expression in Adipozyten

Doctoral thesis for a doctoral degree at the Graduate School of Life Sciences,

Julius-Maximilians-Universität Würzburg,

Section Biomedicine

submitted by

Franziska Ehebauer

from

Nabburg

Würzburg, August 2020

Submitted on: ………………………………………………………………

Members of the Thesis Committee

Chairperson: Prof. Dr. Manfred Gessler

Primary Supervisor: Dr Daniel Kraus

Supervisor (Second): PD Dr. Heike Hermanns

Supervisor (Third): Prof. Dr. Christoph Wanner

Supervisor (Fourth): PD Dr. Dr. Matthias Kroiß

Date of Public Defence: ………………………………………….………

Date of Receipt of Certificates: …………………………………………

Für Julius Ragnar und Daniel

Contents

Abstract

Nicotinamide N-methyltransferase (NNMT) is a new regulator of energy homeostasis. Its expression is increased in models of obesity and diabetes. An enhanced NNMT level is also caused by an adipose tissue-specific knockout of glucose transporter type 4 (GLUT4) in mice, whereas the overexpression of this glucose transporter reduced the NNMT expression. Furthermore, the knockdown of the enzyme prevents mice from diet-induced obesity (DIO) and the recently developed small molecule inhibitors for NNMT reverses the DIO. These previous findings demonstrated the exclusive role of NNMT in adipose tissue and further make it to a promising target in obesity treatment. However, the regulation mechanism of this methyltransferase is not yet clarified.

The first part of the thesis focus on the investigation whether pro-inflammatory signals are responsible for the enhanced NNMT expression in obese adipose tissue because a hallmark of this tissue is a low-level chronic inflammation. Indeed, the NNMT mRNA in our study was elevated in obese patients compared with the control group, whereas the GLUT4 mRNA expression does not differ between lean and obese humans. To analyze whether pro-inflammatory signals, like interleukin (IL-6) and tumor necrosis factor α (TNF-α), regulate NNMT expression 3T3-L1 adipocytes were treated with these cytokines. However, IL-6, TNF-α, and leptin, which is an alternative activator of the JAK/STAT pathway, did not affect the NNMT protein or mRNA level in differentiated 3T3-L1 adipocytes. The mRNA and protein levels were measured by quantitative polymerase chain reaction (qPCR) and western blotting.

In the second part of this study, 3T3-L1 adipocytes were cultivated with varying glucose concentrations to show whether NNMT expression depends on glucose availability. Further studies with activators and inhibitors of AMP-activated protein kinase (AMPK) and mechanistic target of rapamycin (mTOR) signaling pathways were used to elucidate the regulation mechanism of the enzyme.

The glucose deprivation of differentiated 3T3-L1 adipocytes led to a 2-fold increase in NNMT expression. This effect was confirmed by the inhibition of the glucose transports with phloretin as well as the inhibition of glycolysis with 2-deoxyglucose (2-DG). AMPK serves as an intracellular energy sensor and the pharmacological activation of it enhanced the NNMT expression. This increase was also caused by the inhibition of mTOR. Conversely, the activation of mTOR using MHY1485 prevented the effect of glucose deprivation on NNMT. Furthermore, the NNMT up-regulation was also blocked by the different autophagy inhibitors.

Taken together, NNMT plays a critical role in autophagy in adipocytes, because an inhibition of this process prevented the augmented NNMT expression during glucose starvation. Moreover, the effect on NNMT protein and mRNA level depends on AMPK and mTOR. However, pro-inflammatory signals did not affect the expression. Further *in vivo* studies have to clarify whether AMPK activation and mTOR inhibition as well as autophagy are responsible for the increased NNMT levels in obese adipose tissue. In future this methyltransferase emerges as an awesome therapeutic target for obesity.

Zusammenfassung

NNMT ist ein neuer Regler der Energiehomöostase. Seine Expression ist in Adipositas- und Diabetesmodellorgansimen erhöht. Ein verstärktes NNMT Level wird auch durch einen fettgewebs-spezifischen GLUT4 Knockout in Mäusen hervorgerufen, wobei die Überexpression des Glukosetransporters die NNMT Expression reduziert. Des Weiteren schützt der Knockdown von NNMT die Mäuse vor Diät-induzierter Adipositas und die kürzlich entwickelten kleinen Molekülinhibitoren gegen NNMT kehren eine durch die Ernährung bedingte Adipositas wieder um. Neuere Erkenntnisse zeigen die exklusive Rolle von NNMT im Fettgewebe auf und machen das Enzym so zu einem vielversprechenden Target für die Adipositastherapie. Jedoch ist der Regulationsmechanismus dieser Methyltransferase noch nicht geklärt.

Der erste Teil der Arbeit befasst sich mit der Untersuchung, ob pro-inflammatorische Signale verantwortlich sind für die erhöhten NNMT Expression im adipösen Fettgewebe, da sich dieses Gewebe durch eine chronische Inflammation auszeichnet. Tatsächlich war die mRNA in unserer Studie verstärkt exprimiert in adipösen Patienten im Vergleich zur Kontrollgruppe, wobei die GLUT4 mRNA Expression zwischen Schlanken und Adipösen nicht verändert war. Um zu untersuchen, ob pro-inflammatorische Signale, wie IL-6 und TNF-α, die NNMT Expression regulieren, wurden 3T3-L1 Adipozyten mit diesen Zytokinen behandelt. Jedoch beeinflussten IL-6, TNF-α und Leptin, welches ein weiterer Aktivator des JAK/STAT Signalweges ist, NNMT Protein oder mRNA Level in differenzierten 3T3-L1 Adipozyten nicht. Die mRNA und Protein Level wurden mittels qPCR und Western Blot analysiert.

Im zweiten Teil dieser Studie wurden 3T3-L1 Adipozyten mit unterschiedlichen Glukosekonzentrationen kultiviert, um zu zeigen, ob die NNMT Expression von der Glukoseverfügbarkeit abhängig ist. Für die Untersuchung des genauen Regulationsmechanismus von NNMT, wurden weitere Studien mit Aktivatoren und Inhibitoren der AMPK und mTOR Signalwege durchgeführt.

Der Glukosemangel führte zu einem 2-fachen Anstieg der NNMT Expression in differenzierten 3T3-L1 Adipozyten. Dieser Effekt wurde bestätigt durch die Inhibierung der Glukosetransporter mit Phloretin sowie durch die Inhibierung der Glykolyse mit 2-DG. AMPK ist ein intrazellulärer Energiesensor und dessen pharmakologische Aktvierung erhöhte die NNMT Expression. Dieser Anstieg wurde auch verursacht durch die Inhibierung von mTOR. Hingegen verhinderte die Aktivierung von mTOR mithilfe von MHY1485 den Effekt auf NNMT während des

Glukoseentzugs. Des Weiteren wurde die Auswirkungen auf NNMT durch Autophagieinhibitoren unterbunden.

Zusammenfassend spielt NNMT eine kritische Rolle für die Autophagie in Adipozyten, da eine Inhibierung des Prozesses die erhöhte NNMT Expression während eines Glukoseentzugs verhinderte. Darüber hinaus ist der Effekt auf die NNMT Protein und mRNA Level abhängig von AMPK and mTOR. Jedoch beeinflussten pro-inflammatorische Signale die Expression nicht. Weitere *in vivo* Studien müssen klären, ob eine AMPK Aktivierung und eine mTOR Inhibierung sowie die Autophagie in Adipozyten verantwortlich sind für die verstärkte NNMT Expression im adipösen Fettgewebe. Zukünftig wird sich NNMT als ein beeindruckendes Target für die Adipositastherapie herausstellen.

1   Introduction

1.1   NNMT – nicotinamide N-methyltransferase

NNMT (EC 2.1.1.1) is a cytosolic methyltransferase which catalyzes the methylation of nicotinamide to 1-methylnicotinamide (1-MN) (Aksoy et al., 1994). The methyl group is provided by the universal methyl group donor S-adenosyl methionine (SAM) (Rini et al., 1990). After an oxidation step by aldehyde oxidase or cyp2E1 the reaction product 1-MN is excreted via the urine (Felsted and Chaykin, 1967; Real et al., 2013; Rudolphi et al., 2018).

The enzyme NNMT, itself, is primarily expressed in adipose tissue and liver, but also in brain, lung, muscle, and kidney (Cantoni, 1951; Kraus et al., 2014; Pissios, 2017; Rini et al., 1990; Yan et al., 1997). In various cancers the enzyme is up-regulated and contributes to tumorigenicity, e. g. in hepatocellular carcinoma (Li et al., 2019), glioblastoma (Jung et al., 2017), renal cell carcinoma (Tang et al., 2011), esophageal squamous cell carcinoma (Cui et al., 2019), and bladder cancer (Wu et al., 2008). In addition, NNMT expression is increased in mouse models of obesity (Kraus et al., 2014). Therefore, the methyltransferase is a promising target for cancer and obesity treatment. Especially, the second mentioned disease has reached epidemic proportions, because the prevalence has tripled in the last decades (Branca et al., 2007). Overweight is responsible for many diseases, like type 2 diabetes (T2D) and it is a risk factor for ischemic heart disease, and osteoarthritis (Branca et al., 2007). Moreover, obesity is associated with many cancer types, e. g. colorectal cancer, postmenopausal breast cancer, endometrial cancer, and liver cancer (Pischon and Nimptsch, 2016).

1.2   NNMT a target for obesity therapy

The predominant reason for obesity is the overconsumption of inexpensive, calorically dense, inadequately satiating, highly palatable diets and a reduced physical activity (Petersen and Shulman, 2018). The treatment of obesity is great challenge for our society and a successful therapy could also be a big advantage for the cancer and T2D prevention. A part of the solution could be NNMT that is the dominant methyltransferase in white adipose tissue (WAT) (Pissios, 2017), which has a huge influence in the regulation of systemic nutrient and energy homeostasis (Stern et al., 2016). An early association of NNMT and obesity was shown by Lee

and colleagues (Lee et al., 2005). There the enzyme expression was enhanced in obese Pima Indians compared with the non-obese control group (Lee et al., 2005). However, this was not investigated further. Almost 10 years later NNMT was discovered as a novel regulator of energy homeostasis (Kraus et al., 2014). It was not only elevated in db/db and ob/ob mice, but also the knockdown by antisense oligonucleotides (ASOs) against NNMT protects mice form DIO (Kraus et al., 2014). Similar effects were obtained with ASOs conjugated to thyroid hormone T3 (Cao et al., 2017). Furthermore, NNMT$^{-/-}$ female mice fed a Western diet had a reduced weight gain and fat tissue (Brachs et al., 2019). Interestingly, a higher NNMT expression caused by high fat diet (HFD) seems to be exclusive for adipocytes. In hepatocytes, where NNMT is also expressed, the expression is not altered (Hong et al., 2015). This indicates the critical role of this methyltransferase for the adipose tissue. Additionality, patients with T2D showed a higher NNMT expression in WAT and exercise and bariatric surgery reduce the mRNA level (Kannt et al., 2015). The different studies investigated the NNMT expression. However, NNMT is an enzyme and its activity strongly affected the NAD$^+$ and SAM:SAH ratio in fat cells, whereby the cellular metabolome is altered (Kraus et al., 2014). The enzymatic activity is also heightened during DIO in epididymal white adipose tissue (Rudolphi et al., 2018). Therefore, the manipulation of NNMT by small molecules is an interesting target for obesity treatment. Indeed, different developed substances successfully inhibited NNMT (Babault et al., 2018; Chen et al., 2019; Gao et al., 2019; Neelakantan et al., 2018; van Haren et al., 2017). Moreover, such inhibitors could reverse DIO by reducing body weight and adipose tissue mass (Kannt et al., 2018; Neelakantan et al., 2018). Notably, the treatment did not cause observable adverse effects (Neelakantan et al., 2018).

The obtained results clearly show the central and exclusive role of NNMT in adipose tissue and obesity. However, the regulation mechanism of NNMT behind these findings is not analyzed yet. Therefore, the aim of thesis is to investigate how NNMT is regulated in adipocytes. Since a high NNMT expression was discovered in the adipose tissue of a GLUT4 knockdown mouse (Kraus et al., 2014), it would be interesting whether NNMT serves as a glucose or energy sensor for the cell. Obviously, the results obtained by Kraus and colleagues in insulin resistant mice supports that glucose availability is important for NNMT regulation (Kraus et al., 2014). If the enzyme really is an energy sensor, a manipulation of the glucose metabolism at various levels could improve the knowledge of this methyltransferase. After a fully glucose deprivation, the glucose uptake could be analyzed by phloretin, a typical inhibitor of the glucose transporters. Without glucose the glycolysis, an important ATP generating process, could be disrupted. The inhibition by 2-DG could determine the role of the glycolysis for NNMT regulation. To obtain reproducible results, the established adipocyte 3T3-L1 cell line is used in for the experiments.

Nevertheless, pro-inflammatory signals could also contribute to the enhanced NNMT expression in obese individuals and obesity mouse models (db/db and ob/ob), because a hallmark of obese adipose tissue is a low-level chronic inflammation (Hotamisligil, 2006).

1.3   Inflammation in adipose tissue

The macrophages, that accumulated during DIO, contribute to adipose tissue inflammation and mediate insulin resistance in adipocytes (Hotamisligil, 2006). Furthermore, WAT itself produces different pro-inflammatory signals, like TNF-α and IL-6 (Klein et al., 2006). The first is elevated in this tissue type of obese individuals (Gregoire et al., 1998). It reduces the expression of adipocyte genes, as well as may contribute to insulin resistance (Gregoire, et al. 1998). A continuous IL-6 production also causes insulin resistance in adipocytes (Rotter et al., 2003). This cytokine stimulates via STAT3 activation NNMT expression in HepG2, colon cancer cells (Tomida et al., 2008), 3T3-L1 adipocytes (Balhoff and Stephens, 1998), and in human airway smooth muscle cells (Robinson et al., 2015). Therefore, it is possible that pro-inflammatory signals regulate NNMT.

In adipose tissue these signals are typically relayed by the JAK/STAT pathway (Richard and Stephens, 2014). An alternative activator of this pathway is leptin that exerts paracrine and autocrine effects on adipocytes (Kraus et al., 2002). This hormone is primarily produced by this cell type and it binds to its receptors in the hypothalamus (Gregoire et al., 1998). There various studies indicate that leptin is involved in the regulation of energy balance (Harris, 2014). However, the receptors for leptin are also found on many other tissues, including the adipocyte membrane and this highlighted the autocrine function of it for WAT (Stern et al., 2016). Therefore, leptin and pro-inflammatory signals could be responsible for augmented NNMT expression in WAT of obese individuals. To investigate this hypothesis, the 3T3-L1 adipocytes could be treated with IL-6, TNF-α, and leptin. As mentioned above, the inflammation in the adipose tissue could also contribute to insulin resistance (Guilherme, 2008 (Guilherme et al., 2008; Hotamisligil et al., 1996), that leads to an impaired glucose metabolism.

1.4 Insulin resistance and impaired glucose metabolism

The chronic inflammation is not the only hallmark of obesity. T2D and insulin resistance are often associated with this disease. When higher insulin concentrations are necessary to reduce the glucose level, it is called insulin resistance (Petersen and Shulman, 2018). Normally, the binding of this hormone to its receptor leads to the translocation of GLUT4 from intracellular vesicles to the plasma membrane, followed by the glucose uptake into the cell (Im et al., 2007). Microarray analysis of adipose-specific *Glut4*-knockout mice (AG4KO) and adipose-specific *Glut4*-overexpressing mice (AG4Tg) revealed that NNMT is reciprocally regulated (Kraus et al., 2014). Interestingly, NNMT is strongly up-regulated in AG4KO mice (Kraus et al., 2014) (Figure 1.1).
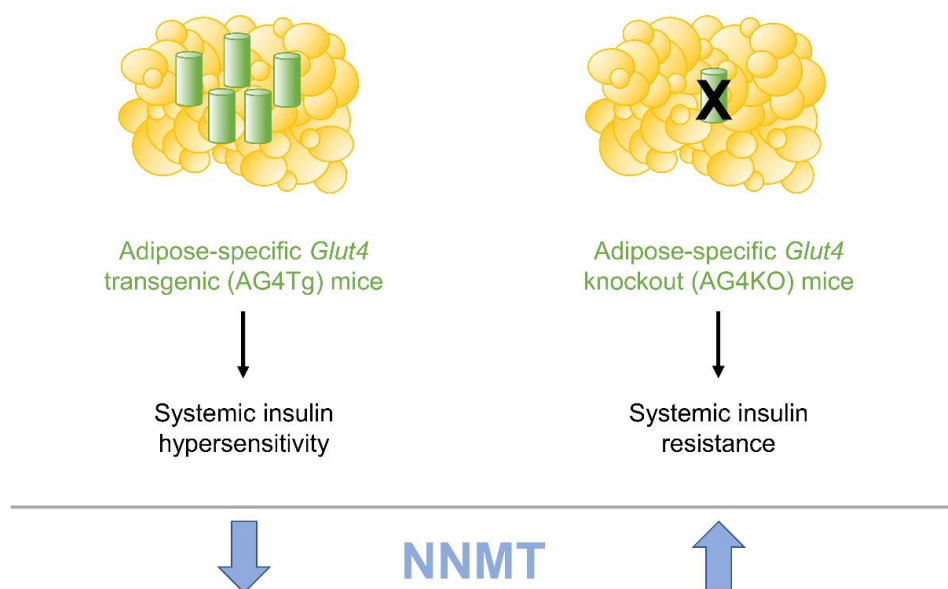
Figure 1.1   **NNMT expression in adipose tissue of AG4Tg and AG4KO mice.**
Adipose-specific *Glut4* transgenic (AG4Tg) mice overexpress GLUT4. They developed a systemic insulin hypersensitivity. The adipose-specific *Glut4*-knockout (AG4KO) mice had a systemic insulin resistance. The NNMT expression was reduced in the adipose tissue of AG4Tg mice, whereby the AG4KO mice showed an enhanced NNMT expression. The glucose transporters of the adipose tissue are shown as green tubes. Based on the results of (Kraus et al., 2014; Yang et al., 2005).

Insulin resistance, diabetes, and obesity are often characterized by a reduced GLUT4 expression in adipose tissue, but not in muscle (Shepherd and Kahn, 1999). Of note, the skeletal muscle predominantly contributes to insulin-dependent glucose disposal, whereas the adipose tissue has only a minor influence (Minokoshi et al., 2003). However, the insulin resistance in WAT is the driving force for T2D (Minokoshi et al., 2003) and an adipose selective reduction of GLUT4 leads to insulin resistance in mice (Abel et al., 2001). This genetic

manipulation negatively affects insulin action in other organs, like muscle and liver (Abel et al., 2001). In contrast to this, the overexpression of GLUT4 in adipose tissue enhanced the glucose uptake and insulin sensitivity (Shepherd et al., 1993). Furthermore, the overexpression in adipocytes could reverse the diabetes in mice lacking muscle GLUT4 (Carvalho et al., 2005). Therefore, the expression of this glucose transporter in adipose tissue plays a critical role in overall glucose metabolism.

To clarify the NNMT regulation during an impaired glucose metabolism, it is necessary to investigate cellular mechanism inside the adipocyte. Consequently, the major metabolic signaling pathways AMPK and mTOR should be investigated. Especially, the AMPK signaling pathway should recognize the reduced ATP concentration, that is strongly affected by the glycolysis, due to the diminished glucose uptake without GLUT4.

1.4.1 AMPK and mTOR signaling pathway

AMPK is the major sensor in adipose tissue for energy status (Ceddia, 2013). It is a trimeric complex that contains a catalytic subunit (α) and the two regulatory subunits (β and γ) (Hardie, 2011). Starvation leads to the activation of this kinase that can quickly modify the uptake, storage, and oxidation status of various compounds in the adipocyte (Gaidhu et al., 2006; Gaidhu and Ceddia, 2009). Therefore, AMPK restores the energy balance at cellular level (Garcia and Shaw, 2017). After the recognition of AMP:ATP and ADP:ATP ratios, AMPK inhibits different anabolic processes, while the numerous catabolic reactions that provide ATP are strengthened (Garcia and Shaw, 2017; Hardie, 2007). Thereby this kinase negatively controls the mechanistic target of rapamycin complex 1 (mTORC1) (Inoki et al., 2012). Figure 1.2 shows both signaling pathways and the controlled cellular processes. Upon activation the catabolic fatty acid oxidation, glucose uptake, and autophagy are promoted. In addition, the anabolic reactions, including protein and fatty acid synthesis are impaired (Tamargo-Gomez and Marino, 2018).

AMPK is activated by two mechanisms. There are two activation mechanisms of this kinase. One depends on phosphorylation of Thr172 by the calcium/calmodulin dependent kinase kinase 2 (CAMKK2) (Hurley et al., 2005). The other way based on the binding of AMP or ADP to the γ subunit and the phosphorylation of Thr172 by liver-kinase B1 (LKB1) (Hawley et al., 2003; Woods et al., 2003). Afterwards, AMPK promoted the translocation of GLUT4 to the membrane (Hardie, 2013; Wu et al., 2013; Zhou et al., 2013). Furthermore, the activated AMPK

phosphorylates acetyl CoA carboxylase 1 and 2, thereby the fatty acid synthesis is blocked (Garcia and Shaw, 2017). Another target of AMPK is mTORC1 (Laplante and Sabatini, 2012). It is the second important signaling pathway that controls energy and lipid metabolism (Caron et al., 2015) and it is an interface between cell growth and starvation (Zoncu et al., 2011).
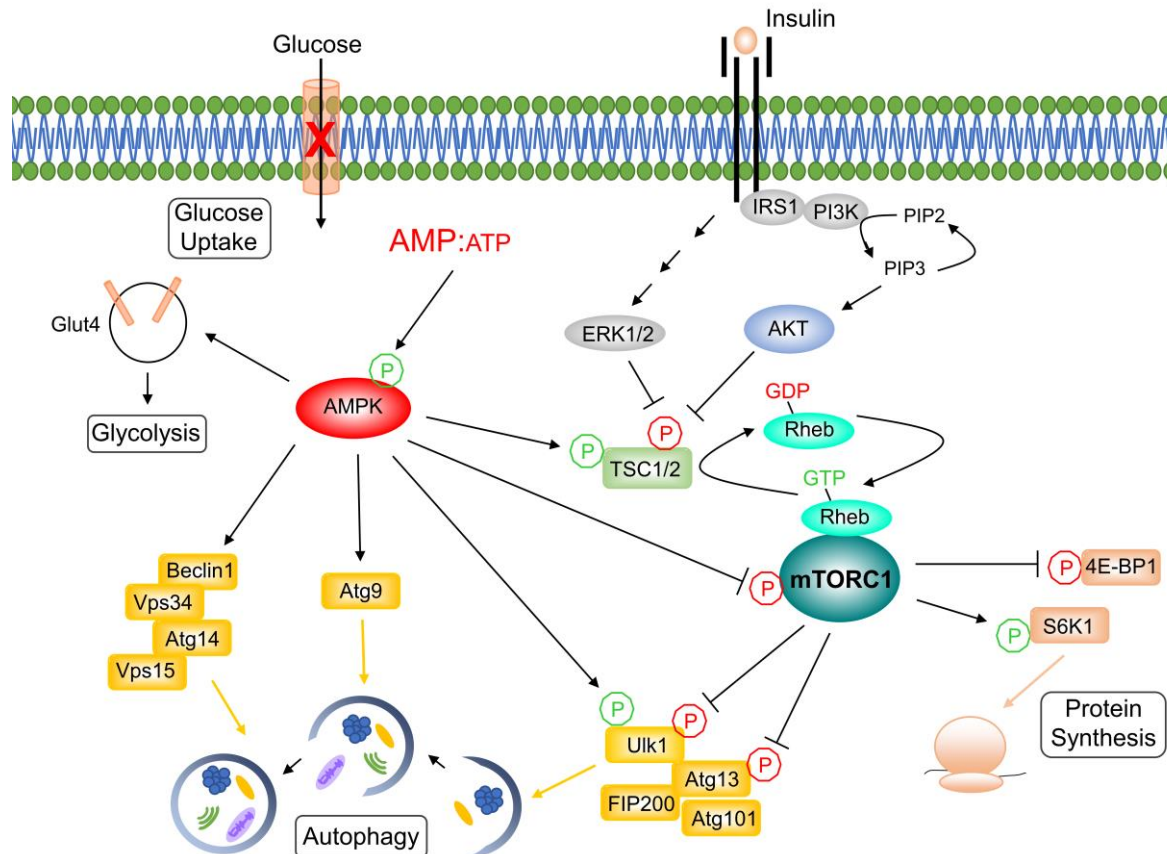
Figure 1.2: **AMPK and mTOR signaling pathway.**
The kinase AMPK is activated by energy stress. This leads to GLUT4 translocation and autophagy induction via different proteins. Under nutrient rich condition AMPK is inactive and mTORC1 is active. Consequently, autophagy is repressed. The autophagy induction requires the inhibition of mTORC1. Thus, mTORC1 activity is directly repressed by AMPK that also phosphorylates TSC1/2, whereby its GTPase activity is raised. When mTORC1 is activated by GTP-Rheb protein synthesis via S6K1 is promoted. For this, the TSC1/2 complex has to be interrupted and the Rheb-GTP is bound to mTORC1. However, after mTORC1 inhibition autophagy is promoted. The activating phosphorylation events are shown in green; the repressing events are colored in red. Redrawn and modified from (Garcia and Shaw, 2017; Kaushal et al., 2020; Laplante and Sabatini, 2009; Saxton and Sabatini, 2017).

Since mTORC1, not mTORC2, is predominantly affected by energy status of the cell (Saxton and Sabatini, 2017), it is investigated in this study and subsequently described. Figure 1.2 shows the mTORC1 signaling pathway in detail. Nutrient rich conditions activate the mTOR signaling pathway, whereas a disruption in energy homeostasis represses it (Laplante and Sabatini, 2013). mTORC1 consists of mTOR, regulatory-associated protein of mTOR (Raptor),

mammalian lethal with Sec13 (mLST8), prolinerich AKT substrate 40 kDa (PRAS40), and DEP-domain-containing mTOR-interacting protein (Deptor) (Peterson et al., 2009). In addition to the energy status, mTORC1 is stimulated by growth factors, amino acids, stress, and oxygen to regulate protein synthesis, lipid metabolism, lysosome biogenesis, energy metabolism, and autophagy (Caron et al., 2015).

Its kinase activity is controlled by Ras homolog enriched in brain (Rheb) that is a GTPase and it is only active when GTP is bound (Zoncu et al., 2011). Rheb, itself, is regulated by the TSC1/2 complex, a GTPase-activating protein, that hydrolyses GTP (Lamming and Sabatini, 2013). Under nutrient rich condition TSC1/2 is interrupted, whereby mTORC1 activation is promoted (Laplante and Sabatini, 2012). For example insulin signaling leads to AKT activation via phosphoinositide 3-kinase (PI3K) (Zoncu et al., 2011). Subsequently TSC1/2 is phosphorylated and inactivated (Laplante and Sabatini, 2012; Zoncu et al., 2011).

In adipocytes an activated mTORC1 complex further promotes lipogenesis (Caron et al., 2015), whereas an inhibited complex promotes catabolic process and autophagy induction under sustained energy depletion (Corona Velazquez and Jackson, 2018; Kaushal et al., 2020; Rabanal-Ruiz and Korolchuk, 2018). Then TSC2 is phosphorylated by AMPK (Garcia and Shaw, 2017; Inoki et al., 2012) and the hydrolysis of the Rheb-bound GTP by TSC1/2 is activated (Laplante and Sabatini, 2009). The inactive mTORC1 could not prevent autophagy due to unc-51-like kinase 1 (Ulk1) and autophagy-related protein 13 (Atg13) phosphorylation (Rabanal-Ruiz and Korolchuk, 2018). Further AMPK promote the autophagy induction by the different components, like vacuolar protein sorting 34 (Vps34) complex (Corona Velazquez and Jackson, 2018; Inoki et al., 2012). The process is more precisely described in the following section because it might be possible that NNMT is up-regulated during autophagy.

It is not known whether the adipocytes of the insulin resistant AG4KO mice induced autophagy. However, before the autophagy induction could be investigated, AMPK and mTOR signaling pathway have to be manipulated. mTOR could be inhibited by rapamycin and AMPK could be activated 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR). Afterwards, the NNMT expression should be determined in 3T3-L1 adipocytes. Later, the autophagy should be closely considered.

1.4.2 Autophagy

Autophagy, a catabolic process, is responsible for the maintenance of cellular homeostasis in response to cellular stresses, like nutrient starvation (Johansen and Lamark, 2011). There are different kinds of it, including macroautophagy, mitophagy, autophagy of the endoplasmic reticulum, and lipophagy (Corona Velazquez and Jackson, 2018). The macroautophagy (hereafter autophagy) is regulated by AMPK and mTORC1 (Figure 1.2).

When the activation of mTORC1 is prevented, the missing phosphorylations at Ulk1 and Atg13 lead to autophagy induction (Jung et al., 2010; Kim et al., 2011; Rabanal-Ruiz and Korolchuk, 2018). The missing Ulk1 phosphorylation allows an interaction with AMPK, whereby this kinase phosphorylates Ulk1 at Ser317 and Ser777 to induce and promote autophagy (Kim et al., 2011; Lee et al., 2010). Atg13, Ulk1, and Vps34 are responsible for autophagy coordination (Kim et al., 2013). In a first step a protein complex including Ulk1, Atg13, Atg101, and focal adhesion kinase family interacting protein 200 kDa (FIP200) is formed (Zachari and Ganley, 2017). Afterwards, the Ulk1 complex regulates the organization of the Vps34-complex (Rabanal-Ruiz and Korolchuk, 2018). It consists of the Vps34, Beclin-1, Vps15, and Atg14-like (Atg14L) (Zachari and Ganley, 2017). Moreover, Vps34 stimulates autophagy due to an AMPK-dependent regulation mechanism (Kim et al., 2013).

After activation of autophagy p62/SQSTM1 induces the nucleation of the autophagosome membrane by the interaction with microtubule-associated proteins 1A/1B light chain 3B, hereafter LC3 (Sánchez-Martín and Komatsu, 2018). The proteins p62 and LC3 are often used as autophagy marker by immunoblotting, because p62 is degraded during this process and LC3 showed two isoforms (Ichimura and Komatsu, 2010; Yoshii and Mizushima, 2017). Under normal conditions LC3 is present in the cytoplasm and is called LC3I (Feng et al., 2015). During autophagy it is processed to LC3II, thereby phosphatidylethanolamine is conjugated to it (Rabinowitz and White, 2010). Thus, the amount of LC3II is an indicator of autophagosomes in the cell (Mizushima and Yoshimori, 2007). After the interaction of p62 with LC3II a phagophore, followed by the autophagosome is formed (Corona Velazquez and Jackson, 2018) (Figure 1.3). Afterwards, the acidification is caused by vacuolar ATPases that originate from the fused endosome (Feng et al. 2015). Thereafter, autolysosomes, a fusion of autophagosome and lysosome, are formed (Rabanal-Ruiz and Korolchuk, 2018). There the cargo is degraded into the individual components that are dispensed into the cytoplasm through permeases (Singh and Cuervo, 2011). The sequestered components are reused to survive the energy stress (Bhattacharya et al., 2018; Rabanal-Ruiz and Korolchuk, 2018).

