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The role of Dicer in adipocyte differentiation and metabolism

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List of abbreviations

5-LO *5-lipoxygenase*
AA/Rot ..... *antimycin A/rotenone*
ACACA ..... *acetyl-CoA carboxylase*
ADicerKO ..... *adipose tissue specific Dicer KO*
ADIPOQ ..... *adiponectin*
AGO ..... *argonaute*
AGrP ..... *agouti-related protein*
AICAR ..... *5-Aminoimidazole-4-carboxamide ribonucleotide*
AMPK ..... *AMP-activated protein kinase*
aP2 ..... *human adipocyte fatty acid-binding protein*
ASC ..... *AT derived mesenchymal stem cells*
AT ..... *adipose tissue*
ATGL ..... *adipose triglyceride lipase*
ATM ..... *adipose tissue macrophage*
ATP ..... *adenosine triphosphate*
ATP5A1 ..... *ATP synthase F1 subunit alpha*
BAT ..... *brown adipose tissue*
BMI ..... *body mass index*
BSA ..... *bovine serum albumin*
C. elegans ..... *Caenorhabditis elegans*
cAMP ..... *cyclic AMP*
Cas9 ..... *CRSIPR associated protein 9*
cDNA ..... *complementary DNA*
CEBPα ..... *CCAAT/enhancer-binding protein alpha*
CEBPβ ..... *CCAAT/enhancer-binding protein beta*
CEBPδ ..... *CCAAT/enhancer-binding protein delta*
CIDEA ..... *cell death activator*
CITED1 ..... *Cbp/p300-interacting transactivator 1*
C-MSC ..... *cardiac mesenchymal stem cells*
CoA ..... *co-enzyme A*
COXII ..... *cytochrome c oxidase subunit 2*
CRISPR ..... *clustered regularly interspaced short palindromic repeats*
CS. ..... *citrate synthase*
DGRC8 ..... *DiGeorge syndrome critical region 8*
DIO2 ..... *type II iodothyronine deiodinase, type II iodothyronine deiodinase*
DMEM ..... *Dulbecco's Modified Eagle Medium*
DMSO ..... *dimethyl sulfoxide*
DNA ..... *deoxyribonucleic acid, desoxyribonucleic acid*
dsRBP ..... *double stranded RNA binding protein*
dsRNA ..... *double stranded RNA*
DTNB ..... *5,5'-dithiobis-2-nitrobenzoic acid*
DUF283 ..... *domain of unknown function 283*
EC ..... *embryonic cells*
ELISA ..... *enzyme-linked immunosorbent assay*
ELOVL3 ..... *elongation of very long chain fatty acids protein 3*

EV ..... *empy vector control*
FASN ..... *fatty acid synthase*
FCCP..... *carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone*
FCS ..... *fetal calf serum*
FGF21 ..... *fibroblast growth factor 21*
GLUT1 ..... *glucose transporter 1*
GLUT4 ..... *glucose transporter 4*
GOF..... *gain of function*
gWAT ..... *gonadal WAT*
HCl ..... *hydrochloric acid*
HFD ..... *high-fat diet*
hg38..... *Genome Reference Consortium Human Build 38*
HIF-1α..... *hypoxia inducing factor 1 alpha*
HIV ..... *human immunodeficiency virus*
hMADS..... *Human multipotent adipose-derived stem cells*
HOMA-IR ..... *homeostasis model assessment of insulin resistance*
HOXC9 ..... *homeobox protein C9*
HPRT ..... *hypoxanthine-guanine phosphoribosyltransferase*
hsa ..... *homo sapiens*
HSL..... *hormone sensitive lipase*
IMM ..... *inner mitochondrial membrane*
IRS1..... *insulin receptor substrate* 1
ITR..... *inverted tandem repeats*
KD ..... *knockdown*
kDa..... *kilodalton*
KO ..... *knockout*
KRH ..... *Krebs-Ringer buffer*
LB..... *Luria Broth*
LOF..... *loss of function*
MCT1 ..... *monocarboxylate transporter 1*
MCT4 ..... *monocarboxylate transporter 4*
MEF..... *mouse embryonal fibroblast, mouse embryonal fibroblast*
MES..... *2-ethanesulfonic acid*
miRNA..... *microRNA*
mRNA ..... *messenger RNA*
mTORc1 ..... *mammalian target of rapamycin complex 1*
MuLE..... *multiple lentiviral expression*
MuSE ..... *multiple sleeping beauty expression*
NaN_3 ..... *soduim azide*
NaOH ..... *sodium hydroxide*
NDUFB8 ..... *NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8*
NT..... *non-targeting control siRNA*
PACT ..... *protein kinase RNA activator*
PAZ ..... *Piwi/Ago/Zwille*
PBS..... *phosphate buffered saline, phosphate buffered saline*
PCR ..... *polymerase chain reaction*
PGC1a ..... *peroxisome proliferator-activated receptor gamma coactivator 1-alpha*
PLIN *perilipin1*

pMuLE ..... *MuLE plasmid*

pMuSE ..... *MuSE plasmid*
PNK ..... *polynucleotide Kinase*
POMC ..... *pro-opiomelanocortin*
PPARγ ..... *peroxisome proliferator-activated receptor gamma*
pre-miRNA ..... *precursor miRNA*
pri-miRNA ..... *primary miRNA*
qRT-PCR ..... *quantitative real-time polymerase chain reaction*
RISC ..... *RNA induced silencing complex*
RLC ..... *RISC loading complex*
RNA ..... *ribonucleic acid*
RNAi ..... *RNA interference*
rpm ..... *revolutions per minute*
SB ..... *sleeping beauty*
SDHA ..... *succinate dehydrogenase subunit alpha*
SDHB ..... *succinate dehydrogenase subunit beta*
SDS-PAGE ..... *sulfate–polyacrylamide gel electrophoresis*
SEM ..... *standard error of the mean*
SGBS ..... *Simpsons Golabi Behmel syndrome*
sgRNA ..... *single-guide RNA*
siDicer ..... *Dicer targeting siRNA transfected cells*
siNT ..... *non-targeting siRNA/control transfected cells*
siRNA ..... *small interfering RNA, small interfering RNA*
SOB ..... *Super Optimal Broth*
SOX4 ..... *SRY-Box transcription factor 4*
T2D ..... *type 2 diabetes*
TBST ..... *TRIS-buffered saline + polysorbate 20*
TIMM23 ..... *mitochondrial import inner membrane translocase subunit Tim23*
TNB ..... *2-nitro-5-benzoic acid*
TRBP ..... *transactivating response element RNA-binding protein*
UCP1 ..... *uncoupling protein 1*
UQCRC2 ..... *cytochrome b-c1 complex subunit 2*
UTR ..... *untranslated region*
WAT ..... *white adipose tissue*
WHO ..... *World Health Organisation*
WT ..... *wildtype*