Of note, the autophagy could be interrupted at different levels. MHY1485 activates mTORC1 and prevents the autophagy-inhibiting phosphorylations on Ulk1 and Atg13. The process of autolysosome formation is blocked by bafilomycin A1 and chloroquine. They inhibited the acidification of the amphisome and the fusion with the lysosome. To investigate the autophagy in cell culture, glucose deprived adipocytes could be treated with the different inhibitors.
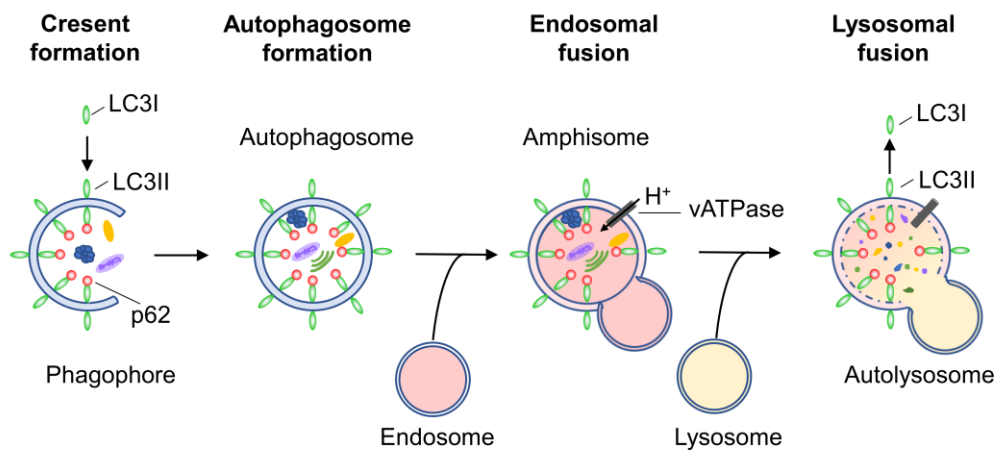
Figure 1.3   **The major steps of autophagy.**
First the phagophore is formed and LC3I is recruited to the phagophore membrane. Through the conjugation to phosphoethanolamine the membrane bound LC3II form is generated. The cargo receptor p62 interacts with LC3II. After phagophore formation it expands into the autophagosome with the typical double membrane. Through the fusion with an endosome an amphisome is formed. It contains vacuolar ATPases (vATPase) that lead to acidification. In a last step the amphisome fuses with a lysosome. The new autolysosome degrades the cargo. Redrawn and modified from (Corona Velazquez and Jackson, 2018; Feng et al., 2015).

1.5   Aim of the study

NNMT is up-regulated in mouse models with obesity (Kraus et al., 2014) and the knockdown of it protects mice from DIO (Cao et al., 2017; Kraus et al., 2014). Moreover, an inhibition of the enzyme with small-molecule inhibitors reversed DIO (Kannt et al., 2018; Neelakantan et al., 2018). Further studies showed the fundamental function of NNMT in energy homeostasis (Brachs et al., 2019; Kannt et al., 2018; Rudolphi et al., 2018). Therefore, this methyltransferase could be an innovative target for obesity treatment and a major advantage in obesity-related cancer prevention. However, it is not known why NNMT is up-regulated during DIO or in obese individuals (Kannt et al., 2015; Kraus et al., 2014; Lee et al., 2005). The analysis of the regulation mechanism may help to develop new targeted therapies for obesity.

To obtain reproducible results the analyses are carried out in 3T3-L1 adipocytes. This established cell line has numerous advantages and they are used in more than 5000 published articles analyzing the biochemistry of adipocytes (Poulos et al., 2010). This cell line expresses NNMT after differentiation into adipocytes and distinct effects could be measured during every experiment. Since the regulation of NNMT is not clarified yet, 3T3-L1 cells are the appropriate for first insight in the regulation mechanism.

The overall goals of the study included:

1. Different studies show that NNMT expression is enhanced by IL-6 (Balhoff and Stephens, 1998; Tomida et al., 2008). Moreover, TNF-α and IL-6 produced by the adipose tissue contributes to the low-level chronic inflammation of obese adipose tissue (Gregor and Hotamisligil, 2011; Klein et al., 2006). Therefore, NNMT expression is investigated after the stimulation of 3T3-L1 adipocytes with these cytokines.
2. Since this enzyme is up-regulated in AG4KO mice (Kraus et al., 2014), analyzes were carried out whether NNMT expression is associated with glucose metabolism. The NNMT levels are also investigated in 3T3-L1 adipocytes to determine the mechanism of regulation. Therefore, glucose metabolism is interrupted at different levels.
   a. First, differentiated 3T3-L1 cells were cultivated under glucose deprivation to reflect the NNMT effects in AG4KO mice.
   b. After the glucose deprivation, the glucose uptake is blocked by phloretin, an inhibitor of the glucose transporters.
   c. Furthermore, 2-DG is used to inhibit the glycolysis.

3. To identify the underlying mechanism of NNMT regulation the AMPK and mTOR signaling pathways are also investigated.

    a. Therefore, the AMPK is activated using AICAR and mTOR is inhibited with rapamycin.

    b. Since both signaling pathways are important for autophagy induction, NNMT expression is also analyzed using autophagy inhibitors.

2 Material

2.1 Reagents and chemicals

Chemical	Manufacturer	Head Office
0.25 % Trypsin-EDTA	Life Technologies	Karlsruhe, Germany
1,4-Dithiothreit (DTT)	Roth	Karlsruhe, Germany
2-Mercaptoehtanol	Sigma-Aldrich	München, Germany
32 % Hydrochloric acid solution	Roth	Karlsruhe, Germany
Acrylamide solution (30%)	Sigma-Aldrich	München, Germany
Ammonium persulfate (APS)	Sigma-Aldrich	München, Germany
Aprotinin	Sigma-Aldrich	München, Germany
Aqua ad injectable	B. Braun	Melsungen, Germany
ATX Ponceau S red staining solution	Sigma-Aldrich	München, Germany
Bicinchoninic acid assay	Sigma-Aldrich	München, Germany
Bromophenol blue	Roth	Karlsruhe, Germany
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	München, Germany
EDTA (0.5 M solution)	Ambion	Carlsbad, USA
Ethanol absolute	Sigma-Aldrich	München, Germany
Ethanol for molecular biology	AppliChem	Darmstadt, Germany
Glycerol	Sigma-Aldrich	München, Germany
Glycine	Roth	Karlsruhe, Germany
IGEPAL® CA-630	Sigma-Aldrich	München, Germany
Isopropyl alcohol	Sigma-Aldrich	München, Germany
Leupeptin	Sigma-Aldrich	München, Germany
Methanol	Sigma-Aldrich	München, Germany
Methylthiazolyldiphenyl-tetrazolium bromide (MTT)	Sigma-Aldrich	München, Germany
Non-fat dry milk powder	Cell Signaling	Frankfurt, Germany
NucleoZOL	Macherey-Nagel	Dürren, Germany
Oil Red O (ORO)	Sigma-Aldrich	München, Germany
PBS tablets	Sigma-Aldrich	München, Germany

Chemical	Manufacturer	Head Office
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich	München, Germany
Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standards	Bio-Rad	München, Germany
Restore Western Blot Stripping Buffer	Thermo Fisher Scientific	Waltham, USA
RNase AWAY®	Molecular Bioproducts	Waltham, USA
Sodium chloride (NaCl)	Sigma-Aldrich	München, Germany
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich	München, Germany
Sodium fluoride (NaF)	Sigma-Aldrich	München, Germany
Sodium hydroxide (NAOH)	Sigma-Aldrich	München, Germany
Sodium orthovanadate (Na_3VO_4)	Sigma-Aldrich	München, Germany
Sodium pyruvate (SP)	Sigma-Aldrich	München, Germany
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich	München, Germany
Tris-Pufferan	Roth	Karlsruhe, Germany
Trizma®-hydrochlorid	Sigma-Aldrich	München, Germany
Tween® 20	Sigma-Aldrich	München, Germany
UltraPure™ DNase/RNase-Free Distilled Water	Invitrogen	Carlsbad, USA
Western Lighting Plus ECL	Perkin Elmer	Waltham, USA

2.2 Antibodies

The secondary anti-rabbit horseradish-linked antibody from Cell Signaling, Cambridge, UK (#7074) was used in the indicated dilutions.

Antibody	Product number	Dilution primary AB	secondary AB	Manufacturer	Head Office
NNMT	15123-1-AP	1:1000	1:5000	Proteintech	Rosemont, USA
actin	ab8227	1:10000	1:10000	abcam	Cambridge, UK
p62	#39149	1:1000	1:5000	Cell Signaling	Cambridge, UK
P-p70 S6K	#9205	1:1000	1:5000	Cell Signaling	Cambridge, UK
AMPK	#5832	1:5000	1:5000	Cell Signaling	Cambridge, UK
P-AMPK	#2535	1:1000	1:5000	Cell Signaling	Cambridge, UK
LC3	14600-1-AP	1:2000	1:5000	Proteintech	Rosemont, USA

2.3 Reaction kits

Kit	Manufacturer	Head Office
Hexokinase Activity Assay Kit (Colorimetric)	Abcam	Cambridge, UK
High-Capacity cDNA Reverse Transcription Kit	Applied Systems	Foster City, USA
iScript™ gDNA Clear cDNA Synthesis Kit	Bio-Rad	München, Germany
MycoAlert™	Lonza	Basel, Switzerland
NucleoSpin® RNA	Macherey Nagel	Düren, Germany
NucleoSpin® RNA Set for NucleoZol	Macherey Nagel	Düren, Germany
SsoAdvancedTM Universal SYBR® Green Supermix	Bio-Rad	München, Germany

2.4   Primers

The primers were purchased from Bio-Rad (München, Germany). They were design as unique assays by Bio-Rad and used in the qPCR with SsoAdvancedTM Universal SYBR® Green Supermix.

Gene	Gene Symbol	Unique Assay ID
Actin, beta	*Actb*	qMmuCED0027505
Eukaryotic translation elongation factor 1 alpha 1	*EEF1A1*	qHSaCED0020436
GLUT4 (human)	*SLC2A4*	qHsaCED0047488
GLUT4 (mouse)	*Slc2a4*	qMmuCED0024734
Importin 8	*IPO8*	qHSaCED0056515
Nicotinamide N-methyltransferase (human)	*NNMT*	qHSaCED0046403
Nicotinamide N-methyltransferase (mouse)	*Nnmt*	qMmuCED0047226
Peptidylprolyl isomerase A (cyclophilin A)	*PPIA*	qHsaCED0038620

2.5   Activators and inhibitors

Substance	Manufacturer	Head Office
2-DG	Sigma-Aldrich	München, Germany
AICAR	Cayman Chemicals	Ann Arbor, USA
Bafilomycin A1	AdipoGen Life Science	San Diego, USA
Chloroquine	Sigma-Aldrich	München, Germany
Cycloheximide	Sigma-Aldrich	München, Germany
DHEA	Sigma-Aldrich	München, Germany
MHY-1485	Sigma-Aldrich	München, Germany
Phloretin	Sigma-Aldrich	München, Germany
Physcion	Cayman Chemicals	Ann Arbor, USA
Rapamycin	AdipoGen Life Science	San Diego, USA

2.6 Cell culture

2.6.1 Cell line

The cell line 3T3-L1 was established from disaggregated Swiss albino mouse embryos (Todaro and Green, 1963; Todaro et al., 1965). The cells can convert from pre-adipocytes to adipocytes, thereby pass from a rapidly dividing to a confluent and contact inhibited state. With serum containing media the fat accumulation is enhanced (Green and Meuth, 1974).

2.6.2 Additives

Additive	Manufacturer	Head Office
3-Isobutyl-1-methylxanthine (IBMX)	Sigma-Aldrich	München, Germany
Dexamethasone (Dexa)	Sigma-Aldrich	München, Germany
FBS Superior	Biochrom	Berlin, Germany
Indomethacin	Sigma-Aldrich	München, Germany
Insulin (Ins)	Sigma-Aldrich	München, Germany
NBCS	Gibco	Karlsruhe, Germany
Rosiglitazone	Sigma-Aldrich	München, Germany

2.6.3 Cell culture media

Medium	Manufacturer	Head Office
DMEM no glucose, glutamine	Gibco	Karlsruhe, Germany
DMEM, GlutaMAX™, high glucose, pyruvate	Gibco	Karlsruhe, Germany
DMEM, no glucose, no glutamine, no phenol red	Gibco	Karlsruhe, Germany

2.6.4 Media formulations

Medium	Composition	
0 mg/ml Glucose		
DMEM, no glucose	10 %	FBS
	1 mM	Sodium pyruvate
	1 µM	Insulin
1.5 mg/ml		
DMEM, no glucose	10 %	FBS
	1 mM	Sodium pyruvate
	1 µM	Insulin
	1.5 mg/ml	Glucose
Basic		
DMEM, GlutaMAX™	10 %	NBCS
Differentiation/4.5 mg/ml		
DMEM, GlutaMAX™	10 %	FBS
	1 µM	Insulin
Freezing		
DMEM, GlutaMAX™	20 %	NBCS
	10 %	DMSO
Induction		
DMEM, GlutaMAX™	10 %	FBS
	1 µM	Insulin
	100 nM	Dexamethasone
	1 µM	Rosiglitazone
	500 µM	IBMX
	250 µM	Indomethacin

2.7   Buffers and Solutions

Buffer/Solution	Composition	
4 x Leammli	0.25 M	Tris-HCl
	0.4 M	DTT
	8 %	SDS
	40 %	Glycerol
	0.004 %	Bromophenol blue
1 x TBS-T		
1 x TBS	0.05 %	Tween® 20
10 x TBS	0.2 M	Tris-Pufferan
	1.49 M	NaCl
10 x Running buffer	0.25 M	Tris-Pufferan
	1.918 M	Glycine
	1 %	SDS
10 x Transfer buffer	0.25 M	Tris-Pufferan
	1.918 M	Glycine
Blocking buffer		
1 x TBS-T	5 %	Non-fat dry milk powder
Lysis buffer	20 mM	Tris-HCl
	5 mM	EDTA
	10 mM	Na_3VO_4
	100 mM	NaF
	1 %	Igepal CA-630
	1 mM	PMSF
	1 µM	Aprotinin
	10 µM	Leupeptin

Buffer/Solution	Composition	
MTT		
Stock	5 mg	MTT
	1 ml	DMEM no phenol red
Working	1:10	Stock in DMEM no phenol red
ORO		
Stock	0.5 %	ORO
	100 %	isopropyl alcohol
Working	0.2 %	in ddH2O
PBS pH 7,4	2.7 mM	KCl
	13.7 mM	NaCl
TBS-T pH 7.4	0.2 mM	Tris-Pufferan
	1.49 mM	NaCl
	0.05 %	Tween 20
Transfer buffer	0.25 mM	Tris-Pufferan
	1.918 mM	Glycin
	20 %	Methanol

2.8   Instruments

Instrument	Manufacturer	Head Office
Accu-jet® pro	Brand	Wertheim, Germany
Analytic scale AE240	Mettler-Toledo	Columbus, USA
Analytic scale PM6100	Mettler-Toledo	Columbus, USA
CO_2 Incubator	Binder	Tuttlingen, Germany

Instrument	Manufacturer	Head Office
CO_2 Incubator	Binder	Tuttlingen, Germany
Criterion Cell	Bio-Rad	München, Germany
Ecomax X Ray Film Processor	Protec GmbH	Oberstenfeld, Germany
Electrophoresis- and blotting chamber	Bio-Rad	München, Germany
Hemocytometer Neubauer	Paul Marienfeld GmbH	Lauda, Germany
Laminar flow type 18	Heraeus	Hanau, Germany
Mastercycler® Gradient	Eppendorf	Hamburg, Germany
Microscope Wilovert 30	Hund	Wetzlar, Germany
Mini Centrifuge	LMS Co.	Tokyo, Japan
Mulitipette®stream	Eppendorf	Hamburg, Germany
NanoDrop2000c spectrophotometer	Thermo Fisher Scientific	Waltham, USA
Optical step tablet	Biostep	Burkhardtsdorf, Germany
Orbital shaker	Heidolph	Schwabach, Germany
Perfect Spin	Peqlab-VWR	Radnor, USA
pH-meter WTW series	inolab	Weilheim, Germany
Pipettes Research® Plus	Eppendorf	Hamburg, Germany
Plate reader UVM 340	ASYS	Dornstadt, Germany
PowerPac® HC	Bio-Rad	München, Germany
Real Time PCR Detection System CFX Connect	Bio-Rad	München, Germany
Thermomixer comfort	Eppendorf	Hamburg, Germany
ULTRA TURRAX® T25 digital	IKA	Staufen, Germany
Vortex-Genie®2	Scientific Industries	Bohemia, USA
Water bath	Memmert	Schwabach, Germany
X-Ray Cassette	Perlux	Calbe, Germany

2.9 Consumables

Consumable	Manufacturer	Head Office
6-well plates	Corning	New York, USA
96-well plate	Sarstedt	Nümbrecht, Germany
Cell culture plates 15 cm	Greiner Bio-One	Frickenhausen, Germany
Cell scraper	Sarstedt	Nümbrecht, Germany
Empty cassettes	Bio-Rad	München, Germany
Filter Bottle Top	Sarstedt	Nümbrecht, Germany
Microseal® 'C' Film	Bio-Rad	München, Germany
Millex-GV 0,22 µM	Merck	Darmstadt, Germany
Nitrocellulose Membrane 0.2 µM	Amersham-GE Healthcare	Buckinghamshire, UK
Nunc™ Cryovails	Thermo Fisher Scientific	Waltam, USA
PCR Tubes 0.2 mL single/8-strip	Eppendorf	Hamburg, Germany
PCR-Plates, 96 well	Bio-Rad	München, Germany
Pipette filter tips	Sarstedt	Nümbrecht, Germany
Pipette tips	Sarstedt	Nümbrecht, Germany
Pipettes serological	Greiner Bio-One	Frickenhausen, Germany
Polypropylene tubes 15 ml / 50 ml	Greiner Bio-One	Frickenhausen, Germany
SafeSeal micro tubes 1.5 ml / 2.0 ml	Sarstedt	Nümbrecht, Germany
Syringes	BD Biosciences	Heidelberg, Germany
Thick Blot Filter Paper	Bio-Rad	München, Germany
X-ray film Fujifilm Super RX-N	Fujifilm	Tokio, Japan

3 Methods

3.1 Cell culture

3T3-L1 preadipocytes were obtained from the American Type Culture Collection via LGC Standards and maintained at 37°C with 5% CO_2 in a humidified atmosphere. A mycoplasma contamination was excluded by MycoAlert™. Medium was changed every 2 to 3 days. The preadipocytes were kept in basic medium containing DMEM GlutaMAX with 4.5 mg/ml glucose and 1 mM sodium pyruvate, supplemented with 10 % newborn calf serum (NBCS). Cells were cultivated in 15 cm cell culture dishes and passaged when 80 % confluence were reached. For differentiation, 8500 cells/cm^2 were seeded on 6-well plates and kept in basic growth medium. Two days after confluence, differentiation into adipocytes was induced using DMEM, 10 % FBS, 1 µmol/l insulin, 100 nmol/l dexamethasone, 1 µmol/l rosiglitazone, 500 µmol/l IBMX, and 250 µmol/l indomethacin. After three days the medium was replaced with DMEM GlutaMAX, 10 % fetal bovine serum (FBS), and 1 µmol/l insulin, and exchanged every 2 days thereafter. Experiments were performed at least 20 days after induction. The cells were treated with substances, explained in section 3.3. Except where noted otherwise, the duration of the treatment was 10 days.

3.2 Freezing and thawing of 3T3-L1 adipocytes

3T3-L1 cells were removed from the 15 cm cell dish by using 0.25 % Trypsin-EDTA solution. After determining the cell number, the preadipocytes were centrifuged for 5 min at 1200 rpm. The cell pellet was resuspended in freezing medium containing 1 x 10^6 cells per ml and pipetted into cryotubes. These were kept in a cryo-freezing container with isopropyl alcohol and immediately stored at -80 °C for 24 h. For long time storage the cells were transferred into liquid nitrogen.

To thaw the preadipocytes the cryotube was kept in 37 °C water bath. The 3T3-L1 cells were immediately transferred into a 15 cm culture dish with 25 ml warmed basic medium. Overnight the cells adhered, and the medium was replaced to remove the remaining DMSO.

3.3   Cell stimulation and treatment

3.3.1   Pro-inflammatory signals

For the stimulation with pro-inflammatory signals, differentiated 3T3-L1 adipocytes were kept in FBS-free medium overnight and then treated in serum free medium with IL-6, TNF-α, and leptin for 24 h. The used cells were at least 10 d in differentiation media until they were fully differentiated. In Table 3.1 the concentrations of the pro-inflammatory signals are mentioned.

Table 3.1:   **Concentrations of pro-inflammatory signals in cell culture.**

Substance	Concentration
IL-6	1, 10, 10 ng/ml
TNF-α	1, 4, 8 ng/ml
Leptin	0.1, 1, 10 ng/ml

3.3.2   Inhibitors and activators

The substances phloretin, 2-DG, rapamycin, AICAR, and cycloheximide were used in culture medium with 4.5 mg/ml glucose and 10 % FBS. The chemicals MHY1485, bafilomycin A1, and chloroquine were applied in no glucose medium with 10 % FBS. For all experiments stable differentiated cells were used. They were at least 20 d in differentiation medium In Table 3.2 the substances and their concentration are mentioned.

Table 3.2:   **Concentration of inhibitors and activators in cell culture.**

Substance	Concentration	Unit
Phloretin	5, 50, 100	µM
2-DG	1	mM
Rapamycin	100, 500	nM
AICAR	1	mM
MHY1485	10	µM
Bafilomycin A1	20	nM
Chloroquine	25	µM
Cycloheximide	5	µM

3.4   Western blotting

3.4.1   Protein extraction

All handling during the protein isolation was done on ice. First cells were washed 2 times with cold 1 x PBS. Then the adipocytes were harvested by mechanical disruption in 250 µl lysis buffer and transferred into 1.5 ml reaction tube. After an incubation for 10 min on ice, followed by a centrifugation for 20 min at 13 500 rpm (4 °C), total protein content was determined using the bicinchoninic acid assay. 30 µg of total protein was diluted and prepared with 4 x Laemmli buffer for the separation in a 6 - 15% gradient sodium dodecyl sulfate polyacrylamide gel.

3.4.2   SDS-Page (sodium dodecyl sulfate polyacrylamide gel electrophoresis)

A denaturing SDS-Page was used for separation. The SDS gels were prepared according to the indicated formulae in Table 3.3. First the 6 -1 5 % gradient separation gel was cast into Criterion Empty Cassettes. After polymerization the 4 % stacking gel was cast with the formulation in Table 3.3. Before loading the samples on the polymerized gel, they were boiled at 95 °C for 5 min. To determine the protein size the Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standard was used. The electrophoresis was performed with 1 x running buffer, at 100 V for 2 – 3 h.

Table 3.3:   **Formulae for two SDS gels.**

	Stacking Gel	Resolving Gel	
	4 %	6 %	15 %
30 % Acrylamide/bis	1.320 ml	3 ml	7.5 ml
0.5 M Tris-HCl, pH 6.8	2.52 ml	-	-
1.5 M Tris-HCl, pH 8.8	-	3.75 ml	3.75 ml
10 % SDS	100 µl	150 µl	150 µl
diH$_2$O	6 ml	8 ml	3.5 ml
TEMED	10 µl	7.5 µl	7.5 µl
10 % APS	50 µl	75 µl	75 µl
Total Volume	10 ml	15 ml	15 ml

3.4.3 Western blot

The separated proteins were transferred to nitrocellulose membrane (0.2 µm) by wet tank western blot. The transfer was performed in 1 x transfer buffer with 20 % methanol at 55 mA for 1 h. Then the blots were stained with Ponceau to control a successful transfer. After documentation, the membrane was washed in TBS-T and blocked in 5 % non-fat dry milk. All used primary antibodies were polyclonal and incubated at 4 °C overnight. The exact dilution is shown in section 2.2. Afterwards, the membrane was washed 3 times for 10 min in TBS-T. The horse radish peroxidase-linked secondary antibody was diluted, as indicated in section 2.2, in 5% non-fat dry milk and incubated for 1 h at room temperature. Subsequently, the blots were again washed 3 times for 10 min in TBS-T. The peroxidase reaction was initiated by an incubation in Western Lighting Plus ECL for 1 min. Then the blots were developed on X ray films by the Ecomax X Ray Film Processor. The western blot bands were analyzed using a transparency scanner and ImageJ software (NIH) after calibration with an optical step tablet. Beta actin was used as loading control.

3.5   qPCR (quantitative polymerase chain reaction)

3.5.1 Cell culture

RNA from cell culture was extracted using NucleoSpin® RNA according to the manufacturer's instructions. RNA concentration and purity were measured with NanoDrop. Afterwards, 1 µg total RNA was reverse-transcribed with the High-Capacity cDNA Reverse Transcription (RT) Kit. Therefore, the total RNA was diluted in RNase free water in 10 µl total. Then 10 µl prepared RT master mix was added to every reaction. The composition of it is indicated in Table 3.5. After a short centrifugation, the samples were transferred into the Eppendorf Mastercycler Gradient. The used running protocol is shown in Table 3.4.

Table 3.4:   **Running protocol for the RT-PCR.**

Settings	Step 1	Step 2	Step 3	Step 4
Temperature	25 °C	37 °C	85 °C	4 °C
Time	10 min	80 min	5 min	Hold

Table 3.5:   Components of the RT-PCR master mix.

Component	Volume [µl]
10 x RT Buffer	2.0
25 x dNTP Mix (100 mM)	0.8
10 x RT Random Primers	2.0
MultiScribe™ Reverse Transcriptase	1.0
RNase free water	4.2
Total per reaction	10.0

After the cDNA synthesis the 20 µl cDNA was diluted with 80 µl RNase free water and frozen in smaller aliquots. The qPCR was performed with SsoAdvancedTM Universal SYBR® Green Supermix and PrimePCR SYBR® Green Assays for NNMT (qMmuCED0047226), GLUT4 (qMmuCED0024734), and beta actin (qMmuCED0027505) using the protocol in Table 3.6. The detailed composition of one qPCR reaction is show in Table 3.7. NNMT and GLUT4 expression was normalized to beta actin.

Table 3.6:   Running protocol of the qPCR

Step	Temperature	Time	Number of Cycles
Activation	95 °C	2 min	1
Denaturation	95 °C	5 sec	40
Annealing/extension	60 °C	35 sec	
Melt curve	65 ° C to 95 °C (0.5 °C increments)	5 sec/step	1

Table 3.7:   Components of the qPCR reaction.

Component	Volume [µl]
SsoAdvancedTM Universal SYBR® Green Supermix	10.0
PrimePCR SYBR® Green Assays	1.0
RNase free water	4.0
cDNA	5.0
Total per reaction	20.0

3.5.2 Human adipose tissue

RNA from human adipose tissue was extracted with NucleoZOL. Approximately 200 mg of fat tissue was given in a 2.0 ml reaction tube and homogenized in 500 µl NucleoZOL using an ultra turrax. Afterwards, the samples were centrifuged for 5 min at 12 000 x *g*. The fat layer on top was removed and the supernatant was transferred into a new 2.0 ml reaction tube. Then 200 µl RNase free water was added to the sample and vigorously mixed for 15 s. After 15 min incubation at room temperature the samples were centrifuged at 12 000 x *g* for 15 min. 500 µl of the supernatant were transferred into a new tube by not disturbing the DNA/protein pellet. Now the RNA is extracted by the NucleoSpin® RNA Set for NucleoZOL according to manufacturer's instructions. The concentration and purity of the eluted RNA was measured by NanoDrop and 0.5 µg total RNA was reversed-transcribed with iScript™ gDNA Clear cDNA Synthesis Kit. First 14 µl total RNA was digested with 0.5 µl iScript DNase in 1.5 µl iScript DNase buffer per reaction. The DNase reaction protocol is indicated in Table 3.8. Then 4 µl iScript Reverse Transcription Supermix is added to the 16 µl DNase-treated RNA template and finial reversed-transcribed using the protocol in Table 3.9. The qPCR was performed with SsoAdvancedTM Universal SYBR® Green Supermix and PrimePCR SYBR® Green Assays for human NNMT and GLUT4. As reference genes served IPO8, cyclophilin A, and EEF1A1.

Table 3.8:   **DNase reaction protocol for the iScript™ gDNA Clear cDNA Synthesis Kit.**

Step	Temperature	Time
DNA digestion	25 °C	5 min
DNase inactivation	75 °C	5 min
Storage conditions	4 °C	Until RT step

Table 3.9:   **Running protocol of the reverse transcription with the iScript™ gDNA Clear cDNA Synthesis Kit.**

Step	Temperature	Time
Priming	25 °C	5 min
Reverse transcription	46 °C	20 min
Inactivation	95 °C	1 min
Hold	4 °C	∞

3.6 Quantitative Oil Red O staining

This staining was performed based on the protocol of (Kraus et al., 2016). In detail, the cells were washed 2 times with 1 ml 1 x PBS. Before fixation, the cells were removed, like shown in Figure 3.1. Then the cells were fixed using 1 ml 4 % PBS-buffered formalin for 15 min at room temperature. The fixed adipocytes were stained with 1.25 ml per well ORO working solution and incubated for 30 min. After removing the staining solution, the cells were washed 5 times with ddH$_2$O. 100 % isopropyl alcohol was used for dye elution. After 10 min on an orbital shaker the ORO was solved and 200 µl were transferred in duplicates in a 96-well plate. The absorption was measured at 510 nm.
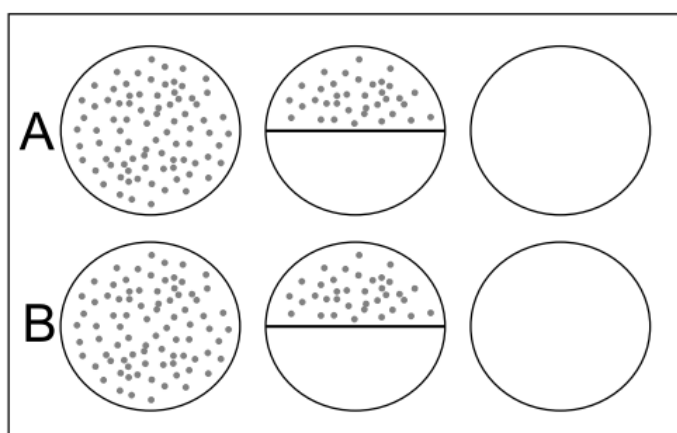
Figure 3.1   **Shema for Oil Red O staining.**
50 % and 100 % of the cells were removed from the wells to show an equal differentiation of the adipocytes.

3.7 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid)

To investigate the viability of cells after treatment, the MTT assay was performed. The yellow MTT working solution is converted into the dark blue, water insoluble MTT formazan by the mitochondrial dehydrogenases of living cells. The differentiated adipocytes were stimulated with the different substances for the longest experimental time. Afterwards, the cells were washed one time with warm, sterile 1 x PBS and incubated with the 1 ml sterile MTT working solution for 3 h at 37 °C with 5 % CO$_2$ in a humidified atmosphere. Then the MTT solution was removed and the MTT formazan is resolved in 1 ml 40 mM HCl isopropyl alcohol on an orbital shaker for 10 min. Afterwards, the solved formazan is transferred into 1.5 ml reaction tube and

centrifuged at 13 000 rpm for 2 min. 100 µl from the supernatant were measured in 96-well plate at 570 nm and 650 nm (reference). The viability is normalized to the control conditions and quantified as percentage. Due to the long-time treatment and the needed autophagy induction, a slight cytotoxicity was always expected. To categorize the cytotoxicity the ISO-10993-5[1] was used as an orientation (Table 3.10).

Table 3.10:   Cytotoxicity categories according to ISO-10993-5.

Cell viability [%]	Valuation
100 – 80	non-cytotoxic
79 – 60	weak cytotoxic
59 – 40	moderate cytotoxic
39 – 0	strong cytotoxic

3.8   Hexokinase activity assay

The colorimetric hexokinase assay kit from abcam (ab136957) is used to measure the production of NADH during the oxidation of glucose-6-phosphate or of 2-DG-6-phosphate in the 2-DG treated adipocytes. The assay is performed according to manufacturer's instructions. Only the sample preparation was modified. Briefly, the cells were lysed in 200 µl ice cold assay buffer, homogenized by pipetting, and centrifuged 5 min at 4 °C at 12 000 rpm. The protein content was analyzed by bicinchoninic acid assay and 40 µg of total protein was used per assay reaction. After performing the assay according to the manufacturer's instructions, the reaction was measured at 20 min and 60 min. The hexokinase activity was calculated according the manufacturer`s instructions, too.

---

[1] Biological evaluation of medical devices - Part 5: Tests for in vitro cytotoxicity (ISO 10993-5:2009); German version EN ISO 10993-5:2009

3.9   Data analysis

Data representing mean ± SEM were analyzed by Wilcoxon test or Kruskal-Wallis test. The analyzes and graph design were performed using R software. Differences with $p < 0.05$ were considered to be statistically significant.

4 Results

4.1 Pro-inflammatory Signals and NNMT regulation

4.1.1 NNMT expression in human adipose tissue

The adipose tissue itself produces a lot of pro-inflammatory signals (Gregoire et al., 1998; Klein et al., 2006) and they contribute to a chronic low-level inflammation, so called metaflammation, in obese adipose tissue (Gregor and Hotamisligil, 2011). IL-6 stimulates via STAT3 activation NNMT expression in HepG2, colon cancer cells (Tomida et al., 2008), 3T3-L1 adipocytes (Balhoff and Stephens, 1998), and in human airway smooth muscle cells (Robinson et al., 2015). These signals could also be associated with NNMT up-regulation in the subcutaneous white adipose tissue of obese, non-diabetic Pima Indians compared to the non-diabetic control group (Lee et al., 2005). Therefore, the NNMT and GLUT4 mRNA levels is investigated in adipose tissue of lean and obese patients.
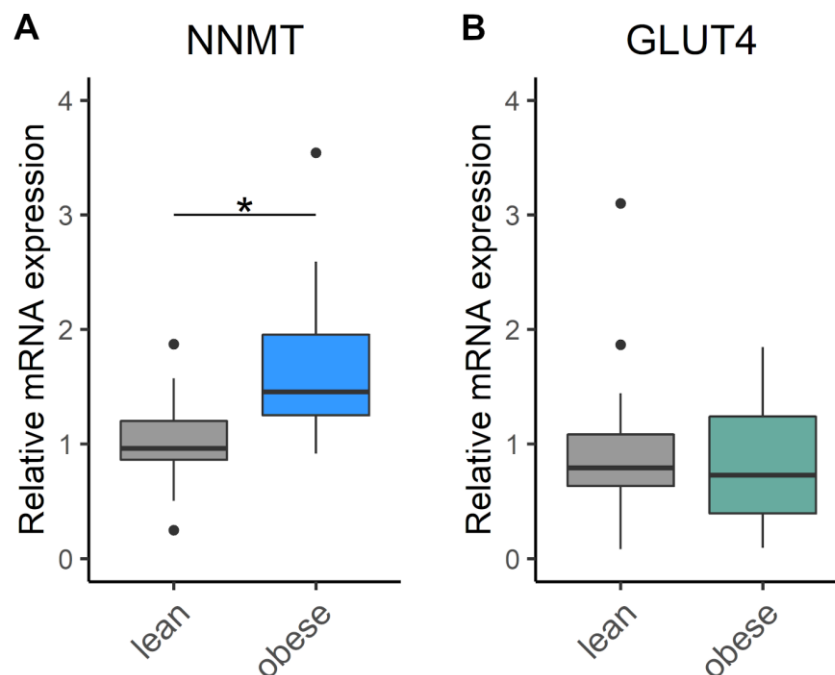
Figure 4.1: **NNMT and GLUT4 expression in human adipose tissue.**
NNMT **(A)** and GLUT4 **(B)** mRNA expression of obese and the lean control group. The expressions were normalized to three reference genes (IPO8, cyclophilin A, and EEFA1A). The relative expression is shown as boxplots (n = 12). *$p < 0.05$.

The NNMT expression was significantly enhanced in obese individuals compared to the control group (Figure 4.1 A), whereas the GLUT4 expression is not affect by obesity (Figure 4.1 B). As mentioned above, the adipose tissue is a source of many pro-inflammatory signals including IL-6 and TNF-α (Klein et al.,2016). In the following these inflammatory cytokines that are critical for the development of T2D and obesity are analyzed. Furthermore, an also important adipokine (leptin) is investigated.

4.1.2 Interleukin 6

The multifunctional cytokine IL-6 is secreted by the adipose tissue, whereby it contributes to approximately 30 % of systemic IL-6 in humans (Klein et al., 2006). It impairs insulin signaling and action by reducing insulin-stimulated glucose uptake (Rotter et al., 2003; Shi et al., 2019). To identify a possible regulation of NNMT by pro-inflammatory signals, differentiated 3T3-L1 cells were incubated with three different IL-6 concentrations in FBS-free media for 24 h.
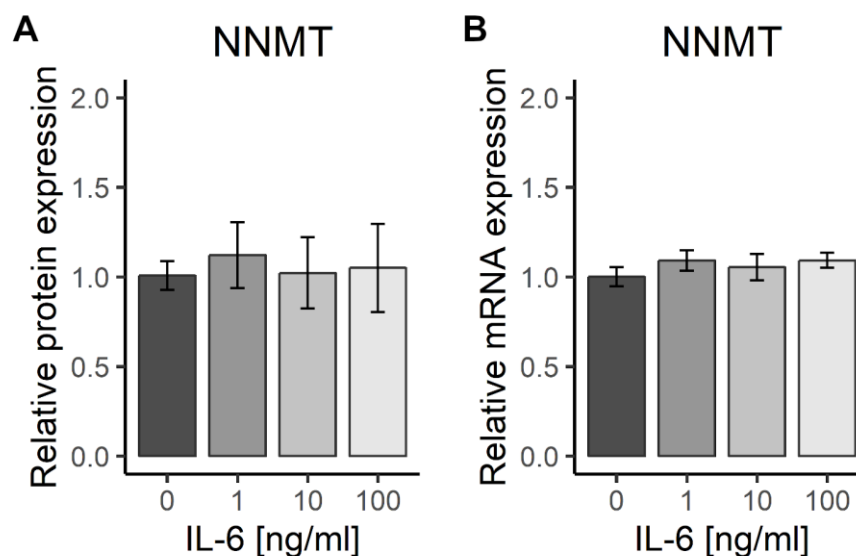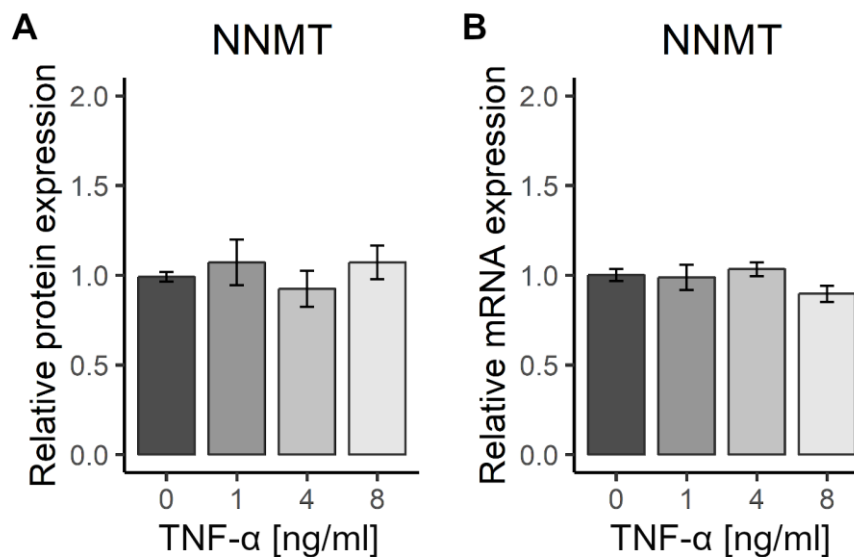

Figure 4.2: **NNMT expression after stimulation with IL-6.**
(A) NNMT relative normalized protein expression and (**B)** NNMT relative normalized mRNA expression of 3T3-L1 adipocytes treated with IL-6 (1, 10, 100 ng/ml) in FBS-free media for 24 h. Data represents relative NNMT mRNA and protein expression normalized to the untreated control and beta actin with standard error of mean (SEM) (n = 3).

Figure 4.2 shows the protein content analyzed by western blot and the mRNA expression of NNMT measured by qRT-PCR. After a 24 h IL-6 stimulation, no difference in NNMT expression was observed even at high concentrations.

4.1.3 Tumor necrosis factor α

TNF-α, a member of TNF family, is produced by macrophages in adipose tissue and is involved in different metabolic disorders (Klein et al., 2006; Shi et al., 2019). Circulating TNF-α concentrations are associated with impaired glucose tolerance and an enhanced T2D risk in humans (Barra et al., 2010; Sun and Liu, 2015). Therefore, 3T3-L1 adipocytes were treated with TNF-α for 24 h. Similar to the IL-6 results, the protein and mRNA levels of NNMT were not affected by the treatment (Figure 4.3).

Figure 4.3: **NNMT expression after stimulation with TNF-α.**
(A) NNMT relative normalized protein expression and **(B)** NNMT relative normalized mRNA expression of 3T3-L1 adipocytes treated with TNF-α (1, 4, 8 ng/ml) in FBS-free media for 24 h. Data represents relative NNMT mRNA and protein expression normalized to the untreated control and beta actin with standard error of mean (SEM) (n = 3).

4.1.4 Leptin

The adipose tissue produces a lot of endocrine signals called adipokines (Stern et al., 2016) and leptin is one of these adipocyte-derived hormones (Klein et al., 2006). It regulates the energy homeostasis through the central nervous system (Pérez-Pérez et al., 2017; Zhang et al., 1994). In addition, it exerts paracrine and autocrine effects on adipocytes (Kraus et al., 2002). Interestingly, the metabolic disorders obesity and T2D showed increased leptin levels

(Shi et al., 2019). Furthermore, higher leptin concentration positively correlated with adipose mass (Wannamethee et al., 2007). It has got another important characteristic: Leptin signals are transmitted by the JAK/STAT cascade (Kraus et al., 2002), and this pathway is also activated by IL-6 in 3T3-L1 cells (Balhoff and Stephens, 1998). The effect of leptin on NNMT expression is analyzed in Figure 4.4.
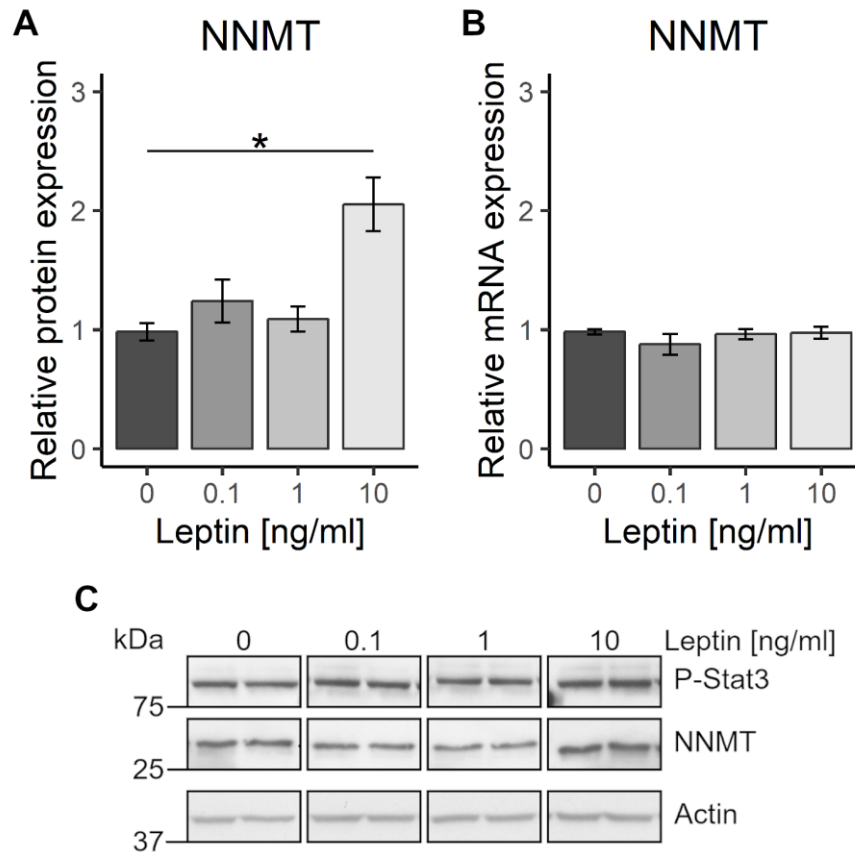

Figure 4.4: **NNMT expression after stimulation with the adiponectin leptin.**
(A) NNMT relative normalized protein expression and **(B)** NNMT relative normalized mRNA expression of 3T3-L1 adipocytes treated with leptin (0.1, 1, 10 ng/ml) in FBS-free media for 24 h. **(C)** Representative western blot out of three independent experiments. Data represents relative NNMT mRNA and protein expression normalized to the untreated control and beta actin with standard error of mean (SEM) (n = 3). *p < 0.05.

After 24 h stimulation, 10 ng/ml leptin significantly elevated the protein level of NNMT (Figure 4.4 A, C). Additionality, the western blot verified that STAT3 is phosphorylated by leptin treatment (Figure 4.4 C). However, the mRNA expression of NNMT did not change as Figure 4.4 B demonstrated. Taken together, it is more obvious that NNMT is regulated in adipocyte cell culture by another mechanism than by pro-inflammatory signals and the JAK/STAT signaling pathway.

4.2   Establishment of 3T3-L1 cell culture for glucose deprivation

4.2.1   Cultivation of stable-differentiated 3T3-L1 adipocytes

A typical marker for 3T3-L1 adipocytes are the lipid droplets that are generated by triacylglycerol synthesis and storage after glucose uptake (Czech et al., 2013). This stored energy, in form of triacylglycerol, is mobilized during energy deprivation (Gregoire et al., 1998). To analyze the effect of glucose starvation in differentiated 3T3-L1 cells it is necessary that they do not de-differentiate into fibroblasts. In literature numerous time points for a complete differentiation are specified. They vary from 7 to 15 d after induction (Green and Meuth, 1974; Zebisch et al., 2012).

Therefore, the stability of differentiation is investigated. In detail, the preadipocytes were kept for 3 d in induction media to induce the differentiation into adipocytes. Afterwards, the 3T3-L1 cells were cultivated for 7 d (short-time) and for 17 d (long-time) in differentiation media. Then these adipocytes were cultivated in 4.5 mg/ml, 1.5 mg/ml, and 0 mg/ml glucose for 10 d. The short differentiation time led to unstable adipocytes in media without glucose, whereas the longer differentiation time did not affect the overall lipid content, that was measured by quantitative ORO staining (Figure 4.5). The method is described in subsection 3.6.
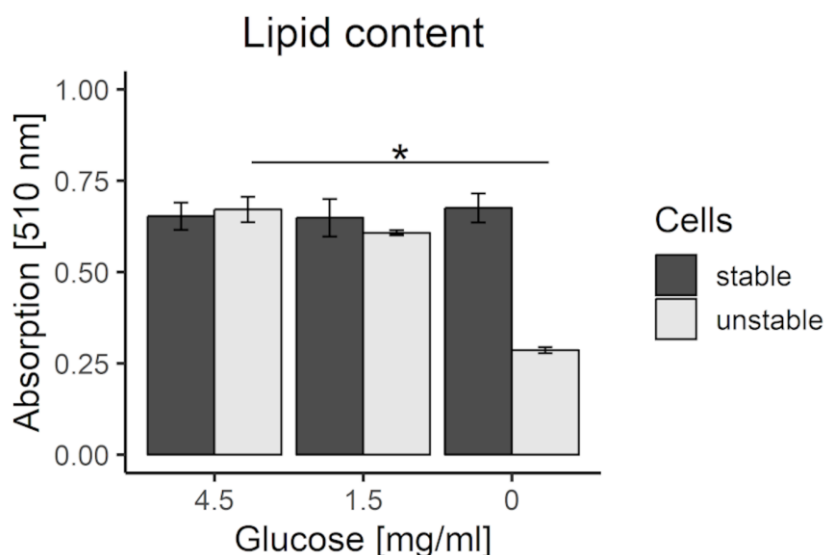
Figure 4.5:   **Quantitative ORO staining after 10 d glucose deprivation.**
All cells were kept for 3 d in induction medium. The unstable adipocytes were cultivated for a short time (7 d) in differentiation medium, whereas the stable adipocytes were kept for a longer time (17 d) in differentiation medium. Afterwards, the adipocytes were incubated in 4.5 mg/ml, 1.5 mg/ml, and 0 mg/ml glucose media. The absorption is shown as mean values with standard error of mean (SEM) (n = 2).

The cultivation of unstable differentiated cells without glucose led to de-differentiation, represented by a decrease of stained lipid droplets. Even 1.5 mg/ml glucose led to an appreciable lipid loss in unstable cells. In contrast to these cells, the stable differentiated 3T3-L1 adipocytes were not affected by glucose deprivation. Therefore, all experiments were performed with long-time differentiated cells.

4.2.2 Differentiation in various FBS batches

To ensure that the results are reproducible and valid, the differentiation of 3T3-L1 cells have to be uniform and stable during the used passages. Before differentiation, these cells have a fibroblast-like morphology. After a successful differentiation they accumulated the typical lipid droplets. However, this requires a suitable protocol. One important factor is the right FBS for the cell culture (Baker, 2016), beside the differentiation time (section 4.2.1). Therefore, the performance of various FBS batches were initially investigated. The examined FBS batches are shown in Table 4.1. 10 d after induction the differentiation of one passage was analyzed my light microscopy (Figure 4.6).
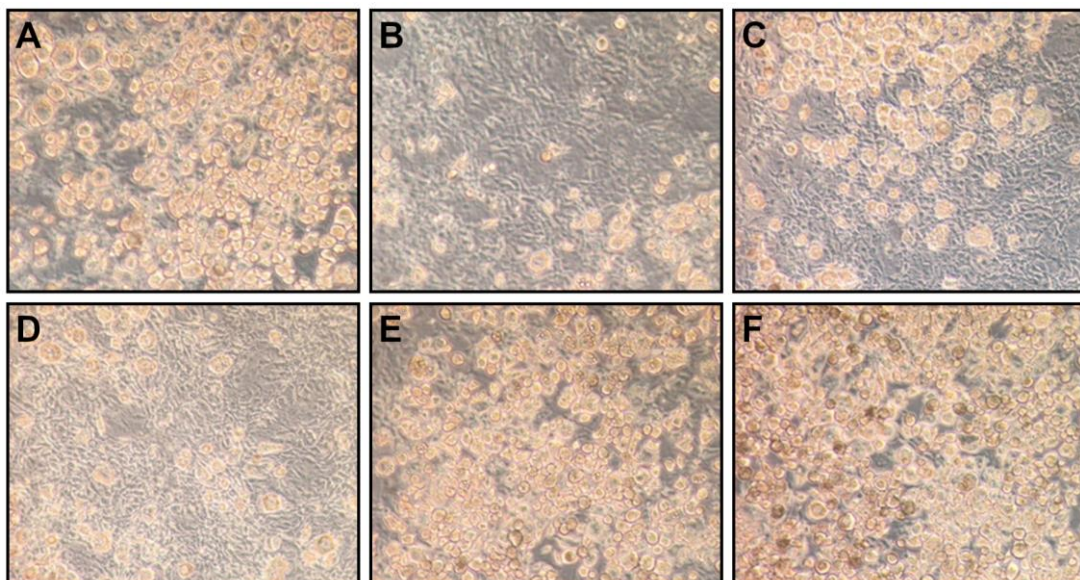
Figure 4.6:   **Differentiation of 3T3-L1 cells in six FBS batches.**
Three wells of passage p+11 were cultivated in differentiation media containing one of the six FBS batches. 10 d after induction the differentiation of the middle well was analyzed by light microcopy. A to D were sera purchased from Gibco. E and F were standardized sera from PAA and Biochrom.

Table 4.1: **Analyzed FBS batches for differentiation**.

	Manufacturer	Batch
A	Gibco	#1698221
B	Gibco	#1671047
C	Gibco	#1667399
D	Gibco	#1515306
E	PAA	PAA-Gold, standardized
F	Biochrom	Superior, standardized

The light microscopy confirmed that the differentiation differs between the FBS batches. Only the Gibco serum A led to a satisfying result, whereas the cells only differentiated to almost 50 % in serum C, B, and D. These three sera were purchased from Gibco and they showed a very slight differentiation potential. However, the both standardized sera E and F showed a good performance, whereas the Superior serum from Biochrom (F) worked best. Therefore, this serum was used in cell culture for all experiments and to ensure an equal differentiation only the passages p+6 to p+14 were used, because the differentiation potential into adipocytes declines with increasing passages numbers (Poulos et al., 2010).

4.3   Glucose deprivation affected NNMT expression

The methyltransferase NNMT has a fundamental role in energy metabolism. It is up-regulated in different models of obesity and a knockdown of NNMT in adipose tissue by antisense oligonucleotides protects mice from diet induced obesity (Kraus et al., 2014). Currently, it is not known how NNMT is regulated. However, it is known that NNMT mRNA levels are increased in insulin-resistant adipose-specific *Glut4*-knockout mice and reduced in insulin-sensitive adipose-specific *Glut4*-overexpressing mice (Kraus et al., 2014). Due to this fact, I hypothesized that glucose availability is responsible for NNMT expression in adipocytes.

4.3.1 Glucose deprivation

In order to investigate the hypothesis fully and stable differentiated 3T3-L1 cells were cultivated under glucose deprivation. This caused an increase in NNMT protein (Figure 4.7 A, D) and mRNA (Figure 4.7 B) levels after 10 d.
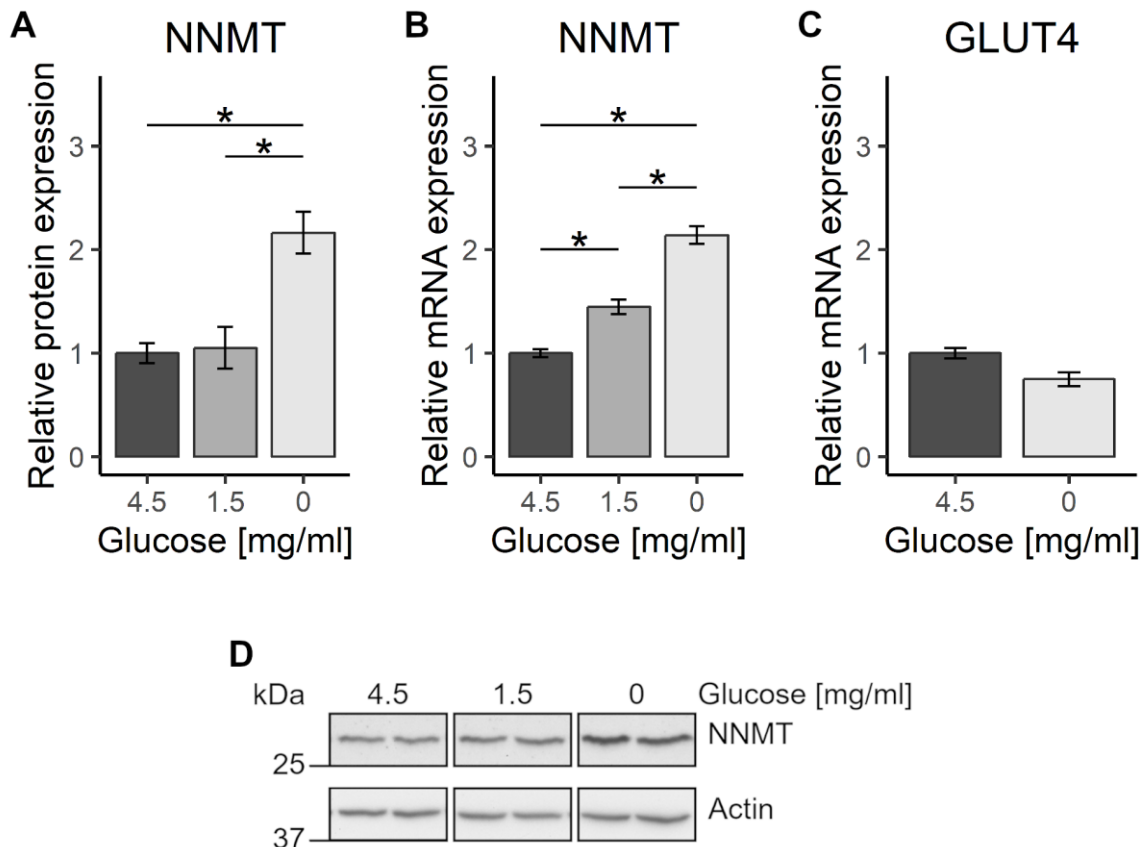

Figure 4.7: **NNMT expression during glucose deprivation.**
(A) NNMT protein and **(B)** NNMT mRNA levels in 3T3-L1 adipocytes cultivated in medium containing different glucose concentrations (4.5 mg/ml, 1.5 mg/ml, 0 mg/ml) for 10 d, whereas 4.5 mg/ml was the standard condition. **(C)** Expression of GLUT4 mRNA after starvation. **(D)** Representative western blot out of three independent experiments. The mRNA and protein were normalized to the untreated control and to beta actin. The relative expression is shown as mean values with standard error of mean (SEM) (n = 3). * $p < 0.05$.

Interestingly, the qPCR results even show a dose-dependent increase in mRNA expression (Figure 4.7 B), whereas the mRNA of GLUT4 is only slightly diminished (Figure 4.7 C). As expected, NNMT expression could be regulated by glucose availability.

4.3.2 Phloretin treatment

Glucose availability could also be manipulated by phloretin treatment.It is a nonspecific glucose transport inhibitor (Zhao and Keating, 2007) and it is used as a conformation of the results in section 4.3.1. To test the cytotoxicity of this inhibitor an MTT assay was performed (Figure 4.8).
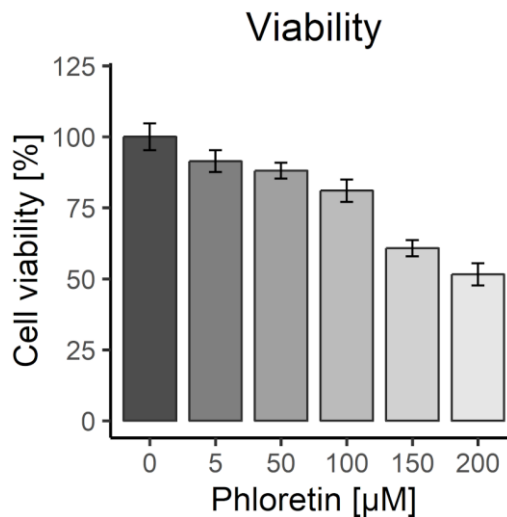

Figure 4.8: **MTT assay of phloretin treatment.**
3T3-L1 adipocytes were cultivated in medium containing 4.5 mg/ml glucose and phloretin (5, 50 100, 150, 200 µM) for 10 d. The cell viability is shown as mean values in percent with standard error of mean (SEM) (n = 2).

A treatment with 5 to 100 µM for 10 d is not cytotoxic. 100 µM phloretin showed a viability of 81 %. However, the last concentrations (150, 200 µM) are obviously toxic for the adipocytes. In line with the MTT, to confirm the glucose deprivation results adipocytes were treated with 5, 50, and 100 µM phloretin for 10 d. The NNMT protein and mRNA expression were analyzed by western blot and qRT-PCR (Figure 4.9).
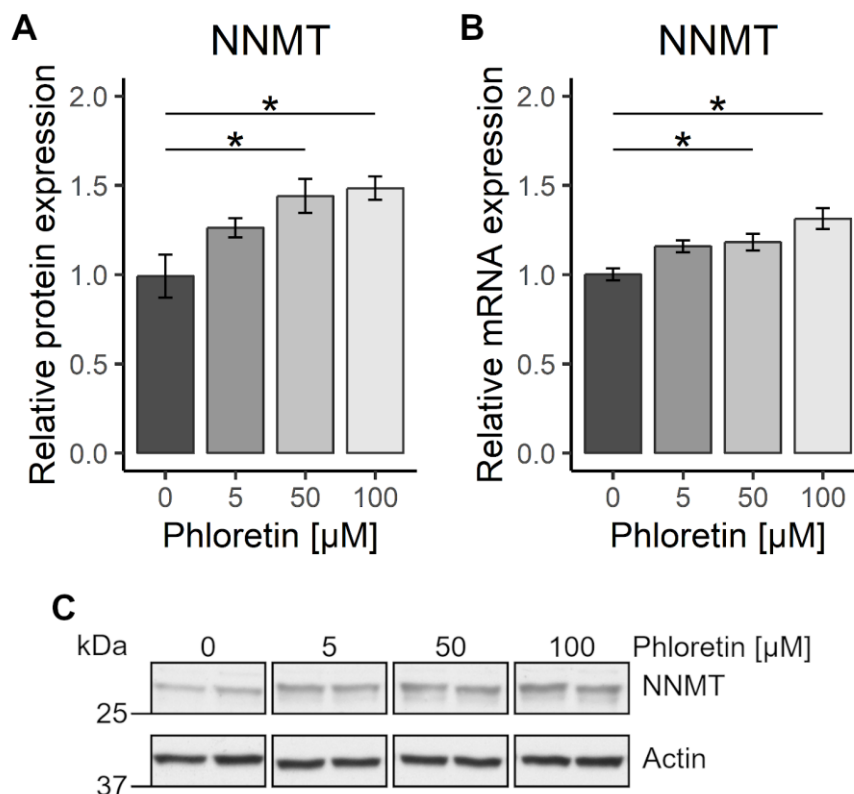
Figure 4.9: **NNMT expression after phloretin treatment.**
(A) NNMT protein and **(B)** NNMT mRNA levels in 3T3-L1 adipocytes cultured in medium containing 4.5 mg/ml glucose and phloretin (5, 50 100 µM) for 10 d. **(C)** Representative western blot out of three independent experiments. The NNMT mRNA and protein were normalized to the untreated control and to beta actin. The relative expression is shown as mean values with standard error of mean (SEM) (n = 3). * $p < 0.05$.

Indeed, the protein expression was significantly enhanced with higher phloretin concentrations. The mRNA level was also affected by 50 µM and 100 µM phloretin. A higher inhibitor concentration for a stronger effect could not be used due to the toxicity that was determined by MTT (Figure 4.8).

4.3.3 Glycolysis inhibition

Based on the result in glucose starved and phloretin treated cells, as well as in the study with GLUT4 knockout mice, it is interesting whether the glycolysis is involved in NNMT regulation. Normally, glucose is converted into pyruvate for ATP production (Heinrich et al., 2014). The availability of this energy source is diminished when glucose uptake or glycolysis is interrupted.