1. Introduction

1.1 Obesity – a persistent worldwide pandemic

Ever since historical records, human beings were periodically exposed to global pandemics. Infectious diseases such as the Black Death, the Spanish Flu or the recent COVID-19 pandemic were responsible for millions of deaths all over the world entailing devastating consequences. Most pandemics endured only for a limited period of time, except for one.

The obesity pandemic silently arose in the middle of the twentieth century, grew extraordinarily fast and still persists until today [1]. According to the World Health Organization (WHO), obesity and its comorbidities costs millions of lives every year (WHO, Obesity and overweight, June 2021).

Obesity is defined as a pathological increase of adipose tissue (AT) in a living organism [1]. It is a consequence of an imbalance between energy consumption and energy intake – a condition that usually sustains over a considerable time. Per definition, a person with a body mass index (BMI) higher than 30 kg/m² is considered to be obese, whereas those with a BMI between 25 and 30 kg/m² are classified as overweight [3]. In 2016, approximately 650 million adults and 340 million children were obese as stated in a report from the WHO (WHO, Obesity and overweight, June 2021). Yet, it should be mentioned that the BMI is just a simplified measure and is not necessarily representative in general.

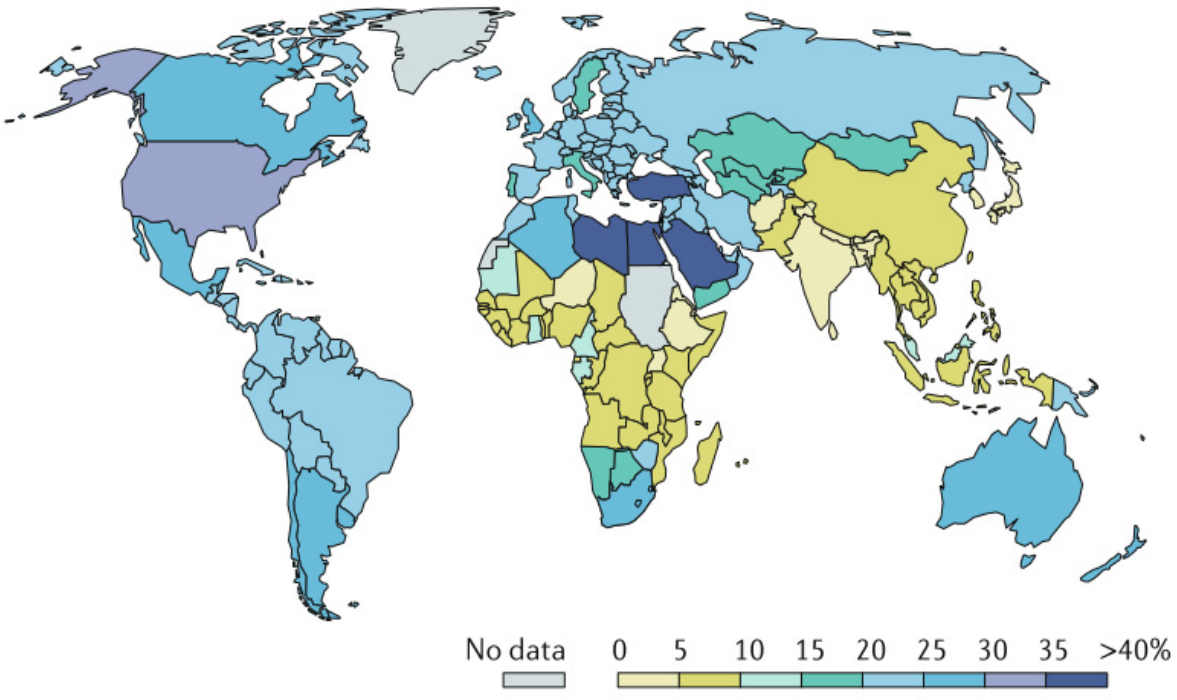


Figure 1 - Global obesity pandemic. *This world map indicates the global prevalence of obesity in adults as for 2014. While in certain countries of Southeast Asia and Central Africa there is relatively moderate percentage of obese individuals ranging from 0 to 10 %, especially in Europe, Oceania, and both American continents more than 20 % of the total adult population is defined as obese. Highest obesity percentages were observed in Turkey, Saudi-Arabia, Egypt and Libya reaching more than 40 %. For Greenland, there is no data available. From Blüher, Matthias. 2019. "Obesity: Global Epidemiology and Pathogenesis." Nature Reviews Endocrinology 15(5): 288–98. Used with permission of Springer Nature, permission conveyed through Copyright Clearance Center, Inc.*

Reasons for the development of an abnormal accumulation of fat are diverse and, in most cases, co-exist with another. Lifestyle is one major contributor to this phenotype as it accounts for both: daily energy needed as well as the intake of energy-rich nutrients on each day. However, there are multiple circumstances and factors that are involved in this process including also genetical components and sex differences [1,4,5]. A summary is provided in Figure 1.