To investigate the impact of the glycolysis on NNMT expression, this pathway is inhibited in the following experiment. The cells are treated for 10 d with 1 mM 2-DG, which early interferes the glycolysis between hexokinase and glucose-6-phosphate isomerase (Urakami et al., 2013). In Figure 4.10 a schematic view of the glycolysis is shown.
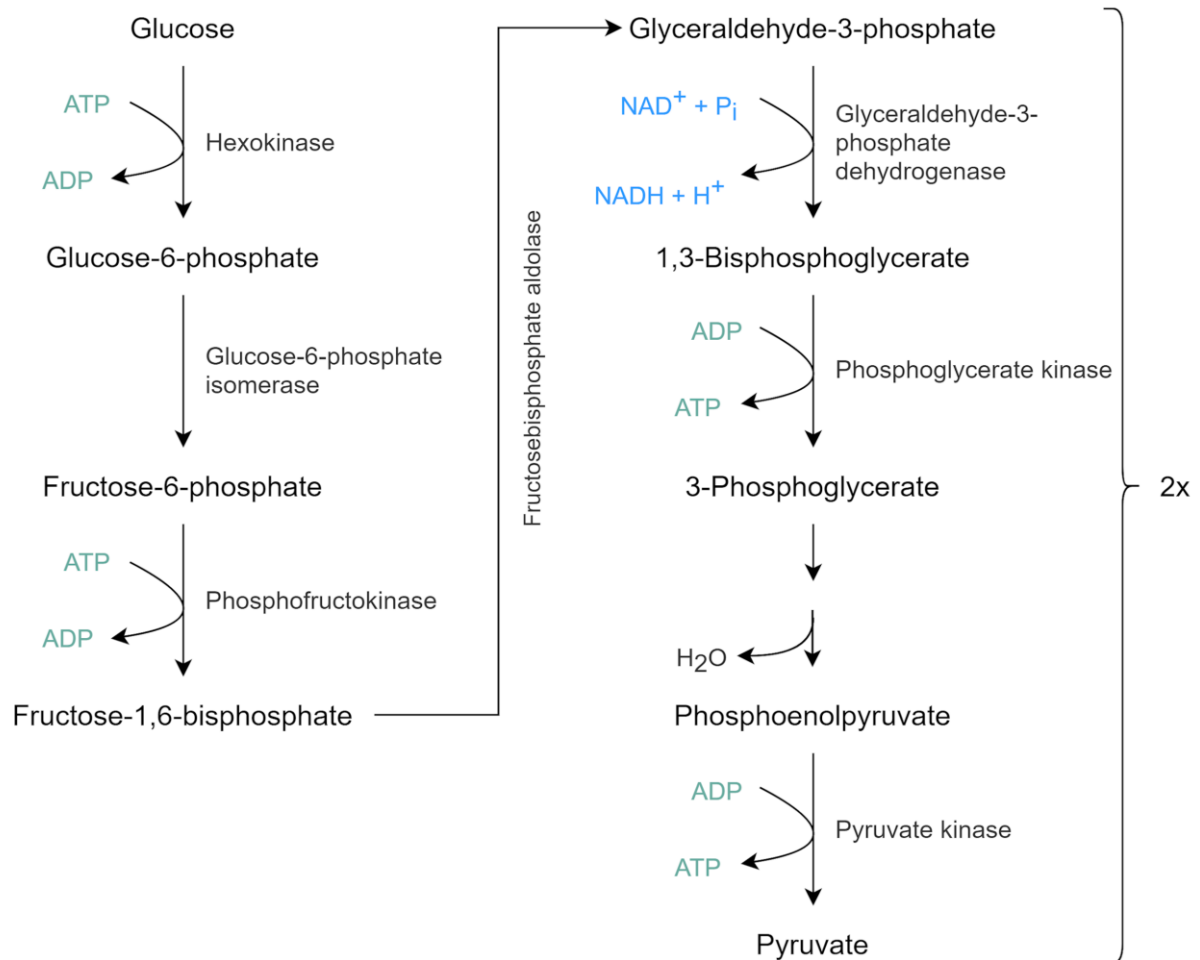

Figure 4.10: **Shematic view of glycolysis.**
Important steps of the glycolysis with enzymes and co factors. Redrawn and simplified from (Heinrich et al., 2014).

2-DG increased NNMT protein in a similar manner as glucose deprivation (Figure 4.11). This effect appeared to be enhanced by removal of sodium pyruvate, the glycolysis end product, although this effect was not statistically significant. Nevertheless, this slight increase is also detectable between the suitable conditions without glucose.
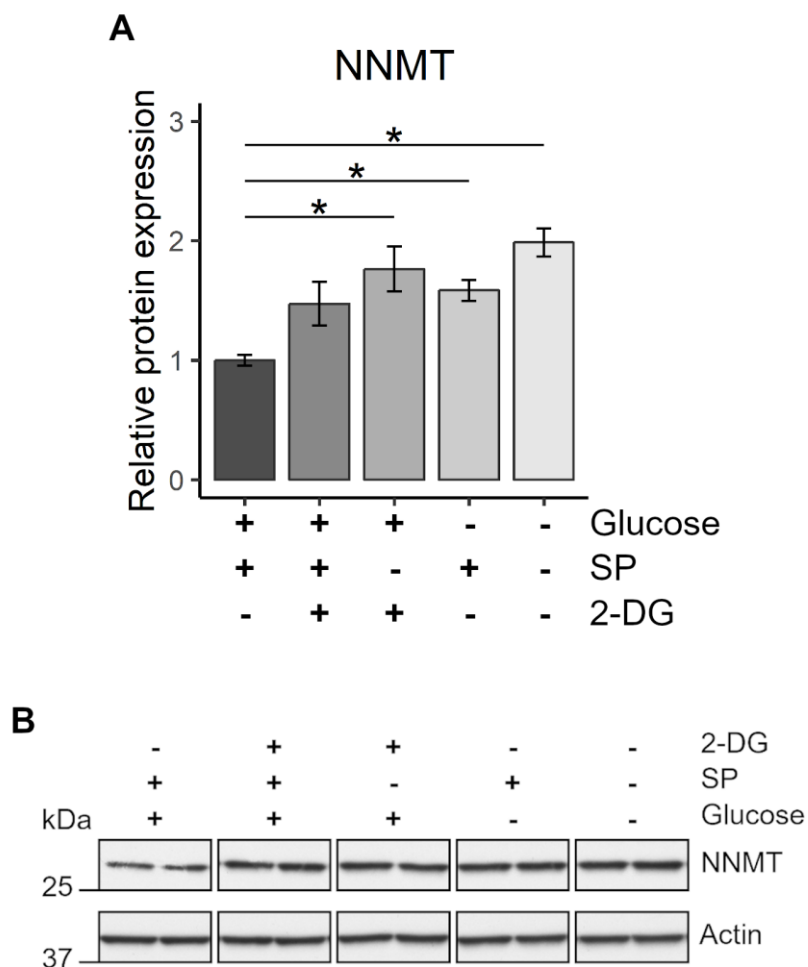
Figure 4.11: NNMT expression after 2-DG treatment.
(A) NNMT protein level in 3T3-L1 adipocytes treated with 1 mM 2-DG in medium with or without 1 mM SP or glucose for 10 d. (B) Representative western blot out of three independent experiments. The protein expression was normalized to the untreated control and to beta actin. The relative expression is shown as mean values with standard error of mean (SEM) (n = 3). * $p < 0.05$.

An MTT assay was carried out to analyze the viability after 2-DG treatment. 1 mM 2-DG was not cytotoxic, whereas the higher concentration (6 mM) with 38 % viability compared to control cells was strong cytotoxic (Figure 4.12 A). In addition, to verify a successful 2-DG uptake a commercially available hexokinase activity assay was performed (Figure 4.12 B, C). Normally, glucose is phosphorylated to glucose-6-phosphate (G6P) during the first step of the glycolysis. G6P is further metabolized by glucose-6-phosphate isomerase and exactly here 2-DG acts as an inhibitor (Urakami et al., 2013). 2-DG is also phosphorylated by hexokinase. However, this new product 2-DG-6-phosphate (2DG6P), could not be processed by glucose-6-phosphate isomerase (Xi et al., 2014).
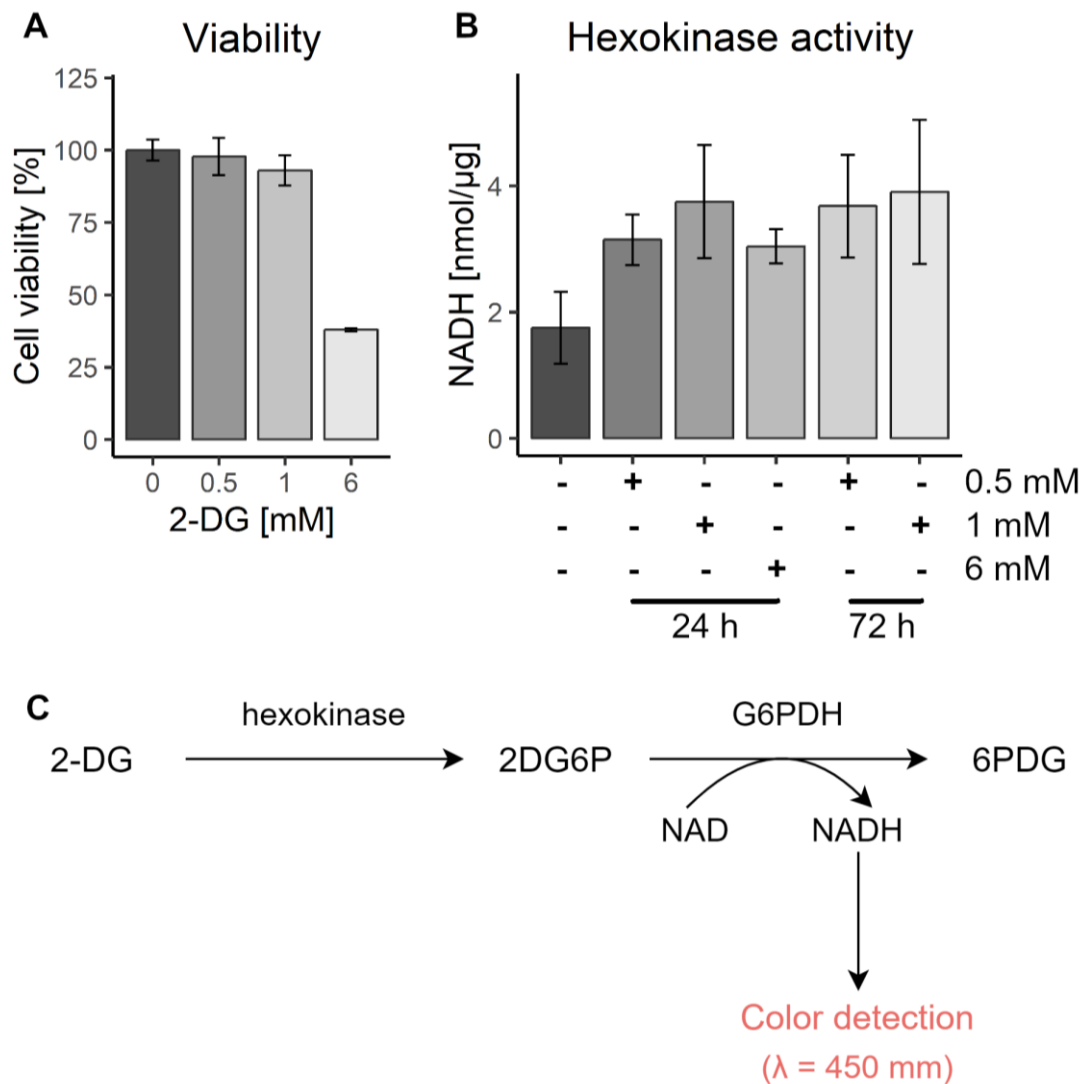
Figure 4.12: Cell viability and hexokinase activity after 2-DG treatment.
(A) 3T3-L1 adipocytes were cultured in medium containing 4.5 mg/ml glucose and 2-DG (0.5, 1, 6 mM) for 10 d. The cell viability is shown as mean values in percent with standard error of mean (SEM) (n = 2). **(B)** Hexokinase activity was determined by NADH production after 24 h and 72 h cultivation with 2-DG (0.5, 1, 6 mM). The hexokinase activity is shown as mean values with standard deviation (SD) (n = 1). **(C)** Schematic reaction of the hexokinase activity assay.

Consequently, 2DG6P accumulated in the cytoplasm of 3T3-L1 adipocytes. Because of the not metabolized 2DG6P, the hexokinase activity assay indicated a higher activity after the 2-DG treatment (Figure 4.12 B). In detail, the assay measured the production of NADH that is part of the reducing reaction during the oxidation of 2DG6P to 6PDG by the glucose-6-phosphate dehydrogenase (G6PDH) (Figure 4.12 C). The cells were treated 24 h and 72 h to ensure that 2-DG works even 72 h after stimulation, that was the longest time without a media change. Even after the longer time the inhibitory effect of 2-DG was present in the cells.

4.3.4 Pentose phosphate pathway inhibition

As shown in subsection 4.3.3, glucose is phosphorylated to glucose-6-phosphate (G6P) by hexokinase and during glycolysis G6P is further processed. This sugar is also part of the pentose phosphate pathway that generates the reducing agent NADPH (Figure 4.13) (Cole and Kramer, 2016; Stincone et al., 2015). Therefore, this pathway could also be involved in NNMT regulation.
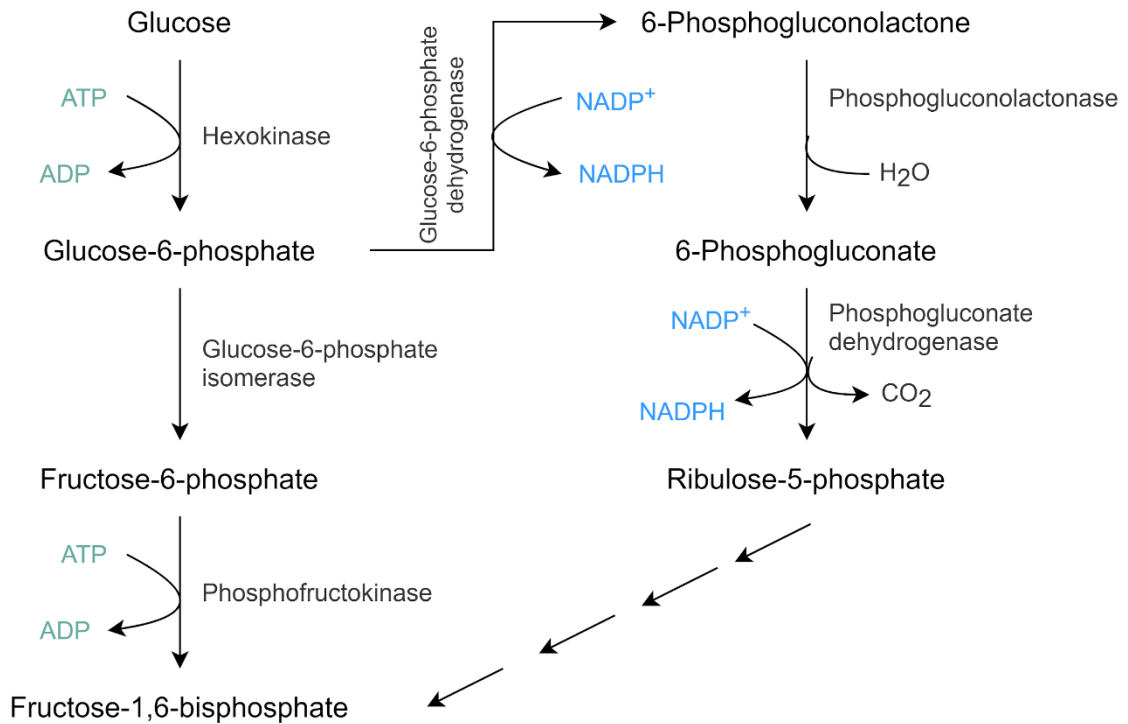

Figure 4.13: Shematic view pentose phosphate pathway.
Important steps of the pentose phosphate pathway with enzymes and co factors. Redrawn and simplified from (Heinrich et al., 2014).

The inhibition of this pathway with dehydroepiandrosterone (DHEA), an inhibitor of G6PDH (Mercaldi et al., 2014), and physcion, an inhibitor of phosphogluconate dehydrogenase (Lin et al., 2015), could not enhance NNMT expression (Figure 4.14).
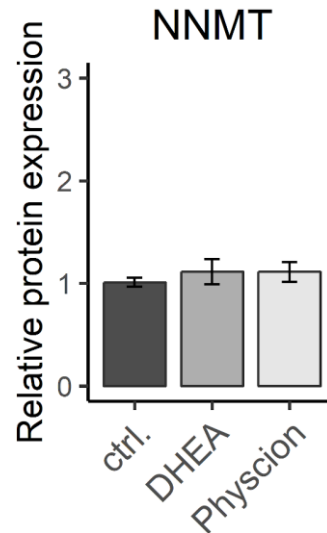
Figure 4.14: NNMT expression after pentose phosphate pathway inhibition.
NNMT protein level in 3T3-L1 adipocytes treated with 200 µM DHEA or 20 µM physcion for 10 d. The protein expression was normalized to the untreated control and to beta actin. The relative expression is shown as mean values with standard error of mean (SEM) (n = 3). *p < 0.05.

4.3.5 Insulin and FBS

However, there are two remaining factors that could also play an important role in NNMT regulation. The first is insulin. It is responsible for the membrane translocation of GLUT4, that is the limiting factor for GLUT4 mediated glucose uptake (Augustin, 2010). To confirm whether GLUT4 alone is responsible for increased NNMT expression, differentiated 3T3-L1 adipocytes were cultivated with and without insulin for 10 d (Figure 4.15 A). The second factor is FBS, that is essential for the differentiation of 3T3-L1 cells (Figure 4.6). FBS contains a lot of different growth factors, simple nutrients like amino acids and vitamins, stability factors for nutrients, etc. Some of these components could also affect the cell metabolism. Therefore, fully differentiated adipocytes were cultivated without FBS for 10 d (Figure 4.15 B).
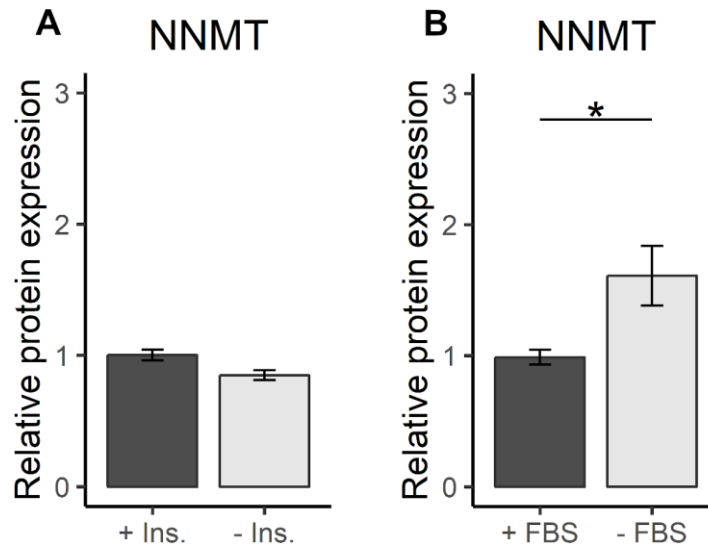
Figure 4.15*:* **NNMT expression of cells cultivated without Insulin or without FBS.**
(A) NNMT protein level in 3T3-L1 adipocytes cultivated without insulin (- Ins.) for 10 d.
(B) NNMT protein expression in 3T3-L1 cells cultivated without FBS (-FBS) for 10 d. The protein expression was normalized to the untreated control and to beta actin. The relative expression is shown as mean values with standard error of mean (SEM) (n = 3). *p < 0.05.

A cultivation without insulin did not change the NNMT expression at all (Figure 4.15 A). Thus, the basal glucose uptake supplies enough energy for the cell, whereas a removal of FBS led to a significantly increased NNMT expression (Figure 4.15 B). This result indicates that NNMT is not exclusively regulated by glucose deprivation. Since FBS and glucose are essential nutrient sources, their deprivation affects the cellular energy status, which could change the activity of the AMPK and mTOR signaling pathways.

4.4   Metabolic signaling pathways

Glycolysis and the pentose phosphate pathway provide substrates for cellular energy metabolism but do not directly affect gene expression. To elucidate the mechanistic link between glucose availability and NNMT expression, two major signaling pathways, that are involved in metabolism, were investigated. In the following section the AMPK pathway, that serves as an energy stress sensor, and the mTOR pathway, which is closely linked to cell metabolism, are analyzed.

4.4.1 AMPK

The cellular energy status is recognized by AMPK that controls different metabolic pathways and processes (Inoki et al., 2012). Under glucose deprivation the increase in AMP and ADP in relation to ATP activates AMPK (Hardie, 2011). This kinase could be pharmacologically activated by AICAR, too. It is transported into the cell by adenosine transporters and phosphorylated to the AMP-mimetic (ZEP) (Corton et al., 1995; Sullivan et al., 1994). ZEP binds to the AMPKγ subunit and does not change ADP:ATP ratio (Kim et al., 2016). Nevertheless, the kinase is active and can regulate the different branches of the signaling pathway (Figure 1.2). To mimic a starvation status after glucose deprivation, adipocytes were cultivated with 1 mM AICAR for 3 d and 9 d (Figure 4.17). Since AICAR led to a direct activation of AMPK signaling pathway, without a reduction of the ATP:AMP ratio, a shorter incubation time (3 d) was also investigated. A longer treatment than 9 d was not possible due to the cytotoxicity (Figure 4.16 B).
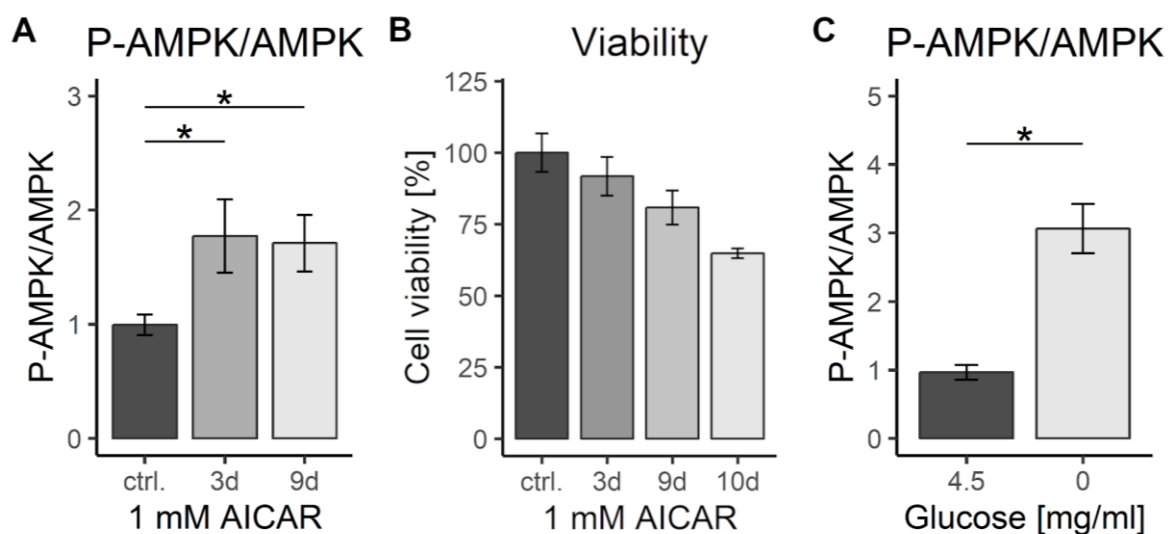

Figure 4.16: AMPK activation of AICAR treated cells, starved cells, and cell viability.
(A) P-AMPK to AMPK ratio of 3T3-L1 adipocytes cultivated with 1 mM AICAR for 3 d and 9 d in 4.5 mg/ml glucose medium. (B) The cell viability after 1 mM AICAR is shown as mean values in percent with standard error of mean (SEM) (n = 2). (C) P-AMPK to AMPK ratio of 3T3-L1 cells under glucose deprivation. The protein expression was normalized to the untreated control and to beta actin. The relative expression is shown as mean values with standard error of mean (SEM) (n = 3). *p < 0.05.

To ensure that AMPK is activated during the treatment the ratio P-AMPK to AMPK was determined by western blot. Indeed, AMPK phosphorylation was affected after 3 d and 9 d AICAR treatment (Figure 4.16 A). Interestingly, an increased P-AMPK/AMPK ratio was also detected in starved adipocytes (Figure 4.16 C).
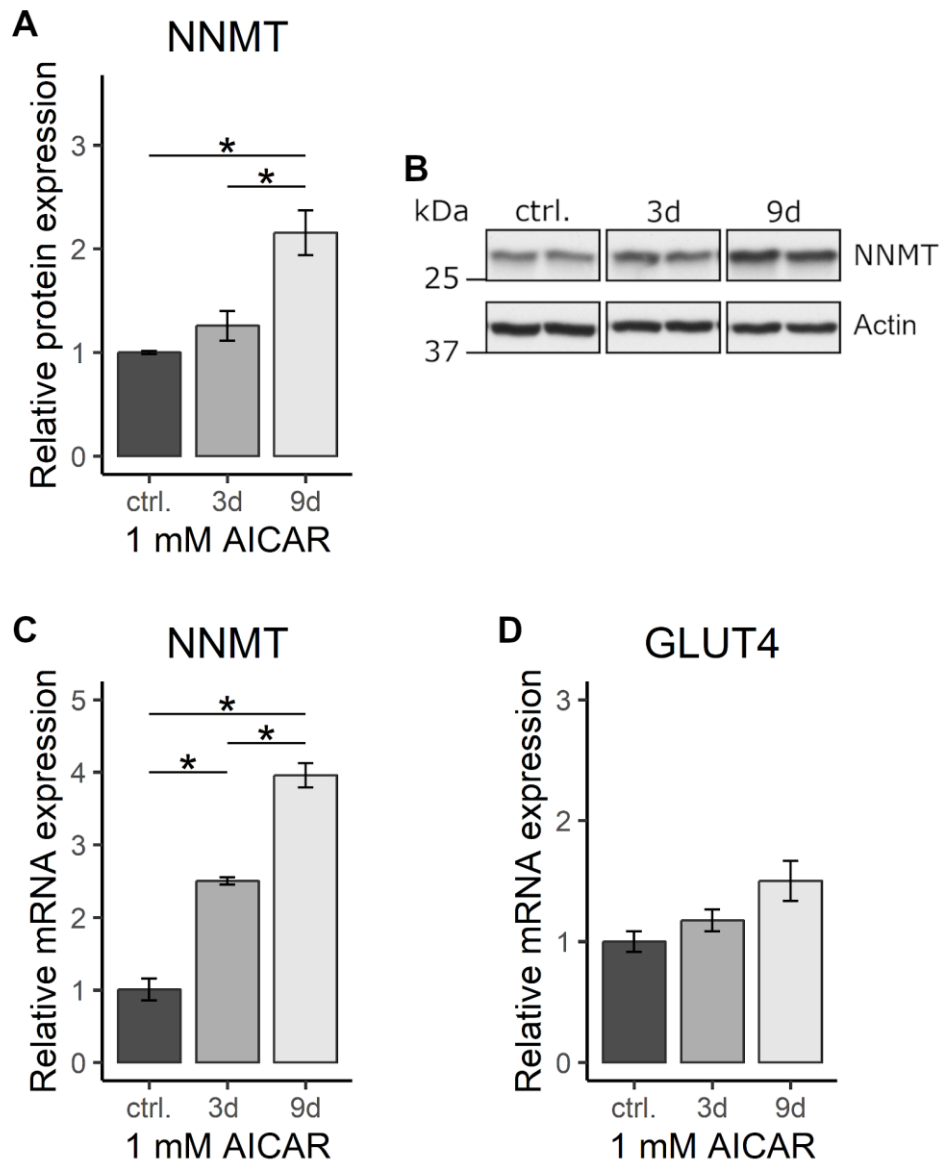

Figure 4.17: NNMT expression after AICAR treatment.
(A) NNMT protein level in 3T3-L1 adipocytes cultivated with 1 mM AICAR for 3 d and 9 d in 4.5 mg/ml glucose medium. (B) Representative western blot out of three independent experiments. (C) NNMT mRNA expression in 3T3-L1 cells cultivated with 1 mM AICAR for 3 d and 9 d. (D) GLUT4 mRNA expression after AICAR treatment. The protein and mRNA expression were normalized to the untreated control and to beta actin. The relative expression is shown as mean values with standard error of mean (SEM) (n = 3). *p < 0.05.

The incubation with 1 mM AICAR for 3 d and 9 d led to a significant increase in NNMT protein (Figure 4.17 A, B). According to this the mRNA expression of NNMT is also augmented in the treated adipocytes (Figure 4.17 C). Furthermore, after 9 d AMPK activation the GLUT4 mRNA expression was slightly elevated (Figure 4.17 D).

Due to the strong activation of AMPK even after AICAR treatment and in starved adipocytes, the mTOR pathway that is an important target of AMPK is investigated (Figure 1.2).

4.4.2 mTOR inhibition

The previous subsection describes AMPK as regulator of NNMT. In the following experiments the mTORC1, which is a major target of AMPK, is analyzed. Under nutrient rich conditions mTORC1 promotes biosynthesis of proteins and lipids (Laplante and Sabatini, 2009) and during glucose deprivation the mTORC1 activity is controlled by AMPK (Garcia and Shaw, 2017). The signaling pathway, shown in Figure 1.2, describes the inhibition of mTORC1 due to AMPK activation. To analyze whether mTORC1 could also be involved in the regulation of NNMT expression, mTORC1 was inhibited by 500 nM rapamycin. Like during glucose deprivation, the treatment time was 10 d.

After this treatment a significant higher NNMT expression was detected (Figure 4.18). The NNMT protein level was augmented more than 2-fold by mTORC1 inhibition (Figure 4.18 A, B), whereas the mRNA expression was only slightly increased (Figure 4.18 C). To verify a successful mTORC1 inhibition the phosphorylation of p70 S6K, a typical mTOR target, was examined by western blot. The missing phosphorylation of p70 S6K confirmed the rapamycin effect (Figure 4.18 B). Thus, the mTORC1 activity was blocked. Moreover, the mRNA analyses of mTORC1 inhibition did not affect GLUT4 expression (Figure 4.18 D).
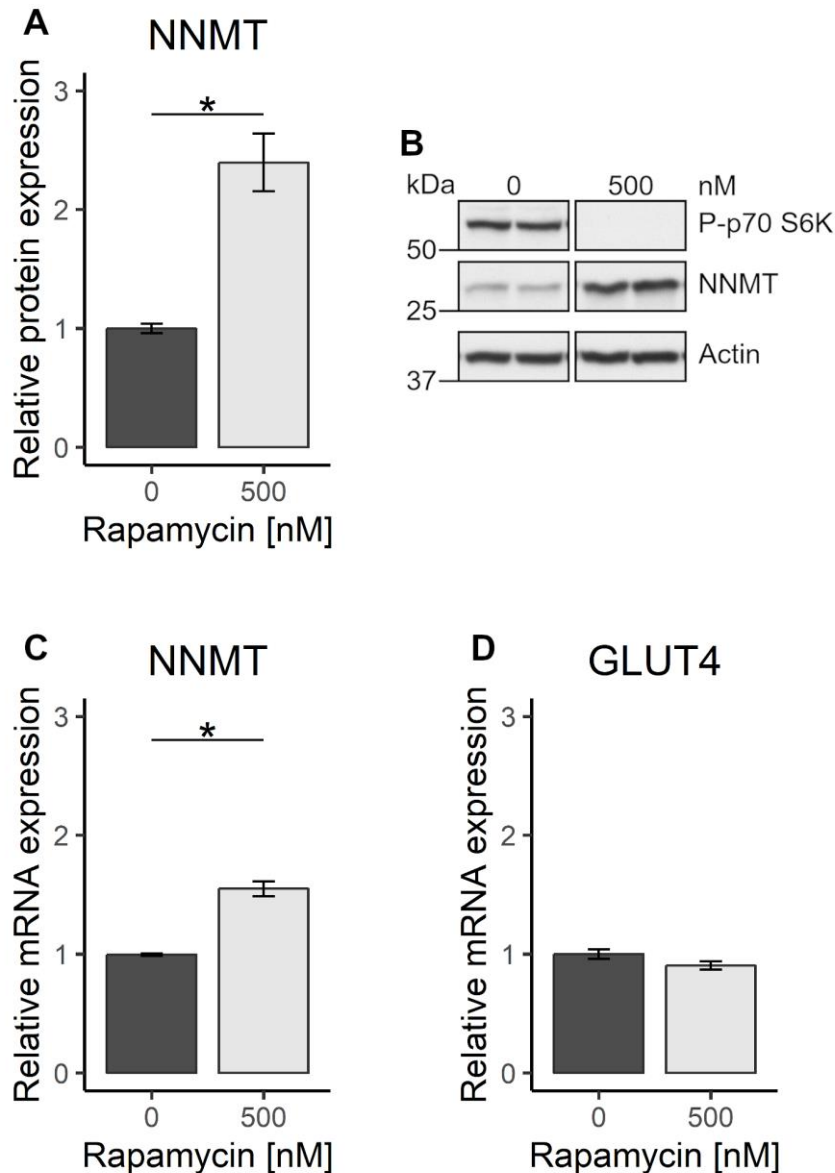
Figure 4.18: NNMT expression after rapamycin treatment.
(A) NNMT protein level in 3T3-L1 adipocytes cultivated with 500 nM rapamycin for 10 d in 4.5 mg/ml glucose medium. (B) Representative western blot out of three independent experiments. (C) NNMT mRNA expression in 3T3-L1 cells cultivated with 500 nM rapamycin for 10 d in 4.5 mg/ml glucose medium. (D) GLUT4 mRNA expression after 10 d 500 nM rapamycin treatment. The protein and mRNA expression were normalized to the untreated control and to beta actin. The relative expression is shown as mean values with standard error of mean (SEM) (n = 3). *$p < 0.05$.

Additionally, a 4 d inhibition of mTORC1 was also be investigated, because the direct manipulation of the signaling pathway could affect the NNMT expression even after a shorter time. Already this manipulation led to an augmented NNMT protein level (Figure 4.19 A), as well as seen after the short-time AICAR treatment (Figure 4.17 A). However, the 4 d rapamycin treatment was not statistical significant. To analyze the mTOR signaling pathway under

glucose starvation, the phosphorylation of p70 S6K was measured in glucose deprived adipocytes. After 10 d, even a slight reduction of this kinase was seen in these cells although the lower activity of mTOR was not statistically significant (Figure 4.19 B). Moreover, the experiments were not cytotoxic for the adipocytes, as the MTT assay indicates (Figure 4.20 A).
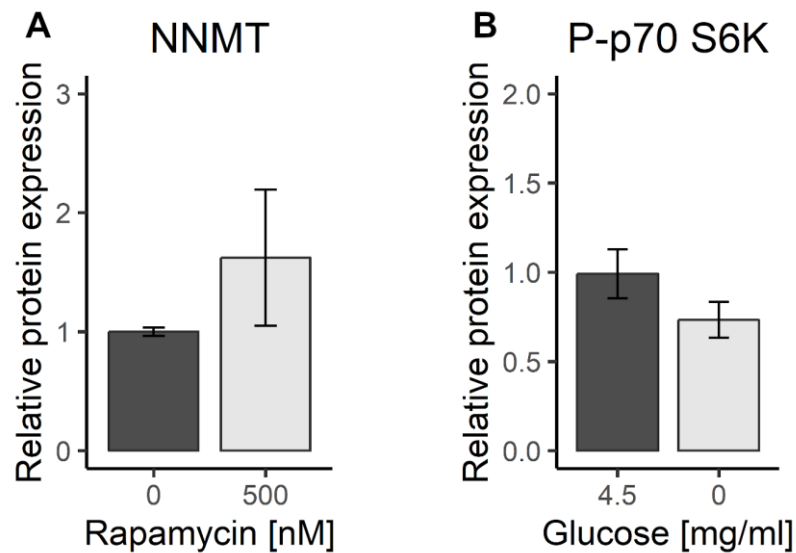

Figure 4.19: **NNMT expression after 4 d rapamycin treatment and P-p70 S6K protein in glucose starved adipocytes.**
(A) NNMT protein level in 3T3-L1 adipocytes cultivated with 500 nM rapamycin for 4 d in 4.5 mg/ml glucose medium. **(B)** Phosphorylation of p70 S6K glucose deprived 3T3-L1 cells compared to control cells cultivated in 4.5 mg/ml glucose. The protein expressions were normalized to the untreated control and to beta actin. The relative expression is shown as mean values with standard error of mean (SEM) (n = 3).

Actually, mTORC1 is a master regulator of translation and an inhibition of this complex should reduce the protein expression in the cell, rather than increase it. Thus, it was also studied the effect of an inhibitor of protein translation. Indeed, in the presence of cycloheximide, mTORC1 inhibition with rapamycin failed to augment NNMT expression (Figure 4.20 B, C). Therefore, mTORC1 regulated NNMT via translation, rather than altered protein half-life.
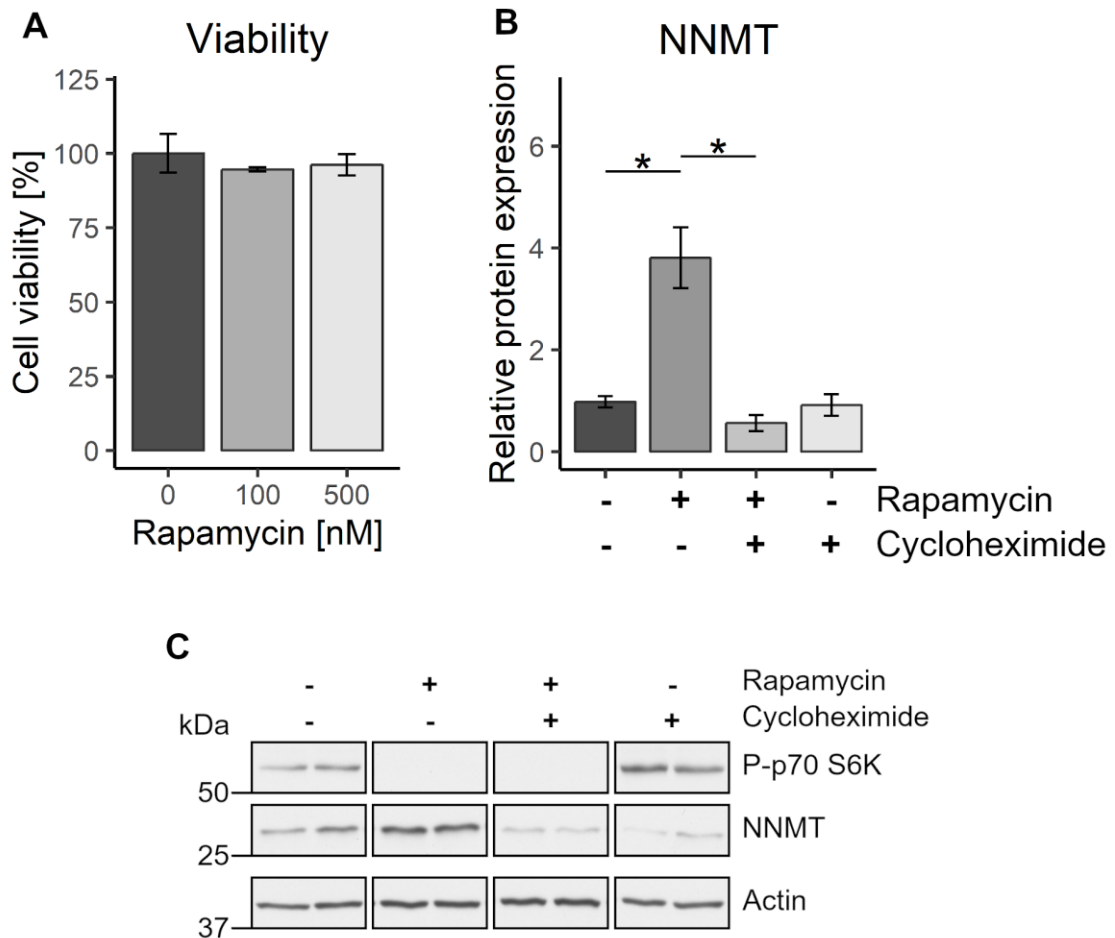
Figure 4.20: Cell viability for rapamycin and NNMT expression after inhibition of translation.
(A) The cell viability after 100 nM and 500 nM rapamycin treatment is shown as mean values in percent with standard error of mean (SEM) (n = 2). (B) NNMT protein level in 3T3-L1 adipocytes cultivated with rapamycin (100 nM) and cycloheximide (5 µM) for 10 d. (C) Representative western blot out of three independent experiments. The protein expression was normalized to the untreated control and to beta actin. The relative expression is shown as mean values with standard error of mean (SEM) (n = 3). *p < 0.05.

4.4.3 mTOR activation

As seen in 4.4.2, the inhibition of mTOC1 enhanced the NNMT level. Furthermore, the mTOC1 activity is slightly reduced under glucose deprivation (Figure 4.19 B), whereas the NNMT expression is significantly increased (Figure 4.7). Therefore, the NNMT expression was investigated under mTORC1 activation in 3T3-L1 adipocytes that were kept again in culture medium without glucose. As mTOR activator MHY1485 was used (Choi et al., 2012; Vizza et al., 2018).
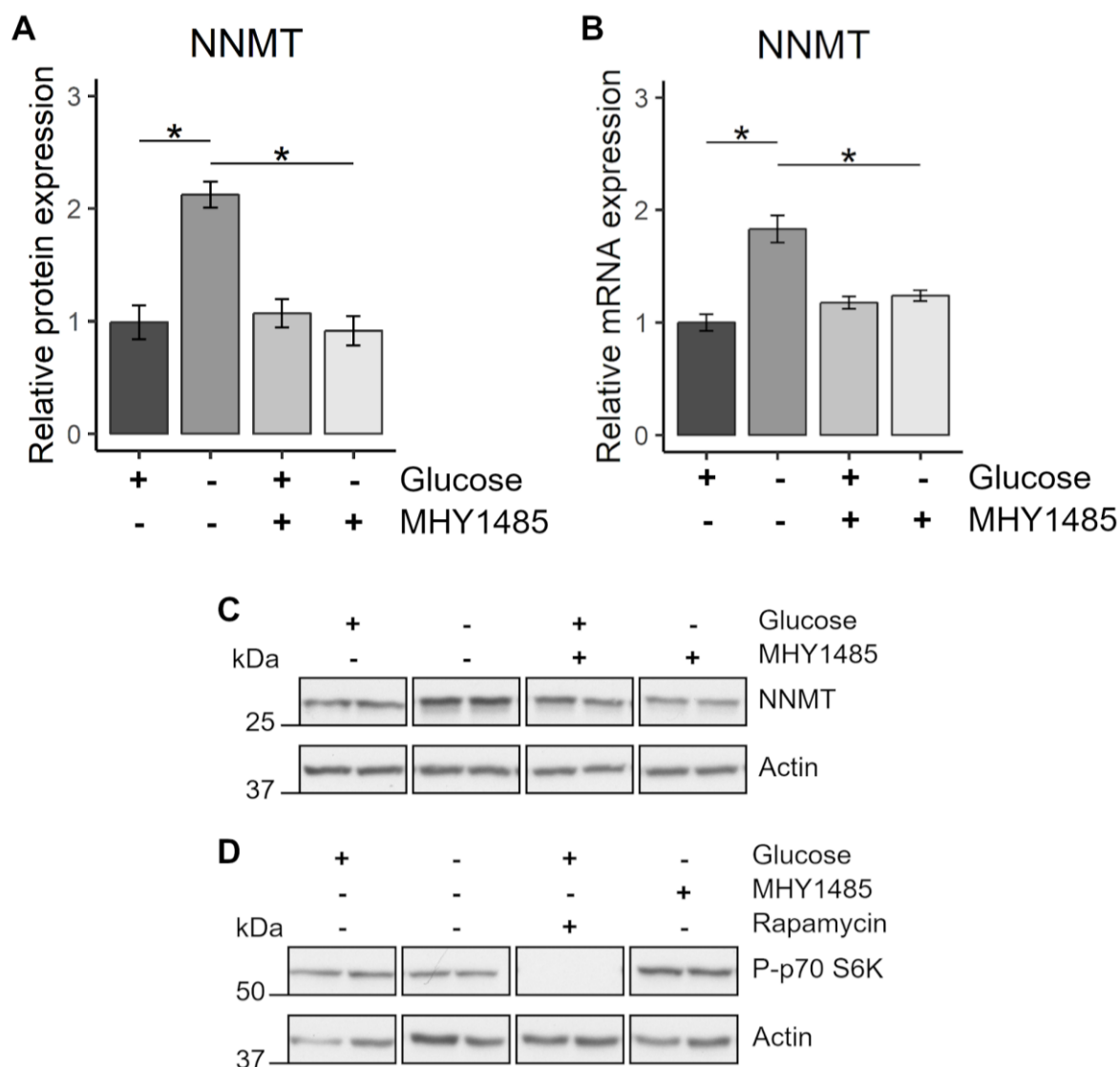
Figure 4.21: Activation of mTORC1 by MHY1485.
(A) NNMT protein and (B) NNMT mRNA levels in 3T3-L1 adipocytes cultured in medium without glucose and MHY1485 (10 µM) for 10 d. (C) Representative western blot out of three independent experiments, that showed the NNMT expression after MHY1485 treatment in glucose starved cells. (D) Representative western blot out of three independent experiments, that confirmed the mTORC1 activation. The protein expression was normalized to the untreated control and to beta actin. The relative expression is shown as mean values with standard error of mean (SEM) (n = 3). *p < 0.05.

In the presence of MHY1485, glucose deprivation no longer caused an increase in NNMT mRNA or protein levels (Figure 4.21 A, B, C). Of note, when adipocytes were cultivated in glucose containing medium, MHY1485 did not alter NNMT expression. The stronger phosphorylation of p70 S6K confirmed the successful activation of mTORC1 in starved adipocytes treated with MHY1485 (Figure 4.21 D). Taken together, a reduced mTOR activity is responsible for the regulation of NNMT expression by glucose deprivation.

4.4.4 Autophagy inhibition

Figure 4.21 demonstrated clearly that the starvation effect on NNMT is reversible by MHY1485. This activator of mTORC1 is also an autophagy inhibitor (Choi et al., 2012). Furthermore, the signaling pathways mTOR and AMPK are known regulators of autophagy (Codogno and Meijer, 2005). Interestingly, starvation is one of the most important stimuli of it (Singh and Cuervo, 2011) and the necessary lysosomal breakdown of the cytoplasm and organelles generates the energy for cell survival (Rabinowitz and White, 2010). To assess whether NNMT is up-regulated during autophagy, this process is inhibited with bafilomycin A1 and chloroquine (Bechor et al., 2017; Chinni and Shisheva, 1999; Mauthe et al., 2018).

Both autophagy inhibitors and MHY1485 abrogated the increase of NNMT protein that was caused by glucose deprivation (Figure 4.22). The inhibition of autophagy could be determined by the autophagic adaptor proteins p62 and LC3 (Johansen and Lamark, 2011). LC3 shows in western blot two bands. The upper one is called LC3I and it is in the cytosol, whereas the lower band with the name LC3II is conjugated to autophagosome membrane (Choi et al., 2012). Figure 1.3 showed the process in detail. During autophagy p62 is normally degraded and LC3II is recycled (Corona Velazquez and Jackson, 2018). The three autophagy inhibitors interrupted this process (Bechor et al., 2017; Chinni and Shisheva, 1999; Mauthe et al., 2018). Thus, the stabilization of p62 and LC3II strongly suggests that autophagy is inhibited in the 3T3-L1 cells (Figure 4.22 B).
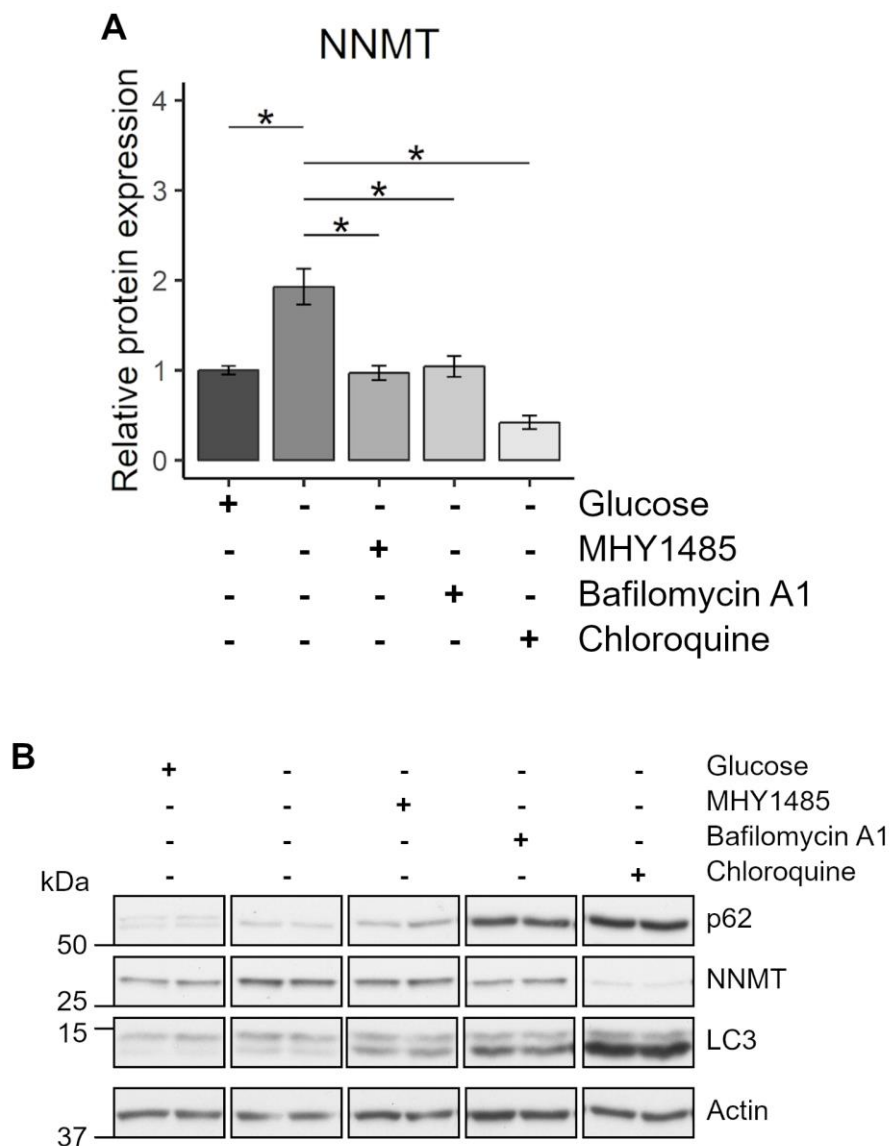
Figure 4.22: NNMT expression after autophagy inhibition.
(A) NNMT protein expression of starved adipocytes cultivated with 10 µM MHY1485, 20 nM bafilomycin A1, and 25 µM chloroquine for 10 d. (B) Representative western blot out of three independent experiments. LC3 and p62 western blot band serve as control for autophagy inhibition. The protein expression was normalized to the untreated control and to beta actin. The relative expression is shown as mean values with standard error of mean (SEM) (n = 3). *p < 0.05.

According to the protein results, the reducing effect was also detectable on mRNA level (Figure 4.23 A). However, the significant reduction of cells treated with chloroquine was not so pronounced as seen in western blot. An MTT assay showed only a slight viability reduction during autophagy inhibition (Figure 4.23 B).
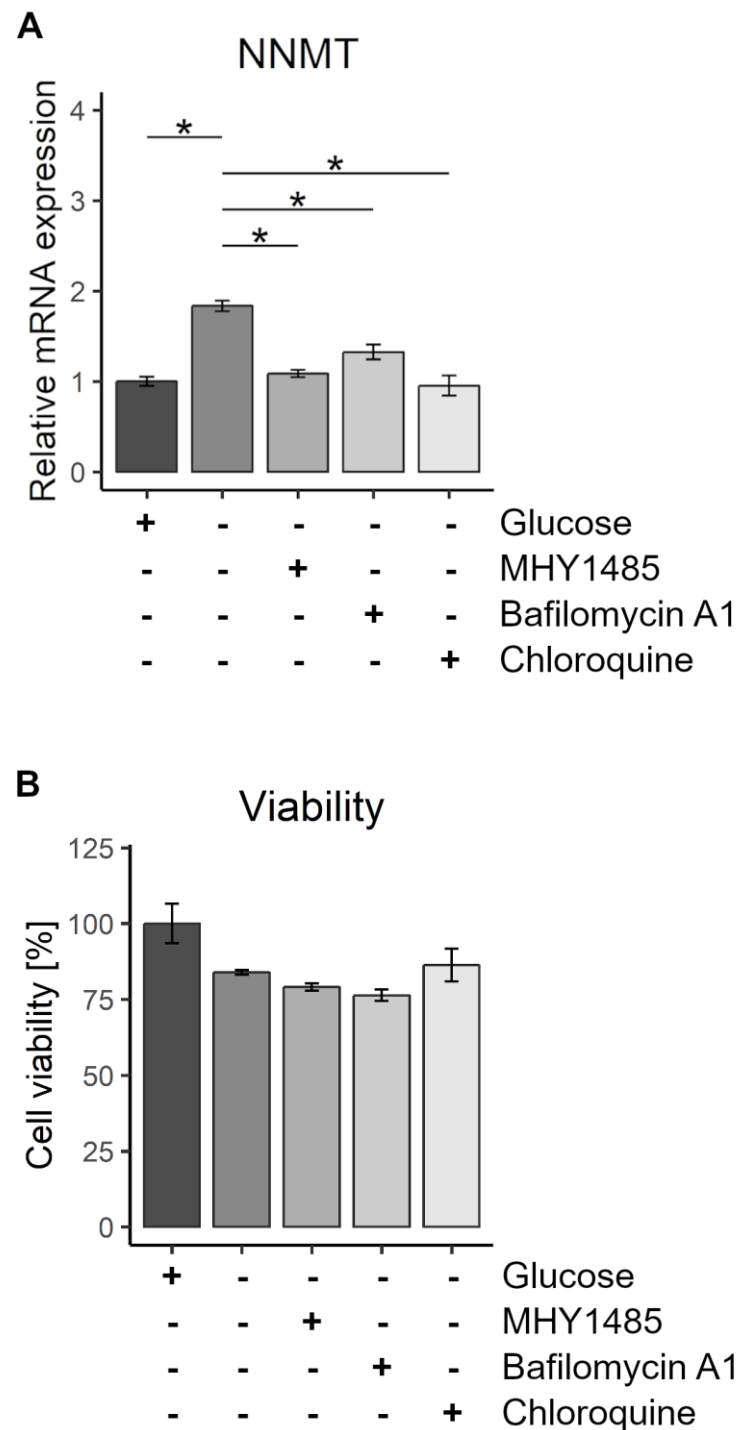
A

B

Figure 4.23: NNMT mRNA expression and cell viability after autophagy inhibition .
(A) NNMT mRNA expression of starved adipocytes cultivated with 10 µM MHY1485, 20 nM bafilomycin A1, and 25 µM chloroquine for 10 d. **(B)** MTT assay of cells treated with the different autophagy inhibitors for 10 d (n = 2). The mRNA expression was normalized to the untreated control and to beta actin. The relative expression is shown as mean values with standard error of mean (SEM) (n = 3). *p < 0.05.

Taken together NNMT is up-regulated during autophagy that is induced by the glucose deprivation. An inhibition of this process by three inhibitors prevented the NNMT up-regulation. Thereby NNMT could serve as an energy sensor and play an important role for cell survival under harmful conditions.

5   Discussion

5.1   Glucose availability regulates NNMT expression

The expression of the methyltransferase NNMT is increased in obesity and diabetes (Kraus et al., 2014). The knockdown or the inhibition of this enzyme prevents obesity (Brachs et al., 2019; Cao et al., 2017; Kannt et al., 2018; Kraus et al., 2014; Neelakantan et al., 2018). Additionally, this methyltransferase was even enhance in human muscle during caloric restriction (Ström et al., 2018).

So far, the distinct regulation mechanism of NNMT is not known when the energy homeostasis is disturbed. This study showed that glucose availability is important for NNMT expression in adipocytes. Indeed, after 10 d glucose deprivation NNMT protein and mRNA level were significantly enhanced (Figure 4.7). Thereby the cultivation without glucose did not cause a de-differentiation of the 3T3-L1 adipocytes (Figure 4.5). Even if the cells were de-differentiated, it would be more likely that the NNMT expression is reduced, rather than enhanced, as NNMT is itself a marker for adipocyte differentiation (Riederer et al., 2009). The same effect on this methyltransferase is measured in adipose tissue of mice that are unable to take up glucose in an insulin insensitive manner due to a knockout of GLUT4 (AG4KO) and the constitutionally augmented GLUT4 causes a reduction in NNMT expression (AG4Tg) (Kraus et al., 2014).

While this results were obtained in a GLUT4 knockdown study (Kraus et al., 2014), GLUT4 mRNA expression in 3T3-L1 cells was not affected by glucose starvation (Figure 4.7 C). In contrast to this, a starvation of 3T3-L1 increased GLUT4 at the cell-surface (Kozka et al., 1991). However, the analyses of the transporter protein levels are difficult by western blot, because GLUT4 is always recycled between the plasma membrane and intracellular storage vesicles (Shepherd and Kahn, 1999). It is not possible to distinguish, whether a GLUT4 transporter is bound in its active from in the plasma membrane or in its inactive form in a vesicle using western blot. Of course, there are methods for investigation of GLUT4 location. Kozka and colleagues used a method that based on photolabeling to analyze the cell-surface location (Kozka et al., 1991). However, in 3T3-L1 cells the basal glucose uptake could also be responsible for enough glucose transport into the cell, whereby the glucose uptake is predominantly mediated by insulin responsive GLUT4 in adipose tissue. Furthermore, in this study the cells were cultured in media without glucose and nevertheless the same effect could be measured as during a GLUT4 knockout (Kraus et al., 2014). Therefore, this is strong indication that the glucose deprivation and consequently the effect of a GLUT4 knockdown

cause the augmented NNMT expression in mice. Furthermore, the inhibition of the glucose transporter by phloretin confirmed the augmented NNMT expression due to glucose deprivation (Figure 4.9).

Additionally, the signaling pathways that regulate expression and trafficking of the glucose transporters could also be manipulated to affect glucose metabolism. For example, GLUT1 trafficking is strictly regulated by phosphatidylinositol 3-kinase and an inhibition of this enzyme prevented GLUT1 mediated glucose uptake (Bentley et al., 2003). mTOR and AMPK are involved in GLUT1 and GLUT4 expression and membrane translocation (Buller et al., 2008; Fryer et al., 2002). Therefore, they are also good targets to analyze the transporters and glucose metabolism.

Moreover, when GLUT4 are considered in a study, the insulin effects on 3T3-L1 adipocytes should also been investigated, since the GLUT4 is an insulin-sensitive glucose transporter (Shepherd and Kahn, 1999). A cultivation without insulin did not affect the NNMT protein expression (Figure 4.13 A). There a reduced glucose uptake and a higher NNMT expression would be expected. Unfortunately, cultured 3T3-L1 adipocytes contain more GLUT1 transporters as isolated adipocytes (Kozka et al., 1991). Therefore, the basal glucose uptake by GLUT1 could quench the missing GLUT4 glucose uptake. Furthermore, this implies that GLUT4 is generally responsible for the glucose uptake in the adipose tissue of animals and humans. This explains the strong NNMT expression in AG4KO mice (Kraus et al., 2014).

5.2 Glycolysis inhibition confirmed the starvation effect

To address where the glucose deprivation is recognized in adipocytes, the glycolysis has to be investigated, because glucose is the first sugar of this process. The inhibition of the glycolysis reduces the ATP levels (Xi et al., 2011), and consequently a deprivation status occurs in the cell. 2-DG is a well-studied glycolysis inhibitor. 1 mM 2-DG that targets the hexokinase reaction caused an increase in NNMT protein expression (Figure 4.11). Interestingly, this effect was strengthened by the removal of 1 mM sodium pyruvate. Moreover, a cultivation without glucose and without sodium pyruvate led to the highest NNMT expression. Both effects point out that the greater the deprivation is, the stronger the NNMT expression is.

The commercial hexokinase assay demonstrated a successful inhibition of the hexokinase step (Figure 4.12 B). The 2-DG treatment led to the accumulation of 2-DG-6-phosphate that is

only processed by the assay enzymes. Therefore, a higher hexokinase activity is measured by the assay. Both 1 mM and 6 mM effectively inhibited the enzyme. However, the higher concentration was quite cytotoxic (Figure 4.12 A). This was detected in different cell types and occurs even after 24 h (DiPaola et al., 2008; Wang et al., 2011; Xi et al., 2011).

The glycolysis not only generates ATP but also supplies different important intermediates for the cell, like glucose-6-phosphate (TeSlaa and Teitell, 2014). This sugar is also the starting point for the pentose phosphate pathway that generates the reducing agent NADPH (Heinrich et al., 2014). It was necessary to investigate this pathway, because NMMT could also be regulated by the availability of the reducing agents. However, neither physcion nor DHEA changed NNMT expression after 10 d treatment (Figure 4.14). Therefore, the availability of glucose and not NAPDH regulates NNMT.

5.3   AMPK and mTOR are involved in NNMT regulation

As mentioned above to manipulate the GLUT1 and GLUT4 translocation AMPK and mTOR signaling pathway could be examined. Moreover, these pathways regulate the cell metabolism and glucose availability is one of the major inputs (Inoki et al., 2012). Indeed, increased NNMT expression caused by glucose deprivation is mediated by AMPK and mTOR. The cultivation of 3T3-L1 adipocytes without glucose led to the activating phosphorylation of AMPK (Figure 4.16 C). Since the activity of mTORC1 is negatively regulated by AMPK (Figure 1.2), the phosphorylation of p70 S6K is slightly reduced in cell culture (Figure 4.19 B). This indicates that mTOR is less active after glucose deprivation. To investigate the distinct role of AMPK and mTOR in NNMT regulation both complexes were manipulated either with an activator or an inhibitor. The early sensor for glucose deprivation AMPK (Garcia and Shaw, 2017) is activated by AICAR. This caused a significantly higher NNMT expression even after a short time treatment (Figure 4.17 A, B, C).

The GLUT4 mRNA expression is increased after AICAR treatment (Figure 4.17 D). Interestingly, after glucose starvation the GLUT4 mRNA expression is not affected (Figure 4.7 C), although the activated AMPK is known to control GLUT4 mRNA expression (Zheng et al., 2001). However, the mRNA expression is not an evidence for the glucose uptake, more important is the GLUT4 membrane translocation (Shepherd and Kahn, 1999). Furthermore, the underling mechanisms of glucose uptake under pharmacological and

non-pharmacological AMPK activation is not fully understood. In general, this process is affected by the Rab GTPase-activating proteins TBC1D1 (Sakamoto and Holman, 2008) and AS160 that are part of the GLUT4 vesicles (Sakamoto and Holman, 2008). A phosphorylation of TBC1D1 and AS160 by AMPK or Akt promotes the membrane incorporation (Hatakeyama et al., 2019). In literature there are contrary findings concerning the GLUT4 mediated glucose uptake after AICAR administration.

On the one hand, this treatment increased the basal but inhibited the insulin-stimulated glucose transport in 3T3-L1 adipocytes (Salt et al., 2000). On the other one, Yamaguchi and colleagues demonstrated that AICAR accelerates the GLUT4 translocation and glucose uptake (Yamaguchi et al., 2005). This effect was not quenched by an Akt inhibition, whereas only insulin-stimulated glucose transport was strongly decreased by wortmannin, an Akt inhibitor (Yamaguchi et al., 2005). Additionally, the insulin receptor expression is decreased after AICAR treatment in HepG2 cells and is not affected in 3T3-L1 adipocytes (Nakamaru et al., 2005). This indicates the insulin-stimulated glucose uptake is possible during AICAR treatment. These findings show that, there are different GLUT4 regulation mechanism. This could explain why GLUT4 expression is not affected by a normal glucose deprivation, whereby AICAR treatment led to a higher transporter mRNA level.

Furthermore, at least two studies demonstrated that AICAR increases the GLUT1 mediated glucose transport in 3T3-L1 cells (Abbud et al., 2000; Salt et al., 2000). Interestingly, cultured 3T3-L1 adipocytes contain per se more GLUT1 transporters as isolated adipocytes (Kozka et al., 1991). Taken together, it is not important whether the glucose uptake is mediated by GLUT4 after AICAR treatment, because this activator could enhance the uptake by GLUT1 (Abbud et al., 2000; Salt et al., 2000). Consequently, enough glucose is provided for the 3T3-L1 adipocyte that nevertheless showed an augmented NNMT level. Therefore, the NNMT expression is regulated as a downstream target of AMPK.