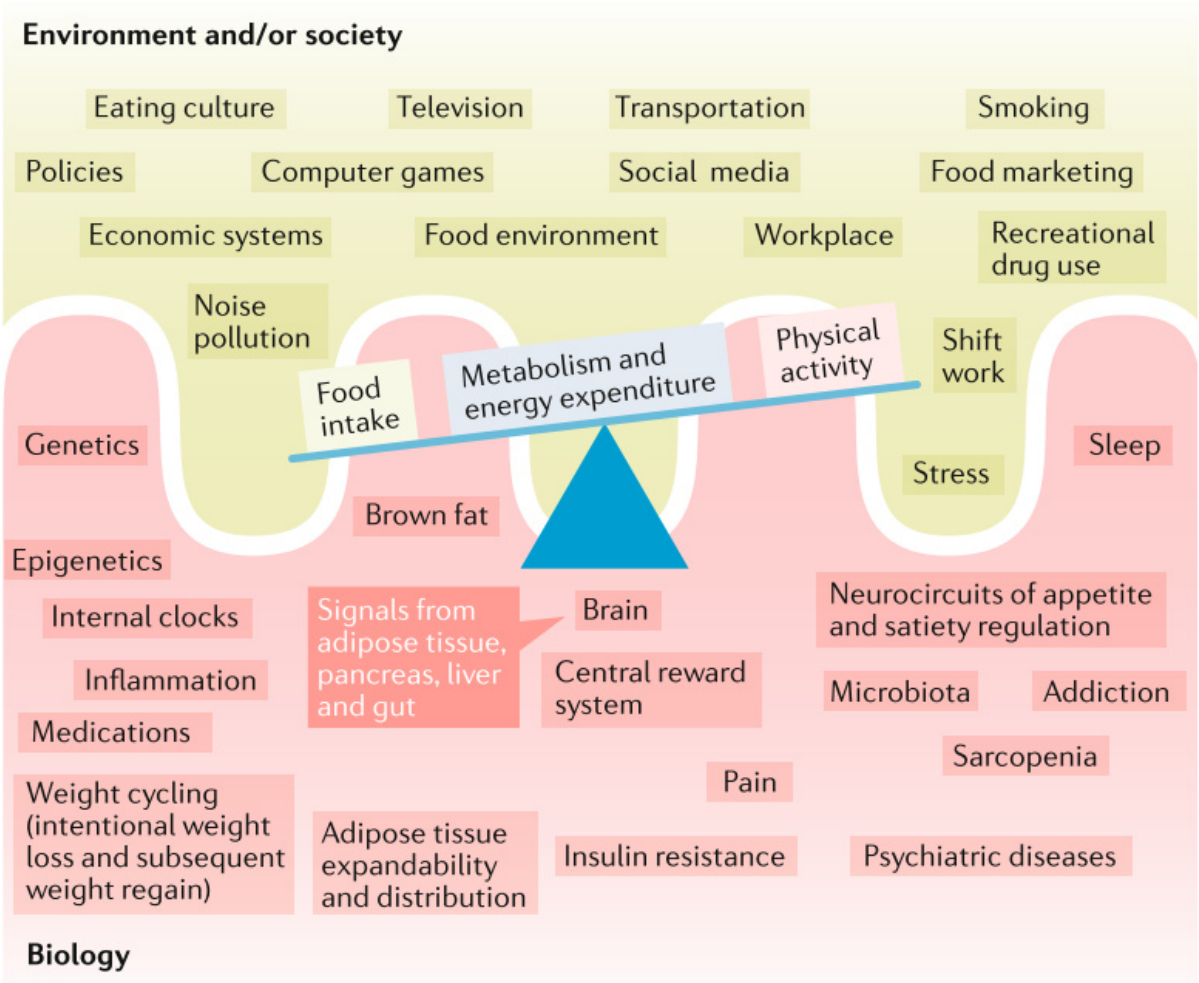


Figure 2 - Influential factors of obesity. *Metabolic maintenance of energy homeostasis is a well-balanced system that can be affected by biological factors, for example genetics, epigenetics or microbiota, and environmental or social factors such as physical activity, eating behavior and stress. Both groups occur simultaneously and contribute to the formation of obesity in a direct or indirect manner. From Blüher, Matthias. 2019. "Obesity: Global Epidemiology and Pathogenesis." Nature Reviews Endocrinology 15(5): 288–98. Used with permission of Springer Nature, permission conveyed through Copyright Clearance Center, Inc.*

Notably, obesity causes severe metabolic alterations as a consequence of prolonged overnutrition. Accordingly, obesity is associated with several comorbidities including chronic subclinical inflammations, insulin resistance, type 2 diabetes (T2D), fatty liver disease, atherosclerosis, hypertension and even certain types of cancer – all of which can lead to reduced life quality [1,5,6].

In addition to the strain for the affected individuals, it is furthermore responsible for causing an overwhelming economic burden to society. Only in the United States of America, about two trillion dollar were spent in 2014 to fight obesity and obesity related diseases [7]. Combined with the severe individual implications including also social disadvantages, it is of utmost common interest to find strategies that help to fight and prevent this global pandemic. As a consequence, obesity was declared as a disease by the scientific community [8].

1.2 Adipose tissue

AT is a specialized organ and important regulator of energy homeostasis. It is a heterogenous tissue that consists of multiple different cell types including for example preadipocytes, mature adipocytes, endothelial and also immune cells. Since excessive intake of nutrients demands the capability of sufficient and safe storage capacity, the main function of AT is to take up excessive calories , store them in form of lipids and to provide them when necessary [9–11].

In human, AT are distributed all over the body including for example the subcutaneous and visceral region. Yet, the individual fat tissues do not only differ with respect to their anatomic location. There are also morphological differences and even more importantly metabolic distinctions that have been identified after investigating fat tissues which led to the discrimination of different varieties of AT.

1.2.1 When adipose tissue is not simply adipose tissue

To date, there are two predominant types of AT classified in mammalian organisms. The classical and widely known fat tissue that primarily acts as major reservoir for lipid storage is called white adipose tissue (WAT). Its main task is to store an oversupply of energy as triglycerides. Besides that, it is also an important endocrine organ secreting adipokines such as leptin and adiponectin which are functionally relevant for regulation of satiety, inflammatory responses, and other cellular processes. [9–11]

The other prominent type of AT is the so-called BAT (brown adipose tissue). This tissue is found for example in the supraclavicular and the paravertebral area. In contrast to WAT, the main function of BAT is to use lipids to generate heat, a process that is called thermogenesis. This process is achieved

due to the presence of a protein named uncoupling protein 1 (UCP1) which is embedded in the inner mitochondrial membrane (IMM). After beta-adrenergic stimulation, fatty acids from BAT are released which then activate UCP1. As a result of its activation, UCP1 shuttles protons from the intermembrane space back into the mitochondrial matrix. Thereby, it counteracts with the build-up of the proton gradient which is generated by the electron transport chain for oxidative phosphorylation. Since the number of mitochondria is very high in BAT compared to WAT, also respiration is elevated resulting in wasting energy for thermogenic processes. Thus, BAT or brown adipocytes are considered to serve as a nutrient sink and are therefore regarded to be metabolically beneficial for overweight and obese individuals. This is why many scientists focus on how activation and new formation of BAT can be triggered best [11–13].

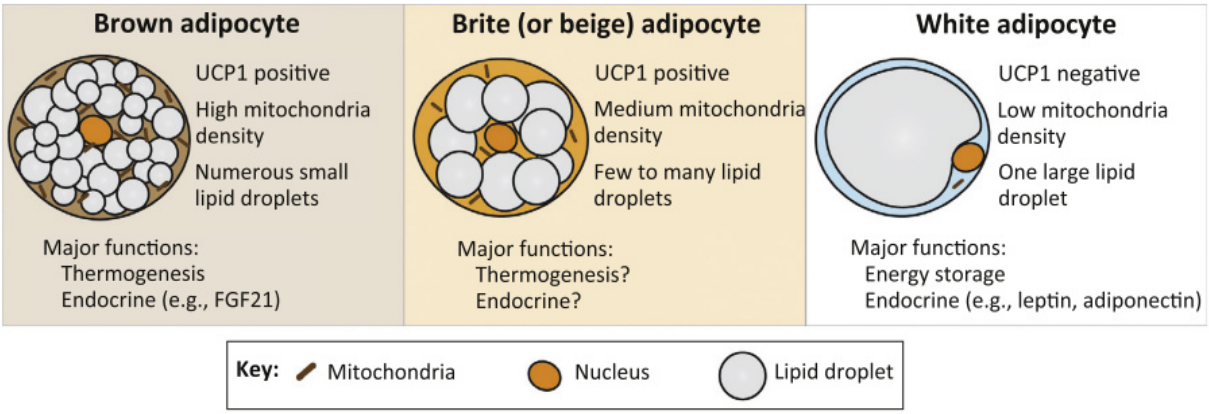


Figure 3 - Different types of adipocytes. *This graph illustrates the cellular characteristics of the different types of adipocytes found in the commonly known forms of adipose tissue. Brown adipocytes (left) are characterized by a large number of mitochondria, while the stored fat consists of several small lipid droplets. They express the BAT hallmark protein UCP1, which is crucial for its thermogenic activity. In contrast, white adipocytes (right) hold a small amount of mitochondria. The incorporated lipid is merged into one large intracellular lipid droplet. White adipocytes' main function is to store lipids for energy purpose and to act as an endocrine organ. Trans-differentiation white adipocytes, termed as brite/beige adipocytes (middle), resemble brown adipocytes and therefore have a brown adipocyte-like phenotype with higher mitochondria density, UCP1 positivity and multiple lipid droplets. Modified from Sanchez-Gurmaches, Joan, Chien Min Hung, and David A Guertin. 2016. "Emerging Complexities in Adipocyte Origins and Identity." Trends in Cell Biology 26(5): 313–26. Used with permission of Elsevier, permission conveyed through Copyright Clearance Center, Inc.*

Remarkably, specific depots of WAT are known to undergo a phenotypic change where white adipocytes develop brown adipocyte features. Accordingly, these beige adipocytes exhibit elevated mitochondrial mass and UCP1 expression. Due to their acquired thermogenic capacity, beige adipocytes ultimately contribute to regulating energy metabolism. This process scientifically termed as beiging or browning. Hence, also browning of WAT has been the focus of investigations in the recent years in order to find appropriate measures to combat obesity [11,14–16].

At this point, it needs to be mentioned that there are also other types of AT that have been identified to date, namely pink and yellow AT [17]. However, since this does not have any relevance in the context of this work, they are not specifically introduced within this thesis.

1.2.2 The limits to growth: increasing adipose tissue storage capacity

As described, AT is the main organ for energy storage. Yet, the accumulation of lipids in cells is limited to a certain degree and therefore requires AT expansion. AT expansion may occur in two different manners. One, by increasing adipocyte size, a process known as hypertrophy. And second, by initiating adipogenic differentiation of AT resident precursor cells – known as preadipocytes – which increases the total number of mature adipocytes within the tissue, a process that is called hyperplasia [10,18,19]. Both events have been shown to occur under *in vivo* condition [19,20]. However, hypertrophy of adipocytes is limited to a certain extent [20,21]. In addition, increased adipocyte diameter negatively correlates with metabolic health suggesting that smaller adipocytes are preferred to maintain appropriate cellular response to environmental changes [22,23].

In this context, many researchers aimed for more profound examinations of cellular signaling processes within the AT including the investigation of microRNAs (miRNAs).