Since AMPK is the most important energy sensor that controls autophagy, lipid metabolism, and protein translation in response to glucose starvation (Kim et al., 2013), it is involved in the regulation of mTORC1, another essential metabolic pathway (Saxton and Sabatini, 2017). After energy depletion AMPK can control the activity of mTORC1 directly or indirectly through TSC2 phosphorylation (Inoki et al., 2003). Subsequently, the active TSC1/TSC2 complex inhibited mTORC1 (Zoncu et al., 2011). Additionally, AMPK phosphorylates the mTOR binding partner raptor and this leads to mTORC1 inhibition (Gwinn et al., 2008). Reduced glucose availability as well as AICAR treatment represses the phosphorylation of p70 S6K (Kimura et al., 2003). To investigate the mTORC1 function in NNMT regulation, the complex was inhibited by rapamycin. Indeed, this led to an increase in protein and mRNA level of NNMT after 10 d

(Figure 4.18 A, B, C). Even when the results were not significant after a short time rapamycin treatment, a trend was visible (Figure 4.19 A). However, AICAR significantly caused a augmented NNMT expression after 3 d (Figure 4.17 A, B, C). All the results confirm that NNMT up-regulation occurs only under extreme conditions, like a constitutively activated AMPK pathway or the 10 d glucose starvation. There, AMPK phosphorylation was significantly increased in 3T3-L1 adipocytes (Figure 4.16 C), whereby mTORC1 activity is slightly reduced in starved cells (Figure 4.19 B). The pharmacological inhibition with rapamycin completely hampered the phosphorylation of p70 S6K by mTORC1. This overall loss of p70 S6K phosphorylation verified that mTORC1 was inhibited during the experimental procedure (Figure 4.18 B). This complex also plays an important role in obesity, because S6K1 null mice are resistant to DIO (Um et al., 2004). Additionally, the higher NNMT expression in mice with DIO (Kraus et al., 2014) might be caused by a reduced S6K1 activity. Thus, NNMT might be regulated by the mTORC1. Furthermore, the activation of AMPK and the subsequent mTORC1 inhibition are targets for different diabetes treatments (Sengupta et al., 2010).

5.4   Increased NNMT protein level even after mTORC1 inhibition

However, another general question must be addressed, now. When mTOR, the master regulator of protein translation, is inhibited, how is it possible that NNMT protein is up-regulated. Therefore, the complete translation has to be interfered at ribosomal level. Indeed, a treatment with rapamycin and cycloheximide, abrogated the NNMT increase (Figure 4.20 B, C). Furthermore, cycloheximide alone reduced the NNMT protein expression. This confirmed that NNMT protein translation is controlled by another regulation mechanism and that the higher protein amount is not due greater protein stabilization. In BJAB lymphoma cells and CTLL-2 T lymphocytes rapamycin induced the expression of nutrient catabolism and energy production genes (Peng et al., 2002). The up-regulated gene profile induced by rapamycin is similar to the gene effects that are caused by amino acid deprivation, not by glucose deprivation (Peng et al., 2002). This could explain the slightly weaker NNMT up-regulation after mTORC1 inhibition, than the strong NNMT expression after AICAR treatment. Furthermore, mTORC1 controls predominantly the translation of mRNA with 5´terminal oligopyrimidine (TOP) motifs (Thoreen et al., 2012). Of note, *in vitro* (NHI 3T3) and *in vivo* rapamycin treatment reduced the cap-dependent, but not the cap-independent translation (Beretta et al., 1996).

There are different mediators that are part of mTORC1 translational control, e. g. the lack of 4E-BPs did not alter cell size, but proliferation was inhibited (Dowling et al., 2010). An active S6K1 regulates different elongation and initiation factors (Dorrello et al., 2006; Wang et al., 2001) as well as S6, a component of the 40S ribosomal subunit (Ruvinsky et al., 2005; Sengupta et al., 2010). They are affected by mTORC1 phosphorylation in a translational promoting manner (Ruvinsky et al., 2005; Sengupta et al., 2010). However, non-phosphorylated S6 can also increases protein synthesis and cell division (Ruvinsky et al., 2005). Since cycloheximide inhibited NNMT protein synthesis during rapamycin treatment, the NNMT protein synthesis is regulated by another mechanism distinct from the typical mTORC1 control.

Cells have to challenge various stresses. Therefore, mRNAs that are part of a stress response evade the typical translation repression (Marques-Ramos et al., 2017; Spriggs et al., 2010). One mechanism are the internal ribosome entry sites (IRESs) (Mitchell et al., 2005). They directly recruit the ribosomes at the 5'UTR and promote the binding of the 40S subunit (Spriggs et al., 2008). Of course, this process is strictly controlled by transacting factors (Powley et al., 2009; Spriggs et al., 2005; Spriggs et al., 2009). This ensures that stress relevant mRNAs are only translated under harmful conditions. A second regulation mechanism for NNMT might involve microRNAs that play a major role in posttranscriptional regulation (Leung and Sharp, 2010). A further explanation for NNMT up-regulation is that the mRNA contains a start codon in the upstream open reading frame (uORFs). In general, these AUG codons have an inhibitory effect during a cap-dependent translation (Spriggs et al., 2010). However, when the normal translation is down-regulated by eIF2 phosphorylation, same stress-important mRNAs are strongly expressed (Spriggs et al., 2010). Interestingly, these uORFs exists in 50 % of all mRNAs (Calvo et al., 2009). One example is ATF4 that is involved in autophagy regulation (Ron and Harding, 2007). It is obvious that long-time glucose starvation, AICAR or rapamycin treatment count to serve stress signals that may allow the NNMT expression by the mentioned translational control. Of course, there are many arguments that a prolonged rapamycin administration is quite harmful for cells. However, various tumors develop a resistance against rapamycin (Gilley et al., 2013), whereby they could maintain their protein synthesis. In addition, multiple injections of rapamycin over 4 weeks did not alter the translational activity in mice, compared to the control group (Garelick et al., 2013). Furthermore, a single administration caused a reduction of the translation process (Garelick et al., 2013). Taken together, there are a lot of possibilities that rescues the protein synthesis during rapamycin treatment or long-time starvation. Further studies are necessary to investigate the NNMT translation in detail, whereby different IRES activity or the uORFs should be analyzed in a new project.

5.5 NNMT expression and autophagy

To confirm whether the reduced mTOR activity is part of the NNMT regulation during a glucose starvation or rapamycin administration, the mTOR activator MHY1485 was used in cell culture. Indeed, 10 µM MHY1485 abrogated the increase in NNMT protein and mRNA (Figure 4.21), whereby the phosphorylation of p70 S6K is enhanced (Figure 4.21 D). MHY1485 is a typical autophagy inhibitor due to its function as mTOR activator (Choi et al., 2012; Lin et al., 2019).

Of note, mTORC1 as well as AMPK are the major regulators of autophagy (Gallagher et al., 2016), that is induced by starvation (Kirkin, 2019). The two signaling pathway also control NNMT expression, as this study shows. Interestingly, the NNMT effect caused by glucose deprivation is reversed by chloroquine and bafilomycin A1 (Figure 4.22 and Figure 4.23 B). Both are strong autophagy inhibitors and they lately interfere this process. Chloroquine not only prevent the increase in NNMT protein level, but even suppress it, whereas the effect of bafilomycin A1 was slightly weaker (Figure 4.22 A). The cause for this discrepancy could be the distinct mechanism of action (Figure 5.1). Chloroquine impairs the fusion with lysosomes (Mauthe et al., 2018) and bafilomycin A1 inhibits the acidification (Mauvezin and Neufeld, 2015). Since a disrupted endosome acidification mimics insulin action on GLUT4 and GLUT1 translocation in 3T3-L1 adipocytes (Chinni and Shisheva, 1999), the bafilomycin A1 effect on NNMT expression might be caused by a possible feedback regulation due to the glucose transporter location. Therefore, this enzyme appears to be sensor for energy depletion.

However, it is more important that mTOR activator and autophagy inhibitor MHY1485 also led to the significant reduction in NNMT protein and mRNA expression. This was similar to the results of bafilomycin A1 and chloroquine. Finally, this demonstrates that NNMT plays a critical role in autophagy, because even the early interruption using MHY1485 prevents the up-regulation of the methyltransferase. The activation of mTORC1 leads to the inhibitory phosphorylation of Ulk1 and Atg13 (Corona Velazquez and Jackson, 2018). Consequently, the autophagosome formation is prevented and less LC3II and p62 is generated than due to bafilomycin A1 and chloroquine administration. Indeed, MHY1485 had a weaker LC3II and p62 signals (Figure 4.22 A and Figure 4.23 A). Additionally, the different amounts of LC3II and p62 protein, shown in western blot (Figure 4.22 B), reflect the effects on NNMT protein level. The strongest p62 and LC3II signals were detected with chloroquine which interferes autophagy at the latest time point. Furthermore, these cells showed the weakest NNMT protein level. The stabilization of p62 and LC3II also indicates a successful autophagy inhibition. The same effect on the LC3II and p62 could be detected in starved 3T3-L1 preadipocytes treated with chloroquine (Igawa et al., 2019).
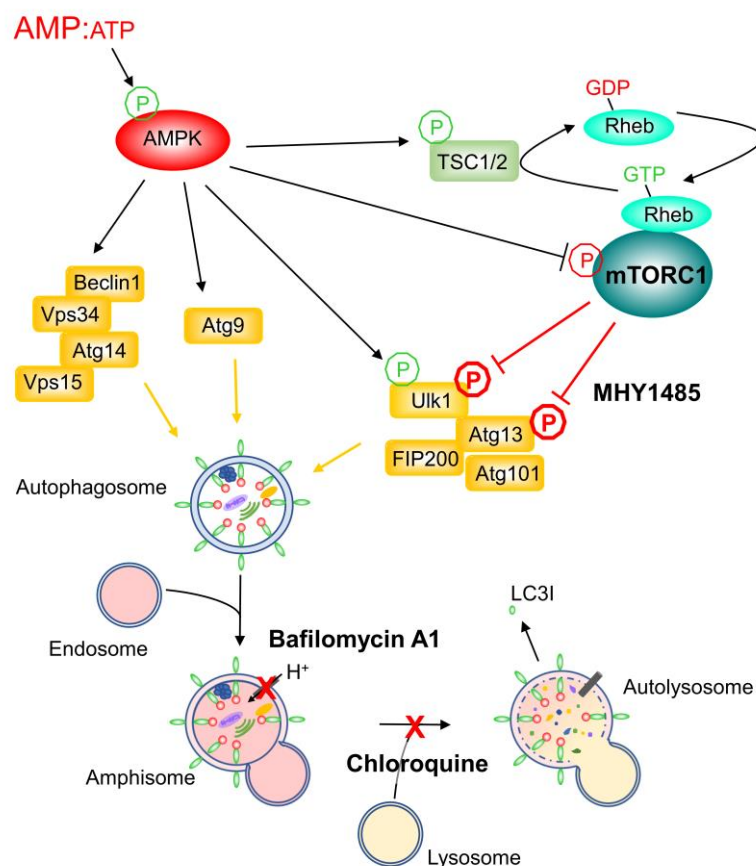
Figure 5.1: **AMPK signaling and mTOR signaling during autophagy.**
The kinase AMPK is activated by energy stress. Simultaneously mTORC1 is repressed. Both promotes autophagy. The inhibited steps of MHY1485, bafilomycin A1, and chloroquine are highlighted. Due to the mTORC1 activation by MHY1485 the phosphorylation of Ulk1 and Atg13 are repressed. Bafilomycin A1 inhibits the vATPase and prevents the necessary acidification. Chloroquine targets the fusion of amphisome and lysosome. Redrawn and modified from (Corona Velazquez and Jackson, 2018; Feng et al., 2015; Garcia and Shaw, 2017; Kaushal et al., 2020; Laplante and Sabatini, 2009; Saxton and Sabatini, 2017).

In further studies the precise role of NNMT must be clarified because autophagy is a cell survival mechanism of diseases, like diabetes and cancer. Early studies already demonstrated that inhibition of this process causes a reduction in white adipose tissue mass (Jung et al., 2008; Singh et al., 2009). This makes NNMT to an interesting target for obesity therapy.

5.5.1 NNMT – important for tumorigenicity and survival under stress conditions

In tumor research autophagy is investigated in different studies, whereas NNMT is becoming more and more important. Its expression correlates always with a great tumorigenicity (Akar et al., 2019; Cui et al., 2020; Eckert et al., 2019; Harmankaya et al., 2020; Li et al., 2019; Shu et al., 2014; Song et al., 2020; Ulanovskaya et al., 2013). However, the distinct role of NNMT is not fully elucidated. Since enough glucose is essential for the tumor tissue, cancer cells developed mechanisms to survive glucose starvation. First it must be mentioned that the tumor tissue consumed 47 to 70 % glucose, whereas the healthy tissue only uses 2 to 18 % (Hirschey et al., 2015); and second the rescue mechanism often involves autophagy (Chen et al., 2018; Endo et al., 2018; Khan et al., 2018; Palorini et al., 2016).

At the moment, there are only few studies that investigated NNMT expression and glucose starvation. In serous ovarian cancer NNMT promotes resistance against glucose starvation (Kanska et al., 2017). Moreover, in pancreatic cancer cells the knockdown of this methyltransferase impairs the resistance against glucose starvation, rapamycin, and 2-DG (Yu et al., 2015). Schmeisser and Parker showed that NNMT overexpression leads to more autophagy, whereas the knockdown reduces it (Schmeisser and Parker, 2018). Additionally, this effect is strengthened by starvation (Schmeisser and Parker, 2018).

Various studies suggest that higher NNMT levels are important for tumor growth, progression, and survival, thereby cancer cells try to escape apoptosis (Mistry et al., 2020; Seta et al., 2018; Xie et al., 2014). Interestingly, a knockdown of NNMT leads to up-regulation of pro-apoptotic genes and down-regulation of anti-apoptotic genes (Zhang et al., 2014). Xie and colleagues demonstrated that the sensitivity to 5-Fluoruracil is increased after NNMT down-regulation (Xie et al., 2016). Even radiation resistance is associated with higher NNMT levels (D'Andrea et al., 2011). These findings show the importance of NNMT for tumor tissue.

As mentioned above autophagy is also a mechanism to survive. Glucose starvation in tumor cells leads to autophagy and the autophagy inhibition leads to cell death (Palorini et al., 2016). Endo and colleagues showed that this autophagy is regulated by AMPK in different cell lines (Endo et al., 2018). Therefore, it would be interesting to investigate which role NNMT has played in both studies. In glioblastoma cells glucose starvation caused autophagy followed by cell quiescence, survival, and chemoresistance (Wang et al., 2018). Of note, the treatment with bafilomycin A1 permitted cell quiescence and survival (Wang et al., 2018). Furthermore, the glucose starved, and bafilomycin A1 treated tumor cells were highly sensitive to carboplatin, a chemotherapeutic agent (Wang et al., 2018). Similar results could be obtained in ovarian cancer (Pagotto et al., 2017). Indeed, NNMT is up-regulated in both cancer types

(Eckert et al., 2019; Jung et al., 2017; Kanska et al., 2017). Recently, in breast cancer cells it was shown that the overexpression of NNMT attenuated autophagy, whereas the knockdown promote autophagy (Yu et al., 2020). There, NNMT suppresses ROS increase, ATP decrease, and AMPK-Ulk1 activation resulting in autophagy inhibition (Yu et al., 2020). Here I show that NNMT expression is again reduced in starved adipocytes after autophagy inhibition. Maybe NNMT is regulated by a feedback loop. Due to an inhibited autophagy NNMT expression is not necessary for the cell and the expression is attenuated.

5.5.2 Autophagy in obesity

The here obtained results show that NNMT also ensures cell survival under low energy conditions in adipocytes. The glucose deprivation led to augmented NNMT expression, that is abrogated by autophagy inhibition. The pharmacological inhibition of autophagy in 3T3-L1 adipocytes represses lipid accumulation, as well as adipocyte specific mouse knockout of Atg7 decreases the white adipose mass and increases insulin sensitivity (Singh et al., 2009). Zhang and colleagues measured the same effect on white adipose tissue. Additionally, they showed that the mutant mice were protected from high-fat-diet-induced obesity (Zhang et al., 2009). Interestingly, a NNMT knockdown had the same effect (Kraus et al., 2014).

Actually, it would be expected that autophagy is reduced during overnutrition and obesity, because one of the major inducers is energy depletion. Indeed, a study suggests that autophagy is more down-regulated in obese mice (Yang et al., 2010). Conversely, interventions in autophagy could have serve consequences for obese individuals. For example, this process contributes to the normalization of lipid metabolism by the elimination of lipid droplets (Singh et al., 2009; Singh and Cuervo, 2012) and an Atg7 haploinsufficiency promotes diabetic pathologies during obesity (Lim et al., 2014). Furthermore, the Atg5 overexpression protects from obesity and insulin resistance (Pyo et al., 2013). Finally, a lot of studies demonstrated that this process is present due to obesity and lipotoxicity in adipose tissue of humans and mice (Jansen et al., 2012; Kovsan et al., 2011; Mei et al., 2011; Nuñez et al., 2013; Ost et al., 2010). Of note, insulin resistance causes a mTORC1 activity repression and a strong up-regulation of autophagy (Ost et al., 2010). The study of Kovsan et al. demonstrated that the autophagic proteins Atg5, LC3I, and LC3II are elevated in omentum majus compared with subcutan fat, whereby the effect was more pronounced in obese samples (Kovsan et al., 2011). Nevertheless, the relationship between obesity and autophagy is not fully understood. NNMT could contribute to a better knowledge.

Taken together, autophagy has got an amazing potential in obesity treatment. The interruption of autophagy decreases adipogenesis, and consequently body weight gain during obesity (Singh et al., 2009; Zhang et al., 2009). To distinguish whether autophagy inhibition is a suitable treatment for a patient, NNMT expression could be a promising marker. Figure 4.1 A shows that NNMT is up-regulated in obese patients. This effect was also measured in non-diabetic, obese Pima Indians (Lee et al., 2005). If the NNMT expression is enhanced in adipose tissue, autophagy inhibition is another therapy, besides the NNMT inhibitors.

5.6   Differentiation of 3T3-L1 cells to stable adipocytes

This study was the first time to investigate the regulation mechanism of NNMT. To measure specific effects the 3T3-L1 cell line was used. In more than 5000 published articles these cells are used to investigate adipogenesis and the biochemistry of adipocytes (Poulos et al., 2010). They were generated through clonal expansion of mouse-derived cells and they contain only one cell type (Poulos et al., 2010). These cells offer further numerous advantages, like mechanistic analysis and high reproducibility of the results. 3T3-L1 preadipocytes have a fibroblast structure before differentiation into adipocytes (Wang et al., 2009). However, a stable and equal differentiation of the cells is important to gain mentioned advantages. In literature various cultivation methods for 3T3-L1 cells are found. Of note, the success of differentiation depends on the media supplements, timing of induction, passage number, and even on the personal handling. Especially, the standardization of the last parameter is quite difficult. Therefore, it is necessary to validate all other aspects for differentiation. Rosiglitazone is one example. Zebisch and colleagues demonstrated that this substance improves the differentiation in early passages, and it is even required in later cell passages (Zebisch et al., 2012). Moreover, Figure 4.6 shows that the FBS is also a critical factor. After the testing of six different sera, only two of them led to 90 % to 100 % 3T3-L1 adipocytes. Because of the almost 100 % differentiation that was reached with the Biochrom Superior serum, it was used for the experiments of this study. The differentiation degree was measured by the quantitative ORO protocol, that was developed in our laboratory (Kraus et al., 2016). The typical lipid droplets are stained by ORO and after dilution of the dye the differentiation status is quantitatively determined. A long-time starvation of unstable or short time differentiated adipocytes led to the loss of lipid and consequently to a de-differentiation (Figure 4.5). Therefore, the differentiation time was expanded to gain stable 3T3-L1 adipocytes.

5.7 Inflammation

5.7.1 IL-6 and TNF-α did not regulate NNMT in 3T3-L1 cells

The adipose tissue is a complex organ. The chronic metaflammation has huge influence on adipocyte gene expression (Hotamisligil, 2006; Hotamisligil, 2017). Indeed, there are alternative pathways for NNMT regulation. IL-6 and LIF control NNMT via STAT3 in cancer cells and HEK293 (Tomida et al., 2008). Further investigations in IL-6 knockout mice showed reduced levels of 1-MN, the reaction product of the methyltransferase, compared with control animals (Sternak et al., 2015). However, the stimulation with IL-6 or TNF-α did not affect NNMT protein level in 3T3-L1 adipocytes (Figure 4.2 and Figure 4.3). Nevertheless, the inflammatory signals could influence NNMT *in vivo*. One problem is that NNMT is up-regulated only after a long-time glucose deprivation or treatment. Unfortunately, TNF-α and IL-6 have to be administered in FBS free media. Figure 4.15 B shows the obvious problem. A longer cultivation without FBS led to augmented NNMT protein levels. Moreover, the inflammatory signal cascade differs between *in vivo* and *in vitro*. Therefore, the next studies concerning NNMT should focus on the investigation of autophagy and inflammation in adipose tissue.

Very interesting studies of both processes are done *in vivo*. For example, an insufficiency in autophagy leads to insulin resistance and higher lipid loading, whereby the results provide evidence for a higher inflammasome activation in Atg7^{+/-} - ob/ob mice (Lim et al., 2014). In obese and insulin resistant adipose tissue LC3 is also increased (Jansen et al., 2012). Additionally, the autophagy inhibition with 3-methylalanine enhance the IL-6 secretion of human and animal explants (Jansen et al., 2012). Nuñez and colleagues found that obesity caused by overfeeding is associated with a defective autophagy regulation (Nuñez et al., 2013). Furthermore, they demonstrated that the pro-inflammatory signals TNF-α and IL-6 are evaluated in adipose tissue of obese individuals (Nuñez et al., 2013). Since none of these studies investigated NNMT, more experiments are necessary to elucidate the role of inflammation on NNMT regulation.

5.7.2 The adiponectin leptin affected NNMT protein expression

Leptin is secreted by adipocytes and mediates its function through the nerve system into the hypothalamus (Stern et al., 2016). It also is an alternative activator of JAK/STAT pathway and it exerts paracrine and autocrine effects on adipocytes (Kraus et al., 2002). Interestingly, the NNMT protein level is elevated by 10 ng/ml leptin (Figure 4.4 A, C), whereas the pro-inflammatory signals did not chance the expression (Figure 4.2 and Figure 4.3). Thus, leptin regulates NNMT not due to its similarity to pro-inflammatory signals, it is more because leptin is an adiponectin with autocrine effects. This hormone impairs insulin-stimulated glucose-uptake in cultured primary rat adipocytes (Müller et al., 1997). Moreover, in muscle leptin activates AMPK (Minokoshi et al., 2002) and hyperleptinemia increased phosphorylation of AMPK in rats (Orci et al., 2004). Therefore, the effect of leptin on NNMT could be attributed to an AMPK dependent mechanism, like shown under glucose starvation in this study. The discrepancy between mRNA and protein cannot be explained. The short time treatment of 24 h could be part of the solution. Maybe the higher NNMT protein content is caused by reduced degradation of NNMT in these adipocytes. However, this has to be addressed in a new study, because this hormone plays an important role during obesity.

Normally, it prevents overnutrition in presence of insulin by inhibition of AMPK in the hypothalamus of lean individuals (Jeon, 2016). In contrast, it is elevated in obese humans and animals (Gregoire et al., 1998). In this study a higher NNMT expression was measured in obese patients (Figure 4.1 A). An equal effect was seen in AG4KO mice, ob/ob, db/db, and DIO mice (Kraus et al., 2014) as well as in obese, non-diabetic Pima Indians (Lee et al., 2005).

Interestingly, the inhibition of autophagy repressed the expression of leptin (Hwang and Lee, 2020). The same positive effect on this process is caused by fasting in obese subjects (Boden et al., 1996). Furthermore, leptin stimulates autophagy of adipocytes shown by elevated expression of Atg5 and LC3II (Goldstein et al., 2019). This could explain the augmented NNMT protein levels after a high concentration of leptin. However, more studies *in vivo* will explain the association between NNMT, autophagy, and leptin.

5.8   Perspective

Of course, further studies are necessary to investigate NNMT regulation in detail, especially *in vivo*. However, I am convinced that NNMT and autophagy are very promising targets for obesity treatment because the glucose deprivation led to increased NNMT expression, that is abrogated by autophagy inhibition. The enzyme is up-regulated in the adipose tissue of obese individuals (Figure 4.1). The same increase was seen in starved 3T3-L1 adipocytes (Figure 4.7), obese Pima Indians (Lee et al., 2005), in AG4KO, ob/ob, db/db, and DIO mice (Kraus et al., 2014).

Indeed, this is the first study that shows a connection between NNMT and autophagy that is necessary for healthy adipose tissue metabolism. The pharmacological inhibition of autophagy in 3T3-L1 adipocytes inhibited lipid accumulation, as well as adipocyte specific mouse knockout of Atg7 decreased the white adipose mass and increased insulin sensitivity (Singh et al., 2009). Zhang and colleagues measured the same effect on white adipose tissue. Additionally, they showed that the mutant mice were protected from high-fat-diet-induced obesity (Zhang et al., 2009). These studies are only a few examples that show that this process is strictly regulated and highly selective. Moreover, not only the obesity therapy could be expedited, but also the treatment of cancer. A combination between autophagy inhibition and glucose deprivation in CR mice could be promising cancer therapy (Lashinger et al., 2016). However, for the development of new treatments in obesity and cancer prevention some important aspects have to be analyzed.

First, the mTOR and AMPK signaling pathway should be investigated in obesity when NNMT expression is increased to demonstrate that NNMT is regulated by these signaling pathways *in vivo*. Then further studies are necessary that analyze NNMT and autophagy. Since there is a growing research of small molecule inhibitors for NNMT, they should be also part of the investigations. In this context, the NNMT genotype also plays an important role because there is an association between a distinct NNMT genotype, the 1-MN concentrations and obesity (Bañales-Luna et al., 2020). This study shows how important the genetic background is and it could be a partial explanation of the differential regulation of NNMT expression and activity (Brachs et al., 2019; Rudolphi et al., 2018). In future studies the enzymatic activity has to be investigated, too.

Second, the insulin resistance, that is often associated with obesity, cannot be neglected, because various studies show that starvation causes insulin resistance in lean and obese individuals (Duska et al., 2005; Mansell and Macdonald, 1990; Newman and Brodows, 1983). Furthermore, in the adipose tissue the insulin sensitive GLUT4 is responsible for glucose

uptake (Shepherd and Kahn, 1999). It would be interesting whether autophagy occurs during insulin resistance and how NNMT expression is changed when the insulin sensitivity is restored *in vivo*.

Taken together this study shows that NNMT is a promising target for obesity treatment. The greatest advantage is that NNMT is predominantly expressed in adipose tissue. Therefore, side effects could be rigorously reduced. Furthermore, a successful treatment of obese humans can prevent obesity-related cancer types. Interestingly, the glucose availability appears to regulate NNMT even in cancer. Both adipocytes and rapidly proliferating cancer cells need a lot of energy, whereby increased NNMT levels are detected in obesity and many tumors. Thus, this methyltransferase emerges as an awesome therapeutic target.

References

Abbud, W., Habinowski, S., Zhang, J. Z., Kendrew, J., Elkairi, F. S., Kemp, B. E., Witters, L. A. and Ismail-Beigi, F. (2000). Stimulation of AMP-activated protein kinase (AMPK) is associated with enhancement of Glut1-mediated glucose transport. *Archives of Biochemistry and Biophysics*. **380**, 347–352. doi:10.1006/abbi.2000.1935.

Abel, E. D., Peroni, O., Kim, J. K., Kim, Y. B., Boss, O., Hadro, E., Minnemann, T., Shulman, G. I. and Kahn, B. B. (2001). Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver. *Nature*. **409**, 729–733. doi:10.1038/35055575.

Akar, S., Harmankaya, İ., Uğraş, S. and Çelik, Ç. (2019). Nicotinamide N-methyltransferase expression and its association with phospho-Akt, p53 expression, and survival in high-grade endometrial cancer. *Turkish journal of medical sciences*. **49**, 1547–1554. doi:10.3906/sag-1907-166.

Aksoy, S., Szumlanski, C. L. and Weinshilboum, R. M. (1994). Human liver nicotinamide N-methyltransferase. cDNA cloning, expression, and biochemical characterization. *The Journal of biological chemistry*. **269**, 14835–14840.

Augustin, R. (2010). The protein family of glucose transport facilitators: It's not only about glucose after all. *IUBMB life*. **62**, 315–333. doi:10.1002/iub.315.

Babault, N., Allali-Hassani, A., Li, F., Fan, J., Yue, A., Ju, K., Liu, F., Vedadi, M., Liu, J. and Jin, J. (2018). Discovery of Bisubstrate Inhibitors of Nicotinamide N-Methyltransferase (NNMT). *Journal of medicinal chemistry* [Epub ahead of print]. doi:10.1021/acs.jmedchem.7b01422.

Baker, M. (2016). Reproducibility: Respect your cells! *Nature*. **537**, 433–435. doi:10.1038/537433a.

Balhoff, J. P. and Stephens, J. M. (1998). Highly specific and quantitative activation of STATs in 3T3-L1 adipocytes. *Biochemical and biophysical research communications*. **247**, 894–900. doi:10.1006/bbrc.1998.8890.

Bañales-Luna, M., Figueroa-Vega, N., Marín-Aragón, C. I., Perez-Luque, E., Ibarra-Reynoso, L., Gallardo-Blanco, H. L., López-Aguilar, I. and Malacara, J. M. (2020). Associations of nicotidamide-N-methyltransferase, FTO, and IRX3 genetic variants with body mass index and resting energy expenditure in Mexican subjects. *Sci Rep*. **10**, 11478. doi:10.1038/s41598-020-67832-7.

Barra, N. G., Reid, S., MacKenzie, R., Werstuck, G., Trigatti, B. L., Richards, C., Holloway, A. C. and Ashkar, A. A. (2010). Interleukin-15 contributes to the regulation of murine adipose tissue and human adipocytes. *Obesity (Silver Spring, Md.)*. **18**, 1601–1607. doi:10.1038/oby.2009.445.

Bechor, S., Nachmias, D., Elia, N., Haim, Y., Vatarescu, M., Leikin-Frenkel, A., Gericke, M., Tarnovscki, T., Las, G. and Rudich, A. (2017). Adipose tissue conditioned media

support macrophage lipid-droplet biogenesis by interfering with autophagic flux. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*. **1862**, 1001–1012. doi:10.1016/j.bbalip.2017.06.012.

Bentley, J., Itchayanan, D., Barnes, K., McIntosh, E., Tang, X., Downes, C. P., Holman, G. D., Whetton, A. D., Owen-Lynch, P. J. and Baldwin, S. A. (2003). Interleukin-3-mediated cell survival signals include phosphatidylinositol 3-kinase-dependent translocation of the glucose transporter GLUT1 to the cell surface. *The Journal of biological chemistry*. **278**, 39337–39348. doi:10.1074/jbc.M305689200.

Beretta, L., Gingras, A. C., Svitkin, Y. V., Hall, M. N. and Sonenberg, N. (1996). Rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap-dependent initiation of translation. *The EMBO Journal*. **15**, 658–664. doi:10.1002/j.1460-2075.1996.tb00398.x.

Bhattacharya, D., Mukhopadhyay, M., Bhattacharyya, M. and Karmakar, P. (2018). Is autophagy associated with diabetes mellitus and its complications? A review. *EXCLI journal*. **17**, 709–720. doi:10.17179/excli2018-1353.

Boden, G., Chen, X., Mozzoli, M. and Ryan, I. (1996). Effect of fasting on serum leptin in normal human subjects. *The Journal of clinical endocrinology and metabolism*. **81**, 3419–3423. doi:10.1210/jcem.81.9.8784108.

Brachs, S., Polack, J., Brachs, M., Jahn-Hofmann, K., Elvert, R., Pfenninger, A., Barenz, F., Margerie, D., Mai, K. and Spranger, J. et al. (2019). Genetic Nicotinamide N-Methyltransferase (Nnmt) Deficiency in Male Mice Improves Insulin Sensitivity in Diet-Induced Obesity but Does Not Affect Glucose Tolerance. *Diabetes* [Epub ahead of print]. doi:10.2337/db18-0780.

Branca, F., Nikogosian, H. and Lobstein, T., eds. (2007). *The challenge of obesity in the WHO European Region and the strategies for response: Summary*. Kopenhagen: World Health Organization Regional Office for Europe.