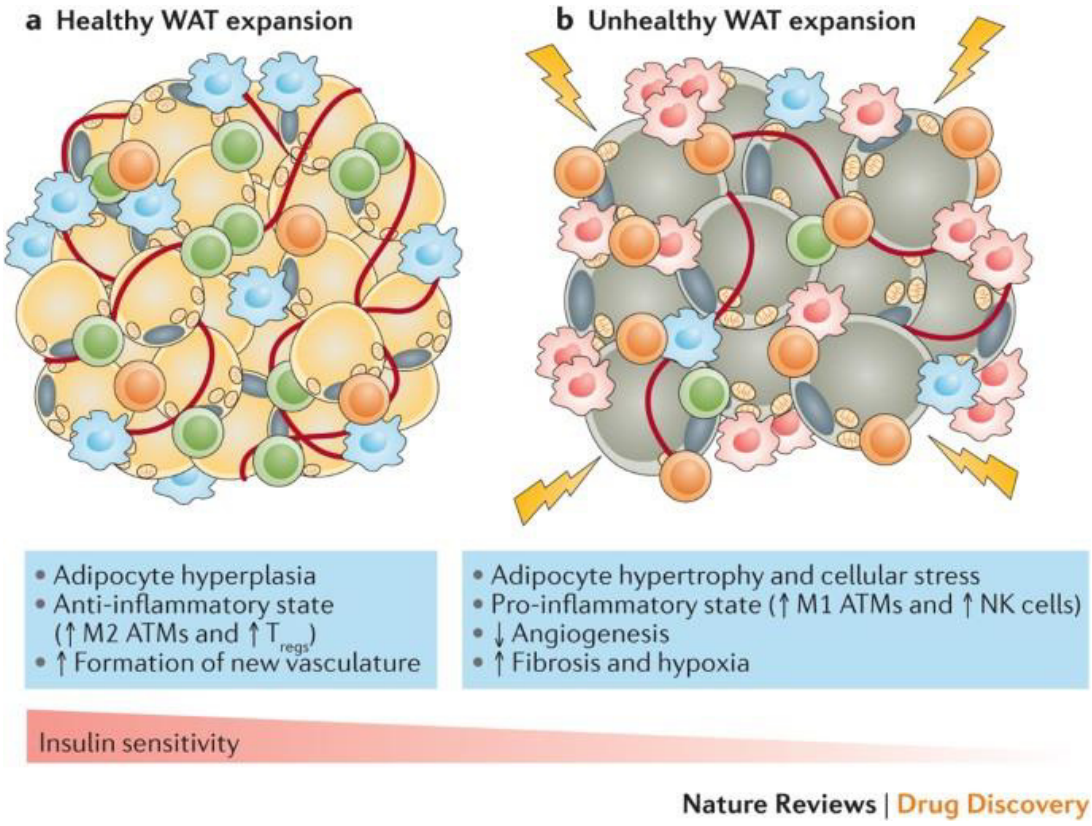


Figure 4 - Expansion of WAT. *The increase in adipose tissue mass can occur in two different manners. One is adipocyte hyperplasia (left), which is characterized by an increase in adipocyte number within the certain tissue. This process favors infiltration of relatively high numbers of M2 macrophages creating an anti-inflammatory environment. Moreover, it promotes tissue vascularization and is characterized with a high insulin sensitivity. This is scientifically considered as healthy expansion of AT. Another way of is depicted on the right. In this scenario, AT size grows by adipocyte hypertrophy. This phenotype causes enlarged cells and is associated with increased cellular stress, combined with infiltration of M1 macrophages and a proinflammatory milieu. Furthermore, reduced angiogenesis results in a hypoxic state and thereby promotes fibrotic changes. As a consequence, adipocyte hypertrophy is stated as unhealthy AT expansion. From Kusminski, Christine M., Perry E. Bickel, and Philipp E. Scherer. 2016. "Targeting Adipose Tissue in the Treatment of Obesity-Associated Diabetes." Nature Reviews Drug Discovery 15(9): 639–60. Used with permission of Springer Nature, permission conveyed through Copyright Clearance Center, Inc.*

1.2.3 miRNAs and their role in adipose tissue and obesity

miRNAs are small non-coding ribonucleic acids (RNAs) that are important regulatory components in the suppression of post-transcriptional gene regulation and gene silencing. With an average length of 22 nucleotides, miRNAs recognize a complementary messenger RNA (mRNA) equivalent and thereby negatively regulate protein levels by either translational repression or destabilization and degradation of their according target. Since their discovery in 1993, more than 2000 miRNAs were identified in human [24–27].

miRNA genes are mostly located in intronic, some other in exonic regions [24,28]. Very few miRNA genes can also be found intergenic holding their own promotors which allows a separate expression independent of other genes [24]. Transcription of miRNA genes is carried out either by RNA polymerase II or III resulting in the formation of a primary miRNA (pri-miRNA). This pri-miRNA is a double stranded RNA (dsRNA) with a hairpin-loop-structure. In a subsequent maturation step, the pri-miRNA binds to a protein complex consisting of Drosha and DiGeorge syndrome critical region 8 (DGRC8) [29]. Here, DGCR8 recognizes and interacts with specific motifs within pri-miRNAs, whilst the ribonuclease Drosha trims the initial dsRNA and simultaneously creates a 3' overhang [24]. This processed pri-miRNA is now called precursor miRNA (pre-miRNA). For a final maturation step via Dicer, pre-miRNAs need to get transported into the cytosol. This is accomplished via an exportin 5/RanGTP dependent transport mechanism [24].

Next, in a helicase-mediated mechanism, both strands are separated from another leading to the formation of two separate, mature miRNA strands. Although, both strands are fully functional, in most cases, there is one strand that predominantly binds to the AGO protein. This strand selectivity determines which of both strands is mainly active. This discrimination depends on the individual stability of each miRNA which is why the more stable strand is referred to as guide strand and the other one as passenger strand [24].

Mature miRNAs are subsequently loaded into AGOs where they recognize and bind a complementary mRNA in the now formed RNA induced silencing complex (RISC) – a process that involves the additional action of chaperones [30].

In general, miRNAs recognize special response elements in the 3'- untranslated region (UTR) of the according mRNA. However, some miRNAs were also identified to bind to the 5'UTR or to coding sequences [24]. Eventually, this miRNA-mRNA interaction causes reduced protein levels as a result of either translational repression or mRNA destabilization and degradation [25,29].
This phenomenon of post-transcriptional gene regulation is considered to equilibrate fluctuations in mRNA transcription [24]. Strikingly, miRNA do not necessarily act intracellularly. Recent findings prove that miRNAs can be integrated into exosomes and thereby exert their function also in distinct tissues [31]. Likewise, recent studies suggest that miRNAs can furthermore be transported under vesicle-free conditions from cell to cell [24]. In addition to miRNAs being shuttled between cells, also Dicer was identified to be transferred among different tissues [32].