Buller, C. L., Loberg, R. D., Fan, M.-H., Zhu, Q., Park, J. L., Vesely, E., Inoki, K., Guan, K.-L. and Brosius, F. C. (2008). A GSK-3/TSC2/mTOR pathway regulates glucose uptake and GLUT1 glucose transporter expression. *American journal of physiology. Cell physiology*. **295**, C836-43. doi:10.1152/ajpcell.00554.2007.

Calvo, S. E., Pagliarini, D. J. and Mootha, V. K. (2009). Upstream open reading frames cause widespread reduction of protein expression and are polymorphic among humans. *PNAS*. **106**, 7507–7512. doi:10.1073/pnas.0810916106.

Cantoni, G. L. (1951). Methylation of nicotinamide with soluble enzyme system from rat liver. *The Journal of biological chemistry*. **189**, 203–216.

Cao, Y., Matsubara, T., Zhao, C., Gao, W., Peng, L., Shan, J., Liu, Z., Yuan, F., Tang, L. and Li, P. et al. (2017). Antisense oligonucleotide and thyroid hormone conjugates for obesity treatment. *Scientific Reports*. **7**, 9307. doi:10.1038/s41598-017-09598-z.

Caron, A., Richard, D. and Laplante, M. (2015). The Roles of mTOR Complexes in Lipid Metabolism. *Annual review of nutrition.* **35**, 321–348. doi:10.1146/annurev-nutr-071714-034355.

Carvalho, E., Kotani, K., Peroni, O. D. and Kahn, B. B. (2005). Adipose-specific overexpression of GLUT4 reverses insulin resistance and diabetes in mice lacking GLUT4 selectively in muscle. *American journal of physiology. Endocrinology and metabolism.* **289**, E551-61. doi:10.1152/ajpendo.00116.2005.

Ceddia, R. B. (2013). The role of AMP-activated protein kinase in regulating white adipose tissue metabolism. *Molecular and cellular endocrinology.* **366**, 194–203. doi:10.1016/j.mce.2012.06.014.

Chen, D., Li, L., Diaz, K., Iyamu, I. D., Yadav, R., Noinaj, N. and Huang, R. (2019). Novel Propargyl-Linked Bisubstrate Analogues as Tight-Binding Inhibitors for Nicotinamide N-Methyltransferase. *Journal of medicinal chemistry.* **62**, 10783–10797. doi:10.1021/acs.jmedchem.9b01255.

Chen, G., Liu, H., Zhang, Y., Liang, J., Zhu, Y., Zhang, M., Yu, D., Wang, C. and Hou, J. (2018). Silencing PFKP inhibits starvation-induced autophagy, glycolysis, and epithelial mesenchymal transition in oral squamous cell carcinoma. *Experimental cell research.* **370**, 46–57. doi:10.1016/j.yexcr.2018.06.007.

Chinni, S. R. and Shisheva, A. (1999). Arrest of endosome acidification by bafilomycin A1 mimics insulin action on GLUT4 translocation in 3T3-L1 adipocytes. *Biochemical Journal.* **339**, 599–606. doi:10.1042/bj3390599.

Choi, Y. J., Park, Y. J., Park, J. Y., Jeong, H. O., Kim, D. H., Ha, Y. M., Kim, J. M., Song, Y. M., Heo, H.-S. and Yu, B. P. et al. (2012). Inhibitory effect of mTOR activator MHY1485 on autophagy: suppression of lysosomal fusion. *PloS one.* **7**, e43418. doi:10.1371/journal.pone.0043418.

Codogno, P. and Meijer, A. J. (2005). Autophagy and signaling: their role in cell survival and cell death. *Cell death and differentiation.* **12 Suppl 2**, 1509–1518. doi:10.1038/sj.cdd.4401751.

Cole, L. A. and Kramer, P. R. (2016). *Human physiology, biochemistry and basic medicine.* Amsterdam: Academic Press is an Elsevier.

Corona Velazquez, A. F. and Jackson, W. T. (2018). So Many Roads: the Multifaceted Regulation of Autophagy Induction. *Molecular and Cellular Biology.* **38**. doi:10.1128/MCB.00303-18.

Corton, J. M., Gillespie, J. G., Hawley, S. A. and Hardie, D. G. (1995). 5-aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells? *European Journal of Biochemistry.* **229**, 558–565. doi:10.1111/j.1432-1033.1995.tb20498.x.

Cui, Y., Yang, D., Wang, W., Zhang, L., Liu, H., Ma, S., Guo, W., Yao, M., Zhang, K. and Li, W. et al. (2020). Nicotinamide N-methyltransferase decreases 5-fluorouracil sensitivity in human esophageal squamous cell carcinoma through metabolic reprogramming and promoting the Warburg effect. *Molecular carcinogenesis.* **59**, 940–954. doi:10.1002/mc.23209.

Cui, Y., Zhang, L., Wang, W., Ma, S., Liu, H., Zang, X., Zhang, Y. and Guan, F. (2019). Downregulation of nicotinamide N-methyltransferase inhibits migration and epithelial-mesenchymal transition of esophageal squamous cell carcinoma via Wnt/beta-catenin pathway. *Molecular and cellular biochemistry* [Epub ahead of print]. doi:10.1007/s11010-019-03573-0.

Czech, M. P., Tencerova, M., Pedersen, D. J. and Aouadi, M. (2013). Insulin signalling mechanisms for triacylglycerol storage. *Diabetologia.* **56**, 949–964. doi:10.1007/s00125-013-2869-1.

D'Andrea, F. P., Safwat, A., Kassem, M., Gautier, L., Overgaard, J. and Horsman, M. R. (2011). Cancer stem cell overexpression of nicotinamide N-methyltransferase enhances cellular radiation resistance. *Radiotherapy and oncology : journal of the European Society for Therapeutic Radiology and Oncology.* **99**, 373–378. doi:10.1016/j.radonc.2011.05.086.

DiPaola, R. S., Dvorzhinski, D., Thalasila, A., Garikapaty, V., Doram, D., May, M., Bray, K., Mathew, R., Beaudoin, B. and Karp, C. et al. (2008). Therapeutic starvation and autophagy in prostate cancer: a new paradigm for targeting metabolism in cancer therapy. *The Prostate.* **68**, 1743–1752. doi:10.1002/pros.20837.

Dorrello, N. V., Peschiaroli, A., Guardavaccaro, D., Colburn, N. H., Sherman, N. E. and Pagano, M. (2006). S6K1- and betaTRCP-mediated degradation of PDCD4 promotes protein translation and cell growth. *Science.* **314**, 467–471. doi:10.1126/science.1130276.

Dowling, R. J. O., Topisirovic, I., Alain, T., Bidinosti, M., Fonseca, B. D., Petroulakis, E., Wang, X., Larsson, O., Selvaraj, A. and Liu, Y. et al. (2010). mTORC1-mediated cell proliferation, but not cell growth, controlled by the 4E-BPs. *Science.* **328**, 1172–1176. doi:10.1126/science.1187532.

Duska, F., Andel, M., Kubena, A. and Macdonald, I. A. (2005). Effects of acute starvation on insulin resistance in obese patients with and without type 2 diabetes mellitus. *Clinical nutrition (Edinburgh, Scotland).* **24**, 1056–1064. doi:10.1016/j.clnu.2005.08.008.

Eckert, M. A., Coscia, F., Chryplewicz, A., Chang, J. W., Hernandez, K. M., Pan, S., Tienda, S. M., Nahotko, D. A., Li, G. and Blazenovic, I. et al. (2019). Proteomics reveals NNMT as a master metabolic regulator of cancer-associated fibroblasts. *Nature.* **569**, 723–728. doi:10.1038/s41586-019-1173-8.

Endo, H., Owada, S., Inagaki, Y., Shida, Y. and Tatemichi, M. (2018). Glucose starvation induces LKB1-AMPK-mediated MMP-9 expression in cancer cells. *Sci Rep.* **8**, 10122. doi:10.1038/s41598-018-28074-w.

Felsted, R. L. and Chaykin, S. (1967). N1-methylnicotinamide oxidation in a number of mammals. *The Journal of biological chemistry*. **242**, 1274–1279.

Feng, Y., Yao, Z. and Klionsky, D. J. (2015). How to control self-digestion: transcriptional, post-transcriptional, and post-translational regulation of autophagy. *Trends in cell biology*. **25**, 354–363. doi:10.1016/j.tcb.2015.02.002.

Fryer, L. G. D., Foufelle, F., Barnes, K., Baldwin, S. A., Woods, A. and Carling, D. (2002). Characterization of the role of the AMP-activated protein kinase in the stimulation of glucose transport in skeletal muscle cells. *Biochemical Journal*. **363**, 167–174. doi:10.1042/0264-6021:3630167.

Gaidhu, M. P. and Ceddia, R. B. (2009). Remodeling glucose and lipid metabolism through AMPK activation: relevance for treating obesity and Type 2 diabetes. *Clinical Lipidology*. **4**, 465–477. doi:10.2217/clp.09.30.

Gaidhu, M. P., Fediuc, S. and Ceddia, R. B. (2006). 5-Aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside-induced AMP-activated protein kinase phosphorylation inhibits basal and insulin-stimulated glucose uptake, lipid synthesis, and fatty acid oxidation in isolated rat adipocytes. *The Journal of biological chemistry*. **281**, 25956–25964. doi:10.1074/jbc.M602992200.

Gallagher, L. E., Williamson, L. E. and Chan, E. Y. W. (2016). Advances in Autophagy Regulatory Mechanisms. *Cells*. **5**. doi:10.3390/cells5020024.

Gao, Y., van Haren, M. J., Moret, E. E., Rood, J. J. M., Sartini, D., Salvucci, A., Emanuelli, M., Craveur, P., Babault, N. and Jin, J. et al. (2019). Bisubstrate Inhibitors of Nicotinamide N-Methyltransferase (NNMT) with Enhanced Activity. *Journal of medicinal chemistry*. **62**, 6597–6614. doi:10.1021/acs.jmedchem.9b00413.

Garcia, D. and Shaw, R. J. (2017). AMPK: Mechanisms of Cellular Energy Sensing and Restoration of Metabolic Balance. *Molecular Cell*. **66**, 789–800. doi:10.1016/j.molcel.2017.05.032.

Garelick, M. G., MacKay, V. L., Yanagida, A., Academia, E. C., Schreiber, K. H., Ladiges, W. C. and Kennedy, B. K. (2013). Chronic rapamycin treatment or lack of S6K1 does not reduce ribosome activity in vivo. *Cell Cycle*. **12**, 2493–2504. doi:10.4161/cc.25512.

Gilley, R., Balmanno, K., Cope, C. L. and Cook, S. J. (2013). Adaptation to chronic mTOR inhibition in cancer and in aging. *Biochemical Society Transactions*. **41**, 956–961. doi:10.1042/BST20130080.

Goldstein, N., Haim, Y., Mattar, P., Hadadi-Bechor, S., Maixner, N., Kovacs, P., Blüher, M. and Rudich, A. (2019). Leptin stimulates autophagy/lysosome-related degradation of long-lived proteins in adipocytes. *Adipocyte*. **8**, 51–60. doi:10.1080/21623945.2019.1569447.

Green, H. and Meuth, M. (1974). An established pre-adipose cell line and its differentiation in culture. *Cell*. **3**, 127–133. doi:10.1016/0092-8674(74)90116-0.

Gregoire, F. M., Smas, C. M. and Sul, H. S. (1998). Understanding adipocyte differentiation. *Physiological Reviews.* **78**, 783–809. doi:10.1152/physrev.1998.78.3.783.

Gregor, M. F. and Hotamisligil, G. S. (2011). Inflammatory mechanisms in obesity. *Annual review of immunology.* **29**, 415–445. doi:10.1146/annurev-immunol-031210-101322.

Guilherme, A., Virbasius, J. V., Puri, V. and Czech, M. P. (2008). Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. *Nat Rev Mol Cell Biol.* **9**, 367–377. doi:10.1038/nrm2391.

Gwinn, D. M., Shackelford, D. B., Egan, D. F., Mihaylova, M. M., Mery, A., Vasquez, D. S., Turk, B. E. and Shaw, R. J. (2008). AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Molecular cell.* **30**, 214–226. doi:10.1016/j.molcel.2008.03.003.

Hardie, D. G. (2007). AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nature reviews. Molecular cell biology.* **8**, 774–785. doi:10.1038/nrm2249.

Hardie, D. G. (2011). AMP-activated protein kinase: an energy sensor that regulates all aspects of cell function. *Genes & development.* **25**, 1895–1908. doi:10.1101/gad.17420111.

Hardie, D. G. (2013). AMPK: a target for drugs and natural products with effects on both diabetes and cancer. *Diabetes.* **62**, 2164–2172. doi:10.2337/db13-0368.

Harmankaya, İ., Akar, S., Uğraş, S., Güler, A. H., Ezveci, H., Aydoğdu, M. and Çelik, Ç. (2020). Nicotinamide N-methyltransferase overexpression may be associated with poor prognosis in ovarian cancer. *Journal of obstetrics and gynaecology : the journal of the Institute of Obstetrics and Gynaecology*, 1–6. doi:10.1080/01443615.2020.1732891.

Harris, R. B. S. (2014). Direct and indirect effects of leptin on adipocyte metabolism. *Biochimica et biophysica acta.* **1842**, 414–423. doi:10.1016/j.bbadis.2013.05.009.

Hatakeyama, H., Morino, T., Ishii, T. and Kanzaki, M. (2019). Cooperative actions of Tbc1d1 and AS160/Tbc1d4 in GLUT4-trafficking activities. *J. Biol. Chem.* **294**, 1161–1172. doi:10.1074/jbc.RA118.004614.

Hawley, S. A., Boudeau, J., Reid, J. L., Mustard, K. J., Udd, L., Mäkelä, T. P., Alessi, D. R. and Hardie, D. G. (2003). Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. *Journal of biology.* **2**, 28. doi:10.1186/1475-4924-2-28.

Heinrich, P. C., Müller, M., Graeve, L. and Löffler, G. (2014). *Löffler/Petrides Biochemie und Pathobiochemie.* Berlin: Springer.

Hirschey, M. D., DeBerardinis, R. J., Diehl, A. M. E., Drew, J. E., Frezza, C., Green, M. F., Jones, L. W., Ko, Y. H., Le, A. and Lea, M. A. et al. (2015). Dysregulated metabolism contributes to oncogenesis. *Seminars in cancer biology.* **35 Suppl**, S129-S150. doi:10.1016/j.semcancer.2015.10.002.

Hong, S., Moreno-Navarrete, J. M., Wei, X., Kikukawa, Y., Tzameli, I., Prasad, D., Lee, Y., Asara, J. M., Fernandez-Real, J. M. and Maratos-Flier, E. et al. (2015). Nicotinamide N-methyltransferase regulates hepatic nutrient metabolism through Sirt1 protein stabilization. *Nature medicine.* **21**, 887–894. doi:10.1038/nm.3882.

Hotamisligil, G. S., Peraldi, P., Budavari, A., Ellis, R., White, M. F. and Spiegelman, B. M. (1996). IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. *Science (New York, N.Y.).* **271**, 665–668. doi:10.1126/science.271.5249.665.

Hotamisligil, G. S. (2006). Inflammation and metabolic disorders. *Nature.* **444**, 860–867. doi:10.1038/nature05485.

Hotamisligil, G. S. (2017). Inflammation, metaflammation and immunometabolic disorders. *Nature.* **542**, 177–185. doi:10.1038/nature21363.

Hurley, R. L., Anderson, K. A., Franzone, J. M., Kemp, B. E., Means, A. R. and Witters, L. A. (2005). The Ca2+/calmodulin-dependent protein kinase kinases are AMP-activated protein kinase kinases. *The Journal of biological chemistry.* **280**, 29060–29066. doi:10.1074/jbc.M503824200.

Hwang, S.-H. and Lee, M. (2020). Autophagy inhibition in 3T3-L1 adipocytes breaks the crosstalk with tumor cells by suppression of adipokine production. *Animal cells and systems.* **24**, 17–25. doi:10.1080/19768354.2019.1700159.

Ichimura, Y. and Komatsu, M. (2010). Selective degradation of p62 by autophagy. *Seminars in immunopathology.* **32**, 431–436. doi:10.1007/s00281-010-0220-1.

Igawa, H., Kikuchi, A., Misu, H., Ishii, K.-A., Kaneko, S. and Takamura, T. (2019). p62-mediated autophagy affects nutrition-dependent insulin receptor substrate 1 dynamics in 3T3-L1 preadipocytes. *Journal of diabetes investigation.* **10**, 32–42. doi:10.1111/jdi.12866.

Im, S.-S., Kwon, S.-K., Kim, T.-H., Kim, H.-i. and Ahn, Y.-H. (2007). Regulation of glucose transporter type 4 isoform gene expression in muscle and adipocytes. *IUBMB life.* **59**, 134–145. doi:10.1080/15216540701313788.

Inoki, K., Kim, J. and Guan, K.-L. (2012). AMPK and mTOR in cellular energy homeostasis and drug targets. *Annual review of pharmacology and toxicology.* **52**, 381–400. doi:10.1146/annurev-pharmtox-010611-134537.

Inoki, K., Zhu, T. and Guan, K.-L. (2003). TSC2 mediates cellular energy response to control cell growth and survival. *Cell.* **115**, 577–590. doi:10.1016/s0092-8674(03)00929-2.

Jansen, H. J., van Essen, P., Koenen, T., Joosten, L. A. B., Netea, M. G., Tack, C. J. and Stienstra, R. (2012). Autophagy activity is up-regulated in adipose tissue of obese individuals and modulates proinflammatory cytokine expression. *Endocrinology.* **153**, 5866–5874. doi:10.1210/en.2012-1625.

Jeon, S.-M. (2016). Regulation and function of AMPK in physiology and diseases. *Experimental & molecular medicine.* **48**, e245. doi:10.1038/emm.2016.81.

Johansen, T. and Lamark, T. (2011). Selective autophagy mediated by autophagic adapter proteins. *Autophagy.* **7**, 279–296. doi:10.4161/auto.7.3.14487.

Jung, C. H., Ro, S.-H., Cao, J., Otto, N. M. and Kim, D.-H. (2010). mTOR regulation of autophagy. *FEBS Letters.* **584**, 1287–1295. doi:10.1016/j.febslet.2010.01.017.

Jung, H. S., Chung, K. W., Won Kim, J., Kim, J., Komatsu, M., Tanaka, K., Nguyen, Y. H., Kang, T. M., Yoon, K.-H. and Kim, J.-W. et al. (2008). Loss of autophagy diminishes pancreatic beta cell mass and function with resultant hyperglycemia. *Cell metabolism.* **8**, 318–324. doi:10.1016/j.cmet.2008.08.013.

Jung, J., Kim, L. J. Y., Wang, X., Wu, Q., Sanvoranart, T., Hubert, C. G., Prager, B. C., Wallace, L. C., Jin, X. and Mack, S. C. et al. (2017). Nicotinamide metabolism regulates glioblastoma stem cell maintenance. *JCI insight.* **2**. doi:10.1172/jci.insight.90019.

Kannt, A., Pfenninger, A., Teichert, L., Tonjes, A., Dietrich, A., Schon, Kloting, N. and Bluher, M. (2015). Association of nicotinamide-N-methyltransferase mRNA expression in human adipose tissue and the plasma concentration of its product, 1-methylnicotinamide, with insulin resistance. *Diabetologia.* **58**, 799–808.

Kannt, A., Rajagopal, S., Kadnur, S. V., Suresh, J., Bhamidipati, R. K., Swaminathan, S., Hallur, M. S., Kristam, R., Elvert, R. and Czech, J. et al. (2018). A small molecule inhibitor of Nicotinamide N-methyltransferase for the treatment of metabolic disorders. *Scientific Reports.* **8**, 3660. doi:10.1038/s41598-018-22081-7.

Kanska, J., Aspuria, P.-J. P., Taylor-Harding, B., Spurka, L., Funari, V., Orsulic, S., Karlan, B. Y. and Wiedemeyer, W. R. (2017). Glucose deprivation elicits phenotypic plasticity via ZEB1-mediated expression of NNMT. *Oncotarget* [Epub ahead of print]. doi:10.18632/oncotarget.15429.

Kaushal, G. P., Chandrashekar, K., Juncos, L. A. and Shah, S. V. (2020). Autophagy Function and Regulation in Kidney Disease. *Biomolecules.* **10**. doi:10.3390/biom10010100.

Khan, M. W., Layden, B. T. and Chakrabarti, P. (2018). Inhibition of mTOR complexes protects cancer cells from glutamine starvation induced cell death by restoring Akt stability. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease.* **1864**, 2040–2052. doi:10.1016/j.bbadis.2018.03.013.

Kim, J., Kim, Y. C., Fang, C., Russell, R. C., Kim, J. H., Fan, W., Liu, R., Zhong, Q. and Guan, K.-L. (2013). Differential Regulation of Distinct Vps34 Complexes by AMPK in Nutrient Stress and Autophagy. *Cell.* **152**, 290–303. doi:10.1016/j.cell.2012.12.016.

Kim, J., Kundu, M., Viollet, B. and Guan, K.-L. (2011). AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nature Cell Biology.* **13**, 132–141. doi:10.1038/ncb2152.

Kim, J., Yang, G., Kim, Y., Kim, J. and Ha, J. (2016). AMPK activators: mechanisms of action and physiological activities. *Experimental & molecular medicine.* **48**, e224. doi:10.1038/emm.2016.16.

Kimura, N., Tokunaga, C., Dalal, S., Richardson, C., Yoshino, K.-i., Hara, K., Kemp, B. E., Witters, L. A., Mimura, O. and Yonezawa, K. (2003). A possible linkage between AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) signalling pathway. *Genes to Cells.* **8**, 65–79. doi:10.1046/j.1365-2443.2003.00615.x.

Kirkin, V. (2019). History of the Selective Autophagy Research: How Did It Begin and Where Does It Stand Today? *Journal of Molecular Biology* [Epub ahead of print]. doi:10.1016/j.jmb.2019.05.010.

Klein, J., Perwitz, N., Kraus, D. and Fasshauer, M. (2006). Adipose tissue as source and target for novel therapies. *Trends in endocrinology and metabolism: TEM.* **17**, 26–32. doi:10.1016/j.tem.2005.11.008.

Kovsan, J., Blüher, M., Tarnovscki, T., Klöting, N., Kirshtein, B., Madar, L., Shai, I., Golan, R., Harman-Boehm, I. and Schön, M. R. et al. (2011). Altered autophagy in human adipose tissues in obesity. *The Journal of clinical endocrinology and metabolism.* **96**, E268-77. doi:10.1210/jc.2010-1681.

Kozka, I. J., Clark, A. E. and Holman, G. D. (1991). Chronic treatment with insulin selectively down-regulates cell-surface GLUT4 glucose transporters in 3T3-L1 adipocytes. *The Journal of biological chemistry.* **266**, 11726–11731.

Kraus, D., Fasshauer, M., Ott, V., Meier, B., Jost, M., Klein, H. H. and Klein, J. (2002). Leptin secretion and negative autocrine crosstalk with insulin in brown adipocytes. *The Journal of endocrinology.* **175**, 185–191. doi:10.1677/joe.0.1750185.

Kraus, D., Yang, Q., Kong, D., Banks, A. S., Zhang, L., Rodgers, J. T., Pirinen, E., Pulinilkunnil, T. C., Gong, F. and Wang, Y. C. et al. (2014). Nicotinamide N-methyltransferase knockdown protects against diet-induced obesity. *Nature.* **508**, 258–262. doi:10.1038/nature13198.

Kraus, N. A., Ehebauer, F., Zapp, B., Rudolphi, B., Kraus, B. J. and Kraus, D. (2016). Quantitative assessment of adipocyte differentiation in cell culture. *Adipocyte*, 1–8. doi:10.1080/21623945.2016.1240137.

Lamming, D. W. and Sabatini, D. M. (2013). A Central role for mTOR in lipid homeostasis. *Cell metabolism.* **18**, 465–469. doi:10.1016/j.cmet.2013.08.002.

Laplante, M. and Sabatini, D. M. (2009). mTOR signaling at a glance. *Journal of cell science.* **122**, 3589–3594. doi:10.1242/jcs.051011.

Laplante, M. and Sabatini, D. M. (2012). mTOR signaling in growth control and disease. *Cell.* **149**, 274–293. doi:10.1016/j.cell.2012.03.017.

Laplante, M. and Sabatini, D. M. (2013). Regulation of mTORC1 and its impact on gene expression at a glance. *Journal of cell science*. **126**, 1713–1719. doi:10.1242/jcs.125773.

Lashinger, L. M., O'Flanagan, C. H., Dunlap, S. M., Rasmussen, A. J., Sweeney, S., Guo, J. Y., Lodi, A., Tiziani, S., White, E. and Hursting, S. D. (2016). Starving cancer from the outside and inside: separate and combined effects of calorie restriction and autophagy inhibition on Ras-driven tumors. *Cancer & metabolism*. **4**, 18. doi:10.1186/s40170-016-0158-4.

Lee, J. W., Park, S., Takahashi, Y. and Wang, H.-G. (2010). The association of AMPK with ULK1 regulates autophagy. *PloS one*. **5**, e15394. doi:10.1371/journal.pone.0015394.

Lee, Y. H., Nair, S., Rousseau, E., Allison, D. B., Page, G. P., Tataranni, P. A., Bogardus, C. and Permana, P. A. (2005). Microarray profiling of isolated abdominal subcutaneous adipocytes from obese vs non-obese Pima Indians: increased expression of inflammation-related genes. *Diabetologia*. **48**, 1776–1783. doi:10.1007/s00125-005-1867-3.

Leung, A. K. L. and Sharp, P. A. (2010). MicroRNA functions in stress responses. *Molecular cell*. **40**, 205–215. doi:10.1016/j.molcel.2010.09.027.

Li, J., You, S., Zhang, S., Hu, Q., Wang, F., Chi, X., Zhao, W., Xie, C., Zhang, C. and Yu, Y. et al. (2019). Elevated N-methyltransferase expression induced by hepatic stellate cells contributes to the metastasis of hepatocellular carcinoma via regulation of the CD44v3 isoform. *Molecular oncology* [Epub ahead of print]. doi:10.1002/1878-0261.12544.

Lim, Y.-M., Lim, H., Hur, K. Y., Quan, W., Lee, H.-Y., Cheon, H., Ryu, D., Koo, S.-H., Kim, H. L. and Kim, J. et al. (2014). Systemic autophagy insufficiency compromises adaptation to metabolic stress and facilitates progression from obesity to diabetes. *Nature Communications*. **5**, 4934. doi:10.1038/ncomms5934.

Lin, R., Elf, S., Shan, C., Kang, H.-B., Ji, Q., Zhou, L., Hitosugi, T., Zhang, L., Zhang, S. and Ho Seo, J. et al. (2015). 6-phosphogluconate dehydrogenase links oxidative PPP, lipogenesis and tumor growth by inhibiting LKB1-AMPK signaling. *Nature Cell Biology*. **17**, 1484–1496. doi:10.1038/ncb3255.

Lin, X., Peng, Z., Wang, X., Zou, J., Chen, D., Chen, Z., Li, Z., Dong, B., Gao, J. and Shen, L. (2019). Targeting autophagy potentiates antitumor activity of Met-TKIs against Met-amplified gastric cancer. *Cell death & disease*. **10**, 139. doi:10.1038/s41419-019-1314-x.

Mansell, P. I. and Macdonald, I. A. (1990). The effect of starvation on insulin-induced glucose disposal and thermogenesis in humans. *Metabolism: clinical and experimental*. **39**, 502–510. doi:10.1016/0026-0495(90)90009-2.

Marques-Ramos, A., Candeias, M. M., Menezes, J., Lacerda, R., Willcocks, M., Teixeira, A., Locker, N. and Romão, L. (2017). Cap-independent translation ensures mTOR expression and function upon protein synthesis inhibition. *RNA (New York, N.Y.)*. **23**, 1712–1728. doi:10.1261/rna.063040.117.

Mauthe, M., Orhon, I., Rocchi, C., Zhou, X., Luhr, M., Hijlkema, K.-J., Coppes, R. P., Engedal, N., Mari, M. and Reggiori, F. (2018). Chloroquine inhibits autophagic flux by decreasing autophagosome-lysosome fusion. *Autophagy.* **14**, 1435–1455. doi:10.1080/15548627.2018.1474314.

Mauvezin, C. and Neufeld, T. P. (2015). Bafilomycin A1 disrupts autophagic flux by inhibiting both V-ATPase-dependent acidification and Ca-P60A/SERCA-dependent autophagosome-lysosome fusion. *Autophagy.* **11**, 1437–1438. doi:10.1080/15548627.2015.1066957.

Mei, S., Ni, H.-M., Manley, S., Bockus, A., Kassel, K. M., Luyendyk, J. P., Copple, B. L. and Ding, W.-X. (2011). Differential roles of unsaturated and saturated fatty acids on autophagy and apoptosis in hepatocytes. *The Journal of pharmacology and experimental therapeutics.* **339**, 487–498. doi:10.1124/jpet.111.184341.

Mercaldi, G. F., Ranzani, A. T. and Cordeiro, A. T. (2014). Discovery of new uncompetitive inhibitors of glucose-6-phosphate dehydrogenase. *Journal of biomolecular screening.* **19**, 1362–1371. doi:10.1177/1087057114546896.

Minokoshi, Y., Kahn, C. R. and Kahn, B. B. (2003). Tissue-specific ablation of the GLUT4 glucose transporter or the insulin receptor challenges assumptions about insulin action and glucose homeostasis. *The Journal of biological chemistry.* **278**, 33609–33612. doi:10.1074/jbc.R300019200.

Minokoshi, Y., Kim, Y.-B., Peroni, O. D., Fryer, L. G. D., Müller, C., Carling, D. and Kahn, B. B. (2002). Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. *Nature.* **415**, 339–343. doi:10.1038/415339a.

Mistry, R. J., Klamt, F., Ramsden, D. B. and Parsons, R. B. (2020). Nicotinamide N-methyltransferase expression in SH-SY5Y human neuroblastoma cells decreases oxidative stress. *Journal of biochemical and molecular toxicology.* **34**, e22439. doi:10.1002/jbt.22439.

Mitchell, S. A., Spriggs, K. A., Bushell, M., Evans, J. R., Stoneley, M., Le Quesne, J. P. C., Spriggs, R. V. and Willis, A. E. (2005). Identification of a motif that mediates polypyrimidine tract-binding protein-dependent internal ribosome entry. *Genes & development.* **19**, 1556–1571. doi:10.1101/gad.339105.

Mizushima, N. and Yoshimori, T. (2007). How to interpret LC3 immunoblotting. *Autophagy.* **3**, 542–545. doi:10.4161/auto.4600.

Müller, G., Ertl, J., Gerl, M. and Preibisch, G. (1997). Leptin impairs metabolic actions of insulin in isolated rat adipocytes. *The Journal of biological chemistry.* **272**, 10585–10593. doi:10.1074/jbc.272.16.10585.

Nakamaru, K., Matsumoto, K., Taguchi, T., Suefuji, M., Murata, Y., Igata, M., Kawashima, J., Kondo, T., Motoshima, H. and Tsuruzoe, K. et al. (2005). AICAR, an activator of AMP-activated protein kinase, down-regulates the insulin receptor expression in HepG2

cells. *Biochemical and biophysical research communications.* **328**, 449–454. doi:10.1016/j.bbrc.2005.01.004.

Neelakantan, H., Vance, V., Wetzel, M. D., Leo Wang, H.-Y., McHardy, S. F., Finnerty, C. C., Hommel, J. D. and Watowich, S. J. (2018). Selective and membrane-permeable small molecule inhibitors of nicotinamide N-methyltransferase reverse high fat diet-induced obesity in mice. *Biochemical Pharmacology* [Epub ahead of print]. doi:10.1016/j.bcp.2017.11.007.

Newman, W. P. and Brodows, R. G. (1983). Insulin action during acute starvation: Evidence for selective insulin resistance in normal man. *Metabolism: clinical and experimental.* **32**, 590–596. doi:10.1016/0026-0495(83)90029-x.