Several miRNAs were discovered to play a tremendous role in the biology of AT being involved in multiple molecular processes that affect proliferation and adipogenesis [33–36]. Moreover, in numerous *in vitro* and *in vivo* experiments miRNAs such as miR-190, miR-30 or miR-155 were identified to contribute to WAT browning [37]. In the context of obesity research, comparison of circulating miRNAs revealed a diverse expression pattern in blood of obese mice versus healthy controls. More interestingly, treatment of lean mice with exosomal miRNAs from obese littermates led to impaired insulin resistance and glucose tolerance suggesting that miRNAs can systemically change a metabolic response to nutrient consumption [38].

The investigations of miRNAs opened a completely new scientific field serving as potential biomarkers and as possible therapeutics for various diseases [39]. It is evident that miRNA undergo a processing procedure in which alterations in expression of key regulators can substantially impair miRNA levels. In this context, one of the most important enzymes in miRNA biogenesis is the endonuclease Dicer [40].

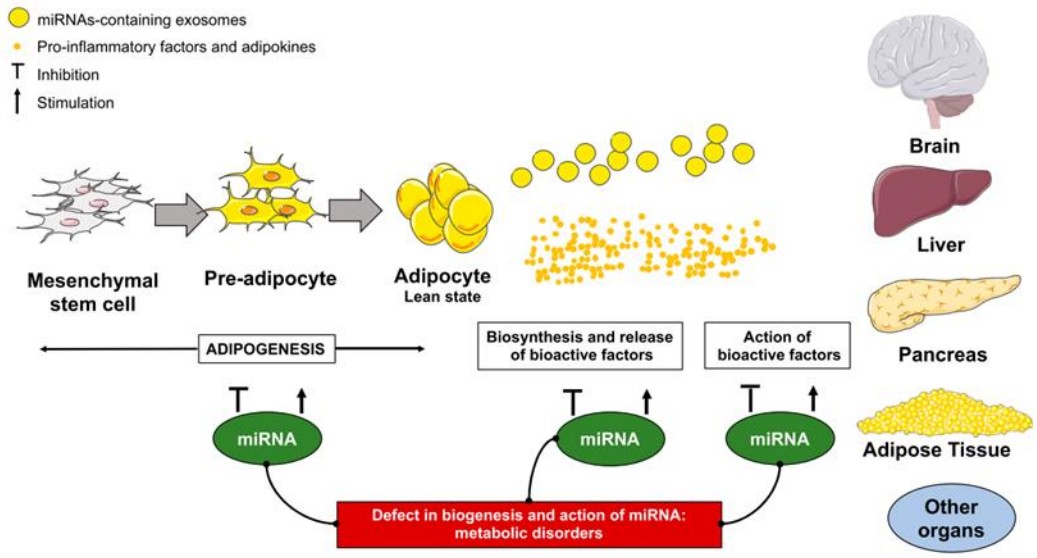


Figure 5 - Role of miRNAs in adipose tissue and other tissues. *Preadipocytes arise from mesenchymal stem cells and can further differentiate into mature adipocytes under given circumstances in a process termed as adipogenesis. Mature adipocytes can synthesize and release adipokines and cytokines. In all of these processes, miRNAs are of utmost importance as they can either have an inhibitory or a stimulatory function. Dysfunction of this central regulatory system may cause metabolic disorders. Adipocytes can furthermore secrete miRNA containing exosomes themselves which can then affect other tissues such as for example brain, liver, pancreas. From Landrier, Jean François, Adel Derghal, and Lourdes Mounien. 2019. "MicroRNAs in Obesity and Related Metabolic Disorders." Cells 8(8). Licensed under CC BY 4.0, https://creativecommons.org/licenses/by/4.0.*

1.3 Dicer

The ribonuclease Dicer is an approximately 200 kilodalton (kDa) enzyme that belongs to the group of class IV RNase III enzymes. It is encoded by the DICER1 gene and is located on the long arm of chromosome 14. Dicer is an important part of the RNA interference machinery with the role of defending against viral infection [41]. Therefore, it is not surprising that its main task is to enzymatically cleave dsRNA [30,42–44].

Knowledge about Dicer gene expression is very limited at this point. This might be because its expression extremely varies between tissues, cell types and differentiation stages. Yet, the promotor region for Dicer was identified to contain a target sequence for p53 and p63 [45,46]. Moreover, it was found that SRY-Box transcription factor 4 (SOX4) induces Dicer gene expression in melanoma cells [47].

1.3.1 Molecular Structure of Dicer

Dicer forms a L-shape protein which can be distinguished into a head, body and a base part. It contains several different domains that are conserved across different species [44].

The DexD/H-box or helicase domain has two important roles which is critical for interaction with either of two double stranded RNA binding proteins (dsRBPs): transactivating response element RNA-binding protein (TRBP) and protein kinase RNA activator (PACT). This interaction with dsRBPs facilitates binding of substrates and enhancing its cleavage activity [30,48,49].

The Piwi/Ago/Zwille (PAZ) domain includes two different pockets for end recognizing of the 3'- and the 5'-end of dsRNAs. Whereas the RNAase IIIa and IIIb domains are required for the catalytical activity, the function of domain of unknown function 283 (DUF283) is not fully clear. In addition, Dicer contains a dsRNA binding domain (RBD) enabling interaction with dsRNA even in the absence of dsRBPs [30,42,44,45].

Notably, Dicer contains an additional nuclear localization sequence. Although it mainly acts in the cytosol, phosphorylation of Dicer initiates a conformational change and enables shuttling into the nucleus [44].

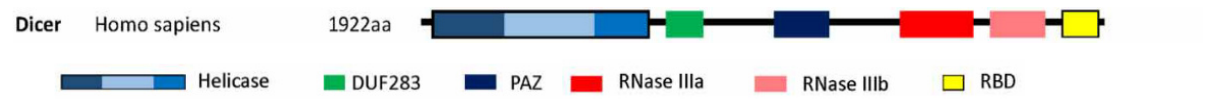


Figure 6 - Functional domains of Dicer. *Dicer consists of 1922 amino acids (aa) and holds five important domains that are specific for its function. These domains illustrated in this figure are namely: helicase, DUF283, PAZ, RNAse IIIa and IIIb domain and the RBD. Modified from Song, Min Sun, and John J. Rossi. 2017. "Molecular Mechanisms of Dicer: Endonuclease and Enzymatic Activity." Biochemical Journal 474(10): 1603–18. Used with permission of Portland Press Limited, permission conveyed through Copyright Clearance Center, Inc.*

1.3.2 Role of Dicer in microRNA biogenesis

The RNase III Dicer is the key regulator of pre-miRNA processing. Situated in the cytosol, it recognizes the duplex structure of the pre-miRNA and removes the hairpin loop resulting in the formation of a mature miRNA duplex [29,30]. This enzymatic cleavage process occurs in the so-called RISC loading complex (RLC). The RLC is a multiprotein-complex that consists of Dicer, an argonaute (AGO) protein and an additional dsRBP, which is either TRBP or PACT [50]. Although not essential for Dicer-mediated processing, both TRBP and PACT are important as they can interact with the DexD/H-box of Dicer. This interaction is crucial to achieve an improved enzymatic dicing efficiency [29,30,49]. Interestingly, since Dicer also processes other types of dsRNA, it is reported that its binding partner seems to dictate the kind of dsRNA that is enzymatically cleaved (H. Y. Lee et al. 2013). The AGO protein is responsible for the pre-processing of the miRNA duplex to facilitate strand dissociation from each other [29].

Dicer is one of the most important enzymes in miRNA biogenesis [25,29]. Therefore, it is no surprise that a loss of function (LOF) in, for example, colorectal cancer cells was reported to lead to a depletion of 96% of mature miRNA *in vitro* [40]. Furthermore, it is important to mention, that a constitutive whole body knockout (KO) of Dicer was reported to be lethal during early development in mice [51].

Of note, although the biogenesis pathway introduced here accounts for the formation of mature miRNAs, many miRNAs have been identified to undergo specific processing in a non-canonical manner [24,29].

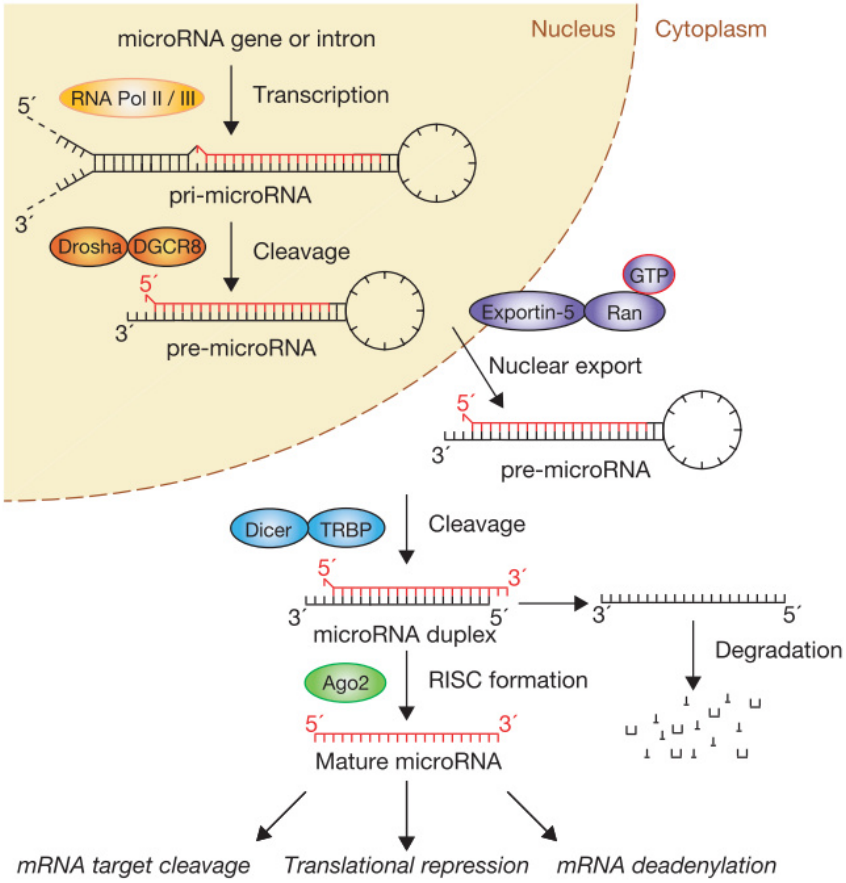


Figure 7 – Canonical miRNA biogenesis pathway. *As depicted, microRNAs are transcribed by the RNA Polymerase II or III in the cell nucleus from either separate microRNA genes or from intronic regions resulting in a pri-miRNA. Trimming via DROSHA-DGCR8 leads to the formation of the pre-miRNA which next gets shuttled into the cytoplasma in an Exportin-5-RanGTP dependent process. Further processing occurs by Dicer-TRBP by cleaving off the hairpin structure. Mature microRNA strands are loaded to the RISC, while unstable microRNA strands are degraded. As a result, mature microRNAs negatively regulate protein biosynthesis via mRNA target cleavage, translational expression or mRNA deadenylation. From Winter, Julia et al. 2009. "Many Roads to Maturity: MicroRNA Biogenesis Pathways and Their Regulation." Nature Cell Biology 11(3): 228–34. Used with permission of Springer Nature, permission conveyed through Copyright Clearance Center, Inc.*

1.3.3 Functional meaning of Dicer beyond miRNA biogenesis

Its most important function besides miRNA biogenesis, is its relevance in an additional RNA interference (RNAi) pathway mechanism mediated by small interfering RNA (siRNA). This second group of non-coding RNAs shares similar characteristics with miRNAs and hence negatively regulates protein levels. Yet, in contrast to miRNAs, each siRNA is fully complementary to its according target mRNA and thus has only one specific mRNA target - therefore being less promiscuous than miRNAs [44,52]. This central role within the RNAi pathway highlights how Dicer can affect crucial cellular mechanisms by altering the abundance of specific proteins in a post-transcriptional manner by either siRNA or miRNA.

Furthermore, Dicer processes transfer RNA-related fragments, pre-ribosomal RNA and holds a protective role against viral infections [44]. Remarkably, in *in vitro* experiments Dicer was reported to enhance 5-lipoxygenase (5-LO) activity by direct interaction suggesting a pivotal role in inflammatory response [53].