Nuñez, C. E., Rodrigues, V. S., Gomes, F. S., Moura, R. F. de, **Victorio, S. C., Bombassaro, B., Chaim, E. A., Pareja, J. C., Geloneze, B. and Velloso, L. A. et al.** (2013). Defective regulation of adipose tissue autophagy in obesity. *International journal of obesity (2005).* **37**, 1473–1480. doi:10.1038/ijo.2013.27.

Orci, L., Cook, W. S., Ravazzola, M., Wang, M.-Y., Park, B.-H., Montesano, R. and Unger, R. H. (2004). Rapid transformation of white adipocytes into fat-oxidizing machines. *Proceedings of the National Academy of Sciences of the United States of America.* **101**, 2058–2063. doi:10.1073/pnas.0308258100.

Ost, A., Svensson, K., Ruishalme, I., Brännmark, C., Franck, N., Krook, H., Sandström, P., Kjolhede, P. and Strålfors, P. (2010). Attenuated mTOR signaling and enhanced autophagy in adipocytes from obese patients with type 2 diabetes. *Molecular medicine (Cambridge, Mass.).* **16**, 235–246. doi:10.2119/molmed.2010.00023.

Pagotto, A., Pilotto, G., Mazzoldi, E. L., Nicoletto, M. O., Frezzini, S., Pastò, A. and Amadori, A. (2017). Autophagy inhibition reduces chemoresistance and tumorigenic potential of human ovarian cancer stem cells. *Cell death & disease.* **8**, e2943. doi:10.1038/cddis.2017.327.

Palorini, R., Votta, G., Pirola, Y., Vitto, H. de, **Palma, S.** de, **Airoldi, C., Vasso, M., Ricciardiello, F., Lombardi, P. P. and Cirulli, C. et al.** (2016). Protein Kinase A Activation Promotes Cancer Cell Resistance to Glucose Starvation and Anoikis. *PLoS genetics.* **12**, e1005931. doi:10.1371/journal.pgen.1005931.

Peng, T., Golub, T. R. and Sabatini, D. M. (2002). The Immunosuppressant Rapamycin Mimics a Starvation-Like Signal Distinct from Amino Acid and Glucose Deprivation. *Molecular and Cellular Biology.* **22**, 5575–5584. doi:10.1128/MCB.22.15.5575-5584.2002.

Pérez-Pérez, A., Vilariño-García, T., Fernández-Riejos, P., Martín-González, J., Segura-Egea, J. J. and Sánchez-Margalet, V. (2017). Role of leptin as a link between metabolism and the immune system. *Cytokine & growth factor reviews.* **35**, 71–84. doi:10.1016/j.cytogfr.2017.03.001.

Petersen, M. C. and Shulman, G. I. (2018). Mechanisms of Insulin Action and Insulin Resistance. *Physiological Reviews.* **98**, 2133–2223. doi:10.1152/physrev.00063.2017.

Peterson, T. R., Laplante, M., Thoreen, C. C., Sancak, Y., Kang, S. A., Kuehl, W. M., Gray, N. S. and Sabatini, D. M. (2009). DEPTOR is an mTOR inhibitor frequently overexpressed in multiple myeloma cells and required for their survival. *Cell.* **137**, 873–886. doi:10.1016/j.cell.2009.03.046.

Pischon, T. and Nimptsch, K. (2016). Obesity and Risk of Cancer: An Introductory Overview. *Recent results in cancer research. Fortschritte der Krebsforschung. Progres dans les recherches sur le cancer.* **208**, 1–15. doi:10.1007/978-3-319-42542-9_1.

Pissios, P. (2017). Nicotinamide N-Methyltransferase: More Than a Vitamin B3 Clearance Enzyme. *Trends in endocrinology and metabolism: TEM.* **28**, 340–353. doi:10.1016/j.tem.2017.02.004.

Poulos, S. P., Dodson, M. V. and Hausman, G. J. (2010). Cell line models for differentiation: preadipocytes and adipocytes. *Experimental biology and medicine (Maywood, N.J.).* **235**, 1185–1193. doi:10.1258/ebm.2010.010063.

Powley, I. R., Kondrashov, A., Young, L. A., Dobbyn, H. C., Hill, K., Cannell, I. G., Stoneley, M., Kong, Y.-W., Cotes, J. A. and Smith, G. C. M. et al. (2009). Translational reprogramming following UVB irradiation is mediated by DNA-PKcs and allows selective recruitment to the polysomes of mRNAs encoding DNA repair enzymes. *Genes & development.* **23**, 1207–1220. doi:10.1101/gad.516509.

Pyo, J.-O., Yoo, S.-M., Ahn, H.-H., Nah, J., Hong, S.-H., Kam, T.-I., Jung, S. and Jung, Y.-K. (2013). Overexpression of Atg5 in mice activates autophagy and extends lifespan. *Nature Communications.* **4**, 2300. doi:10.1038/ncomms3300.

Rabanal-Ruiz, Y. and Korolchuk, V. I. (2018). mTORC1 and Nutrient Homeostasis: The Central Role of the Lysosome. *International journal of molecular sciences.* **19**. doi:10.3390/ijms19030818.

Rabinowitz, J. D. and White, E. (2010). Autophagy and Metabolism. *Science.* **330**, 1344–1348. doi:10.1126/science.1193497.

Real, A. M., Hong, S. and Pissios, P. (2013). Nicotinamide N-Oxidation by CYP2E1 in Human Liver Microsomes. *Drug Metabolism and Disposition.* **41**, 550–553. doi:10.1124/dmd.112.049734.

Richard, A. J. and Stephens, J. M. (2014). The role of JAK-STAT signaling in adipose tissue function. *Biochimica et biophysica acta.* **1842**, 431–439. doi:10.1016/j.bbadis.2013.05.030.

Riederer, M., Erwa, W., Zimmermann, R., Frank, S. and Zechner, R. (2009). Adipose tissue as a source of nicotinamide N-methyltransferase and homocysteine. *Atherosclerosis.* **204**, 412–417.

Rini, J., Szumlanski, C., Guerciolini, R. and Weinshilboum, R. M. (1990). Human liver nicotinamide N-methyltransferase: ion-pairing radiochemical assay, biochemical properties and individual variation. *Clinica chimica acta; international journal of clinical chemistry.* **186**, 359–374. doi:10.1016/0009-8981(90)90322-j.

Robinson, M. B., Deshpande, D. A., Chou, J., Cui, W., Smith, S., Langefeld, C., Hastie, A. T., Bleecker, E. R. and Hawkins, G. A. (2015). IL-6 trans-signaling increases expression of airways disease genes in airway smooth muscle. *American journal of physiology. Lung cellular and molecular physiology.* **309**, L129-38. doi:10.1152/ajplung.00288.2014.

Ron, D. and Harding, H. P. (2007). *eIF2a phosphorylation in cellular stress responses and disease.* Cold Spring Harbor N.Y.: Cold Spring Harbor Laboratory Press.

Rotter, V., Nagaev, I. and Smith, U. (2003). Interleukin-6 (IL-6) induces insulin resistance in 3T3-L1 adipocytes and is, like IL-8 and tumor necrosis factor-alpha, overexpressed in human fat cells from insulin-resistant subjects. *The Journal of biological chemistry.* **278**, 45777–45784. doi:10.1074/jbc.M301977200.

Rudolphi, B., Zapp, B., Kraus, N. A., Ehebauer, F., Kraus, B. J. and Kraus, D. (2018). Body weight predicts Nicotinamide N-Methyltransferase activity in mouse fat. *Endocrine research.* **43**, 55–63. doi:10.1080/07435800.2017.1381972.

Ruvinsky, I., Sharon, N., Lerer, T., Cohen, H., Stolovich-Rain, M., Nir, T., Dor, Y., Zisman, P. and Meyuhas, O. (2005). Ribosomal protein S6 phosphorylation is a determinant of cell size and glucose homeostasis. *Genes & development.* **19**, 2199–2211. doi:10.1101/gad.351605.

Sakamoto, K. and Holman, G. D. (2008). Emerging role for AS160/TBC1D4 and TBC1D1 in the regulation of GLUT4 traffic. *American journal of physiology. Endocrinology and metabolism.* **295**, E29-37. doi:10.1152/ajpendo.90331.2008.

Salt, I. P., Connell, J. M. and Gould, G. W. (2000). 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) inhibits insulin-stimulated glucose transport in 3T3-L1 adipocytes. *Diabetes.* **49**, 1649–1656. doi:10.2337/diabetes.49.10.1649.

Sánchez-Martín, P. and Komatsu, M. (2018). p62/SQSTM1 - steering the cell through health and disease. *Journal of cell science.* **131**. doi:10.1242/jcs.222836.

Saxton, R. A. and Sabatini, D. M. (2017). mTOR Signaling in Growth, Metabolism, and Disease. *Cell.* **168**, 960–976. doi:10.1016/j.cell.2017.02.004.

Schmeisser, K. and Parker, J. A. (2018). Nicotinamide-N-methyltransferase controls behavior, neurodegeneration and lifespan by regulating neuronal autophagy. *PLoS genetics.* **14**, e1007561. doi:10.1371/journal.pgen.1007561.

Sengupta, S., Peterson, T. R. and Sabatini, D. M. (2010). Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. *Molecular cell.* **40**, 310–322. doi:10.1016/j.molcel.2010.09.026.

Seta, R., Mascitti, M., Campagna, R., Sartini, D., Fumarola, S., Santarelli, A., Giuliani, M., Cecati, M., Lo Muzio, L. and Emanuelli, M. (2018). Overexpression of nicotinamide N-methyltransferase in HSC-2 OSCC cell line: effect on apoptosis and cell proliferation. *Clinical oral investigations* [Epub ahead of print]. doi:10.1007/s00784-018-2497-8.

Shepherd, P. R., Gnudi, L., Tozzo, E., Yang, H., Leach, F. and Kahn, B. B. (1993). Adipose cell hyperplasia and enhanced glucose disposal in transgenic mice overexpressing GLUT4 selectively in adipose tissue. *The Journal of biological chemistry*. **268**, 22243–22246.

Shepherd, P. R. and Kahn, B. B. (1999). Glucose transporters and insulin action--implications for insulin resistance and diabetes mellitus. *The New England journal of medicine*. **341**, 248–257. doi:10.1056/NEJM199907223410406.

Shi, J., Fan, J., Su, Q. and Yang, Z. (2019). Cytokines and Abnormal Glucose and Lipid Metabolism. *Frontiers in Endocrinology*. **10**. doi:10.3389/fendo.2019.00703.

Shu, G., Lu, N.-S., Zhu, X.-T., Xu, Y., Du, M.-Q., Xie, Q.-P., Zhu, C.-J., Xu, Q., Wang, S.-B. and Wang, L.-N. et al. (2014). Phloretin promotes adipocyte differentiation in vitro and improves glucose homeostasis in vivo. *The Journal of nutritional biochemistry*. **25**, 1296–1308. doi:10.1016/j.jnutbio.2014.07.007.

Singh, R. and Cuervo, A. M. (2011). Autophagy in the Cellular Energetic Balance. *Cell metabolism*. **13**, 495–504. doi:10.1016/j.cmet.2011.04.004.

Singh, R. and Cuervo, A. M. (2012). Lipophagy: connecting autophagy and lipid metabolism. *International journal of cell biology*. **2012**, 282041. doi:10.1155/2012/282041.

Singh, R., Xiang, Y., Wang, Y., Baikati, K., Cuervo, A. M., Luu, Y. K., Tang, Y., Pessin, J. E., Schwartz, G. J. and Czaja, M. J. (2009). Autophagy regulates adipose mass and differentiation in mice. *J Clin Invest*. **119**, 3329–3339. doi:10.1172/JCI39228.

Song, M., Li, Y., Miao, M., Zhang, F., Yuan, H., Cao, F., Chang, W., Shi, H. and Song, C. (2020). High stromal nicotinamide N-methyltransferase (NNMT) indicates poor prognosis in colorectal cancer. *Cancer medicine*. **9**, 2030–2038. doi:10.1002/cam4.2890.

Spriggs, K. A., Bushell, M., Mitchell, S. A. and Willis, A. E. (2005). Internal ribosome entry segment-mediated translation during apoptosis: the role of IRES-trans-acting factors. *Cell death and differentiation*. **12**, 585–591. doi:10.1038/sj.cdd.4401642.

Spriggs, K. A., Bushell, M. and Willis, A. E. (2010). Translational regulation of gene expression during conditions of cell stress. *Molecular cell*. **40**, 228–237. doi:10.1016/j.molcel.2010.09.028.

Spriggs, K. A., Cobbold, L. C., Ridley, S. H., Coldwell, M., Bottley, A., Bushell, M., Willis, A. E. and Siddle, K. (2009). The human insulin receptor mRNA contains a functional internal ribosome entry segment. *Nucleic acids research*. **37**, 5881–5893. doi:10.1093/nar/gkp623.

Spriggs, K. A., Stoneley, M., Bushell, M. and Willis, A. E. (2008). Re-programming of translation following cell stress allows IRES-mediated translation to predominate. *Biology of the cell*. **100**, 27–38. doi:10.1042/BC20070098.

Stern, J. H., Rutkowski, J. M. and Scherer, P. E. (2016). Adiponectin, Leptin, and Fatty Acids in the Maintenance of Metabolic Homeostasis through Adipose Tissue Crosstalk. *Cell Metabolism.* **23**, 770–784. doi:10.1016/j.cmet.2016.04.011.

Sternak, M., Jakubowski, A., Czarnowska, E., Slominska, E. M., Smolenski, R. T., Szafarz, M., Walczak, M., Sitek, B., Wojcik, T. and Jasztal, A. et al. (2015). Differential involvement of IL-6 in the early and late phase of 1-methylnicotinamide (MNA) release in Concanavalin A-induced hepatitis. *International Immunopharmacology.* **28**, 105–114.

Stincone, A., Prigione, A., Cramer, T., Wamelink, M. M. C., Campbell, K., Cheung, E., Olin-Sandoval, V., Grüning, N.-M., Krüger, A. and Tauqeer Alam, M. et al. (2015). The return of metabolism: biochemistry and physiology of the pentose phosphate pathway. *Biological reviews of the Cambridge Philosophical Society.* **90**, 927–963. doi:10.1111/brv.12140.

Ström, K., Morales-Alamo, D., Ottosson, F., Edlund, A., Hjort, L., Jörgensen, S. W., Almgren, P., Zhou, Y., Martin-Rincon, M. and Ekman, C. et al. (2018). N1-methylnicotinamide is a signalling molecule produced in skeletal muscle coordinating energy metabolism. *Scientific Reports.* **8**, 3016. doi:10.1038/s41598-018-21099-1.

Sullivan, J. E., Brocklehurst, K. J., Marley, A. E., Carey, F., Carling, D. and Beri, R. K. (1994). Inhibition of lipolysis and lipogenesis in isolated rat adipocytes with AICAR, a cell-permeable activator of AMP-activated protein kinase. *FEBS Letters.* **353**, 33–36. doi:10.1016/0014-5793(94)01006-4.

Sun, H. and Liu, D. (2015). Hydrodynamic delivery of interleukin 15 gene promotes resistance to high fat diet-induced obesity, fatty liver and improves glucose homeostasis. *Gene therapy.* **22**, 341–347. doi:10.1038/gt.2014.114.

Tamargo-Gomez, I. and Marino, G. (2018). AMPK: Regulation of Metabolic Dynamics in the Context of Autophagy. *International journal of molecular sciences.* **19**. doi:10.3390/ijms19123812.

Tang, S.-W., Yang, T.-C., Lin, W.-C., Chang, W.-H., Wang, C.-C., Lai, M.-K. and Lin, J.-Y. (2011). Nicotinamide N-methyltransferase induces cellular invasion through activating matrix metalloproteinase-2 expression in clear cell renal cell carcinoma cells. *Carcinogenesis.* **32**, 138–145. doi:10.1093/carcin/bgq225.

TeSlaa, T. and Teitell, M. A. (2014). Techniques to monitor glycolysis. *Methods in enzymology.* **542**, 91–114. doi:10.1016/B978-0-12-416618-9.00005-4.

Thoreen, C. C., Chantranupong, L., Keys, H. R., Wang, T., Gray, N. S. and Sabatini, D. M. (2012). A unifying model for mTORC1-mediated regulation of mRNA translation. *Nature.* **485**, 109–113. doi:10.1038/nature11083.

Todaro, G. J. and Green, H. (1963). Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *The Journal of cell biology.* **17**, 299–313. doi:10.1083/jcb.17.2.299.

Todaro, G. J., Habel, K. and Green, H. (1965). Antigenic and cultural properties of cells doubly transformed by polyoma virus and SV40. *Virology.* **27**, 179–185. doi:10.1016/0042-6822(65)90157-1.

Tomida, M., Ohtake, H., Yokota, T., Kobayashi, Y. and Kurosumi, M. (2008). Stat3 up-regulates expression of nicotinamide N-methyltransferase in human cancer cells. *Journal of cancer research and clinical oncology.* **134**, 551–559. doi:10.1007/s00432-007-0318-6.

Ulanovskaya, O. A., Zuhl, A. M. and Cravatt, B. F. (2013). NNMT promotes epigenetic remodeling in cancer by creating a metabolic methylation sink. *Nature chemical biology.* **9**, 300–306. doi:10.1038/nchembio.1204.

Um, S. H., Frigerio, F., Watanabe, M., Picard, F., Joaquin, M., Sticker, M., Fumagalli, S., Allegrini, P. R., Kozma, S. C. and Auwerx, J. et al. (2004). Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. *Nature.* **431**, 200–205. doi:10.1038/nature02866.

Urakami, K., Zangiacomi, V., Yamaguchi, K. and Kusuhara, M. (2013). Impact of 2-deoxy-D-glucose on the target metabolome profile of a human endometrial cancer cell line. *Biomedical research (Tokyo, Japan).* **34**, 221–229.

van Haren, M. J., Taig, R., Kuppens, J., Sastre Toraño, J., Moret, E. E., Parsons, R. B., Sartini, D., Emanuelli, M. and Martin, N. I. (2017). Inhibitors of nicotinamide N-methyltransferase designed to mimic the methylation reaction transition state. *Organic & biomolecular chemistry.* **15**, 6656–6667. doi:10.1039/c7ob01357d.

Vizza, D., Perri, A., Toteda, G., Lupinacci, S., Perrotta, I., Lofaro, D., Leone, F., Gigliotti, P., La Russa, A. and Bonofiglio, R. (2018). Rapamycin-induced autophagy protects proximal tubular renal cells against proteinuric damage through the transcriptional activation of the nerve growth factor receptor NGFR. *Autophagy.* **14**, 1028–1042. doi:10.1080/15548627.2018.1448740.

Wang, D., Zhou, Y., Lei, W., Zhang, K., Shi, J., Hu, Y., Shu, G. and Song, J. (2009). Signal transducer and activator of transcription 3 (STAT3) regulates adipocyte differentiation via peroxisome-proliferator-activated receptor gamma (PPARgamma). *Biology of the cell.* **102**, 1–12. doi:10.1042/BC20090070.

Wang, L., Shang, Z., Zhou, Y., Hu, X., Chen, Y., Fan, Y., Wei, X., Wu, L., Liang, Q. and Zhang, J. et al. (2018). Autophagy mediates glucose starvation-induced glioblastoma cell quiescence and chemoresistance through coordinating cell metabolism, cell cycle, and survival. *Cell death & disease.* **9**, 213. doi:10.1038/s41419-017-0242-x.

Wang, Q., Liang, B., Shirwany, N. A. and Zou, M.-H. (2011). 2-Deoxy-D-glucose treatment of endothelial cells induces autophagy by reactive oxygen species-mediated activation of the AMP-activated protein kinase. *PloS one.* **6**, e17234. doi:10.1371/journal.pone.0017234.

Wang, X., Li, W., Williams, M., Terada, N., Alessi, D. R. and Proud, C. G. (2001). Regulation of elongation factor 2 kinase by p90(RSK1) and p70 S6 kinase. *The EMBO Journal.* **20**, 4370–4379. doi:10.1093/emboj/20.16.4370.

Wannamethee, S. G., Lowe, G. D. O., Rumley, A., Cherry, L., Whincup, P. H. and Sattar, N. (2007). Adipokines and risk of type 2 diabetes in older men. *Diabetes care.* **30**, 1200–1205. doi:10.2337/dc06-2416.

Woods, A., Johnstone, S. R., Dickerson, K., Leiper, F. C., Fryer, L. G. D., Neumann, D., Schlattner, U., Wallimann, T., Carlson, M. and Carling, D. (2003). LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. *Current biology : CB.* **13**, 2004–2008. doi:10.1016/j.cub.2003.10.031.

Wu, N., Zheng, B., Shaywitz, A., Dagon, Y., Tower, C., Bellinger, G., Shen, C.-H., Wen, J., Asara, J. and McGraw, T. E. et al. (2013). AMPK-dependent degradation of TXNIP upon energy stress leads to enhanced glucose uptake via GLUT1. *Molecular cell.* **49**, 1167–1175. doi:10.1016/j.molcel.2013.01.035.

Wu, Y., Siadaty, M. S., Berens, M. E., Hampton, G. M. and Theodorescu, D. (2008). Overlapping gene expression profiles of cell migration and tumor invasion in human bladder cancer identify metallothionein 1E and nicotinamide N-methyltransferase as novel regulators of cell migration. *Oncogene.* **27**, 6679–6689. doi:10.1038/onc.2008.264.

Xi, H., Kurtoglu, M. and Lampidis, T. J. (2014). The wonders of 2-deoxy-d-glucose. *IUBMB life.* **66**, 110–121. doi:10.1002/iub.1251.

Xi, H., Kurtoglu, M., Liu, H., Wangpaichitr, M., You, M., Liu, X., Savaraj, N. and Lampidis, T. J. (2011). 2-Deoxy-D-glucose activates autophagy via endoplasmic reticulum stress rather than ATP depletion. *Cancer chemotherapy and pharmacology.* **67**, 899–910. doi:10.1007/s00280-010-1391-0.

Xie, X., Liu, H., Wang, Y., Zhou, Y., Yu, H., Li, G., Ruan, Z., Li, F., Wang, X. and Zhang, J. (2016). Nicotinamide N-methyltransferase enhances resistance to 5-fluorouracil in colorectal cancer cells through inhibition of the ASK1-p38 MAPK pathway. *Oncotarget* [Epub ahead of print]. doi:10.18632/oncotarget.9962.

Xie, X., Yu, H., Wang, Y., Zhou, Y., Li, G., Ruan, Z., Li, F., Wang, X., Liu, H. and Zhang, J. (2014). Nicotinamide N-methyltransferase enhances the capacity of tumorigenesis associated with the promotion of cell cycle progression in human colorectal cancer cells. *Archives of Biochemistry and Biophysics.* **564**, 52–66. doi:10.1016/j.abb.2014.08.017.

Yamaguchi, S., Katahira, H., Ozawa, S., Nakamichi, Y., Tanaka, T., Shimoyama, T., Takahashi, K., Yoshimoto, K., Imaizumi, M. O. and Nagamatsu, S. et al. (2005). Activators of AMP-activated protein kinase enhance GLUT4 translocation and its glucose transport activity in 3T3-L1 adipocytes. *American journal of physiology. Endocrinology and metabolism.* **289**, E643-9. doi:10.1152/ajpendo.00456.2004.

Yan, L., Otterness, D. M., Craddock, T. L. and Weinshilboum, R. M. (1997). Mouse Liver Nicotinamide N-Methyltransferase. *Biochemical Pharmacology*. **54**, 1139–1149. doi:10.1016/S0006-2952(97)00325-0.

Yang, L., Li, P., Fu, S., Calay, E. S. and Hotamisligil, G. S. (2010). Defective hepatic autophagy in obesity promotes ER stress and causes insulin resistance. *Cell metabolism*. **11**, 467–478. doi:10.1016/j.cmet.2010.04.005.

Yang, Q., Graham, T. E., Mody, N., Preitner, F., Peroni, O. D., Zabolotny, J. M., Kotani, K., Quadro, L. and Kahn, B. B. (2005). Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. *Nature*. **436**, 356–362. doi:10.1038/nature03711.

Yoshii, S. R. and Mizushima, N. (2017). Monitoring and Measuring Autophagy. *International journal of molecular sciences*. **18**. doi:10.3390/ijms18091865.

Yu, H., Zhou, X., Wang, Y., Huang, X., Yang, J., Zeng, J., Li, G., Xie, X. and Zhang, J. (2020). Nicotinamide N-methyltransferase inhibits autophagy induced by oxidative stress through suppressing the AMPK pathway in breast cancer cells. *Cancer cell international*. **20**, 191. doi:10.1186/s12935-020-01279-8.

Yu, T., Wang, Y.-T., Chen, P., Li, Y.-H., Chen, Y.-X., Zeng, H., Yu, A.-M., Huang, M. and Bi, H.-C. (2015). Effects of nicotinamide N-methyltransferase on PANC-1 cells proliferation, metastatic potential and survival under metabolic stress. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology*. **35**, 710–721. doi:10.1159/000369731.

Zachari, M. and Ganley, I. G. (2017). The mammalian ULK1 complex and autophagy initiation. *Essays in biochemistry*. **61**, 585–596. doi:10.1042/EBC20170021.

Zebisch, K., Voigt, V., Wabitsch, M. and Brandsch, M. (2012). Protocol for effective differentiation of 3T3-L1 cells to adipocytes. *Analytical Biochemistry*. **425**, 88–90. doi:10.1016/j.ab.2012.03.005.

Zhang, J., Wang, Y., Li, G., Yu, H. and Xie, X. (2014). Down-regulation of nicotinamide N-methyltransferase induces apoptosis in human breast cancer cells via the mitochondria-mediated pathway. *PloS one*. **9**, e89202. doi:10.1371/journal.pone.0089202.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L. and Friedman, J. M. (1994). Positional cloning of the mouse obese gene and its human homologue. *Nature*. **372**, 425–432. doi:10.1038/372425a0.

Zhang, Y., Goldman, S., Baerga, R., Zhao, Y., Komatsu, M. and Jin, S. (2009). Adipose-specific deletion of autophagy-related gene 7 (atg7) in mice reveals a role in adipogenesis. *PNAS*. **106**, 19860–19865. doi:10.1073/pnas.0906048106.

Zhao, F.-Q. and Keating, A. F. (2007). Expression and Regulation of Glucose Transporters in the Bovine Mammary Gland1. *Journal of Dairy Science*. **90**, E76-E86. doi:10.3168/jds.2006-470.

Zheng, D., MacLean, P. S., Pohnert, S. C., Knight, J. B., Olson, A. L., Winder, W. W. and Dohm, G. L. (2001). Regulation of muscle GLUT-4 transcription by AMP-activated protein kinase. *Journal of applied physiology (Bethesda, Md. : 1985).* **91**, 1073–1083. doi:10.1152/jappl.2001.91.3.1073.

Zhou, B., Li, H., Xu, L., Zang, W., Wu, S. and Sun, H. (2013). Osteocalcin reverses endoplasmic reticulum stress and improves impaired insulin sensitivity secondary to diet-induced obesity through nuclear factor-kappaB signaling pathway. *Endocrinology.* **154**, 1055–1068. doi:10.1210/en.2012-2144.

Zoncu, R., Efeyan, A. and Sabatini, D. M. (2011). mTOR: from growth signal integration to cancer, diabetes and ageing. *Nature reviews. Molecular cell biology.* **12**, 21–35. doi:10.1038/nrm3025.

List of tables

List of figures

List of abbreviations

%	percent
°C	degree celsius
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar
1-MN	1-methylnicotinamide
2-DG	2-deoxyglucose
2DG6P	2-DG-6-phosphate
AB	antibody
ADP	adenosine diphosphate
AICAR	5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
APS	ammonium persulfate
ASO	antisense oligonucleotide
ATGs	autophagy-related proteins
ATP	adenosine triphosphate
CAMKK2	calcium/calmodulin dependent kinase kinase 2
cm	centimeter
Ctrl	control
d	day
Deptor	DEP-domain-containing mTOR-interacting protein
Dexa	dexamethasone
DHEA	dehydroepiandrosterone
DIO	diet-induced obesity
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DTT	1,4-Dithiothreit
e. g.	exempli gratia (for example)
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
FIP200	focal adhesion kinase family interacting protein 200 kDa

g	relative centrifugal force / G force
G6P	glucose-6-phosphate
G6P	glucose-6-phosphate
G6PDH	glucose-6-phosphate dehydrogenase
GDP	guanosine diphosphate
GLUT4	glucose transporter type 4
GTP	guanosine triphosphate
h	hour
H	hydrogen
HFD	high fat diet
IBMX	3-Isobutyl-1-methylxanthine
IL-6	interleukin 6
Ins	insulin
JAK	Janus kinase
l	liter
LKB1	liver-kinase B1
M	molarity
MAP1LC3B	microtubule-associated proteins 1A/1B light chain 3B
mg	milligram
min	minutes
ml	milliliter
mLST8	mammalian lethal with Sec13
mM	millimolar
mRNA	messenger ribonucleic acid
mTOR	mechanistic target of rapamycin
mTORC1	mechanistic target of rapamycin complex 1
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid
Na_3VO_4	sodium orthovanadate
NaCl	sodium chloride
NADP	nicotinamide adenine dinucleotide phosphate
NAF	sodium fluoride
NAOH	sodium hydroxide
NBCS	newborn calf serum
nM	nanomolar
NNMT	nicotinamide N-methyltransferase
ORO	Oil Red O

P62/SQSTM1	sequestosome-1
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PI3K	phosphoinositide 3-kinase
PMSF	phenylmethylsulfonyl fluoride
PRAS40	prolinerich AKT substrate 40 kDa
Raptor	regulatory-associated protein of mTOR
Rheb	ras homolog enriched in brain
RNA	ribonucleic acid
rpm	rotations per minute
RT-qPCR	reverse transcription quantitative polymerase chain reaction
s	second
SAM	S-adenosyl methionine
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of mean
Ser	serine
SP	sodium pyruvate
STAT	signal transducers and activators of transcription
T2D	type 2 diabetes
TEMED	N,N,N',N'-tetramethylethylenediamine
Thr	threonine
TNF-α	tumor necrosis factor α
TRIS	tris(hydroxymethyl)aminomethane
TSC	tuberous sclerosis complex
ULK1	unc-51-like kinase 1
Vps34	vacuolar protein sorting 34
WAT	white adipose tissue
α	alpha
β	beta
γ	gamma

Danksagung

Publications

Ehebauer, F.*, Ghavampour, S. and Kraus, D. (2020). Glucose availability regulates nicotinamide N-methyltransferase expression in adipocytes. Life Sciences, 117474. doi:10.1016/j.lfs.2020.117474.

Kraus, N. A.*, **Ehebauer, F.**, Zapp, B., Rudolphi, B., Kraus, B. J. and Kraus, D. (2016). Quantitative assessment of adipocyte differentiation in cell culture. Adipocyte, 1–8. doi:10.1080/21623945.2016.1240137.

Rudolphi, B.*, Zapp, B., Kraus, N. A., **Ehebauer, F.**, Kraus, B. J. and Kraus, D. (2018). Body weight predicts Nicotinamide N-Methyltransferase activity in mouse fat. Endocrine research. 43, 55–63. doi:10.1080/07435800.2017.1381972.

*First author

Declaration

The data presented in this thesis have been partially published in the Journal Life Sciences as an original article entitled "Glucose availability regulates nicotinamide N-methyltransferase expression in adipocytes".

Curriculum Vitae

Affidavit

I hereby confirm that my thesis entitled "Regulation of Nicotinamide N-methyltransferase Expression in Adipocytes" is a result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process nether in identical nor in similar form.

_____   _____
Place, Date   Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation „Regulation der Nicotinamide N-methyltransferase Expression in Adipozyten" eigenständig, d. h. insbesondere selbstständig ohne Hilfe eines kommerziellen Promotionsberaters angefertigt und keine anderen als die von mir angegeben Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegt habe.

_____   _____
Ort, Datum   Unterschrift