Moreover, the activity of Dicer was recently found to not be limited to the cytosol. Upon phosphorylation Dicer translocates into the nucleus where it is involved in deoxyribonucleic acid (DNA) damage repair by processing nuclear dsRNA after DNA double-strand breaks [30,54]. Notably, enhanced Dicer phosphorylation is associated with postnatal lethality, accelerated aging and infertility *in vivo* [55]. In addition to this, a truncated form of Dicer was detected in Caenorhabditis elegans (C. elegans). This processed form was reported to participate in apoptosis-induced DNA fragmentation as studies revealed [45,56]. Such a truncated version of Dicer was moreover shown by Sawh and Duchaine 2013 to act as a negative regulator of microRNA biogenesis in this model system.

Taken together, all these roles of Dicer mentioned so far not only represent crucial cellular processes that are essential for survival and other molecular function. Yet, more important than that, they are not limited to selected cells and thus are carried out ubiquitously and also constitutively. These findings illustrates how import it is that Dicer functions properly.

1.3.4 Pathologic implications of altered Dicer function

To date, there are several pathologic implications identified that result from an aberrant activity of Dicer. The best-known herein is summarized as DICER1 syndrome. This disease is a result of inhibitory, autosomal-dominant mutations in the DICER1 gene and is associated with an increased susceptibility to develop multinodular goiter and certain types of cancer. The most common types of tumor emerging

in these patients are cystic nephroma, ovarian Sertoli-Leydig cell tumor and especially pleuropulmonary blastoma, a rare form of lung cancer [29,43,58,59]. Accordingly, a conditional *in vivo* knockdown of Dicer was shown to promote tumor transformation in a murine lung cancer model [60]. Hotspot mutations that occur in the RNAse III domain are associated with a more severe form of this disease resulting in the GLOW syndrome. This acronym stands for its four predominantly observed phenotypes which are: global developmental delay, lung cysts, overgrowth and Wilms tumor [58,61].

Yet, not only the downregulation of Dicer is associated with adverse phenotypes. A gain of function (GOF) is associated to cause diseases such as psoriasis [41]. In addition, Dicer overexpression is related to poor survival in colorectal and prostate cancer [62].

Remarkably, in breast cancer, Dicer was found in exosomes of tumorigenic cells - along with TRBP and AGO2. These exosomes were also shown to be enriched in mature miRNA suggesting increased processing of pre-miRNA. When injected into mice, these exosomes were able to promote tumor formation indicating a tumor boosting effect of tumor cell exosomes [32].

These results highlight that dysregulation of Dicer dramatically harms cellular integrity and stimulates the formation and progression of diseases by mediating pathologic alteration. Due to its pleiotropic effects observed, special emphasize were put on Dicer to identify its specific role in metabolism. The questions that were addressed in this context were: how is Dicer involved with regard to energy metabolism? And is it a suitable target in combating the obesity pandemic?

1.3.5 Crucial role of functional Dicer in energy metabolism

In an initial *in vitro* study investigating the effect of a conditional Dicer KO, Dicer was identified as an important modulator of adipogenic differentiation in primary mouse preadipocytes from WAT origin as well as in mouse embryonal fibroblasts (MEFs), which was confirmed by Mori et al. 2014 in immortalized brown preadipocytes [63,64]. Along with this reduced capacity to generate mature adipocytes from brown preadipocytes, diminished expression of UCP1 was detected whereas levels of Leptin were elevated suggesting a whitening phenotype that goes along with loss of Dicer in brown cells [63]. Assessment of cellular oxygen consumption revealed reduced proton leak respiration in these cells highlighting that reduced UCP1 levels are also functionally relevant in this regard.

Microarray studies with human samples highlighted that Dicer mRNA is reduced in liver and skeletal muscle biopsies of obese individuals with T2D [65]. Following studies performed by Mori et al. 2012,

identified that expression levels of Dicer in AT inversely correlate with age in human and mice. This phenotype was furthermore associated with a decrease in mature miRNAs. Simultaneously, levels of pri- as well as from pre-miRNA were increased suggesting that cells try to compensate for the loss of mature miRNAs that results from Dicer ablation. Moreover, premature senescence and increased susceptibility for stress-induced apoptosis were observed when Dicer activity was hampered.

To get a more precise view onto the function of Dicer, many research groups performed loss of function approaches in multiple different tissues. Here, first *in vivo* studies performed introducing an adipose tissue specific Dicer KO (ADicerKO) in mice revealed pleiotropic effects on multiple metabolic levels. In WAT for example, Dicer deletion led to a lipodystrophy phenotype in subcutaneous, epididymal and mesenteric fat depots [63]. Interestingly, this was in line with findings obtained from AT samples of HIV patients with a lipodystrophy phenotype which also exhibited reduced Dicer expression level [63,67]. In contrast to WAT, BAT was enlarged while gaining a white-like phenotype [63]. Both, WAT and BAT, showed markedly increased adipocyte size. In addition to this, infiltration of macrophages was observed in ADicerKO mice under high-fat diet (HFD) indicating local inflammation within the adipose tissue. These animals had an impaired glucose metabolism and were less responsive to insulin stimulation [63]. Similar findings were also collected by Mudhasani et al. 2011. Furthermore, in contrast to control mice, ADicerKO exhibited impaired oxidative metabolism increased glycolytic activity in inguinal WAT following nutrient restriction [69]. This indicates that metabolic adaptation induced by hypocaloric stress is markedly altered in the absence of Dicer. All of these metabolically unfavorable effects shown in ADicerKO eventually lead to accelerated ageing and enhanced risk for mortality in mutant mice lacking Dicer in adipose tissue [69].

Profiling of exosomal miRNA from ADicerKO mice serum furthermore identified adipose tissue as a major source of circulating miRNAs [31]. Here, Dicer KO led to a general and dramatic loss of exosomal miRNA by 88% (fourfold downregulation). In line with this, investigation of serum samples from congenital generalized lipodystrophy patients and human immunodeficiency virus (HIV) associated lipodystrophy patients in comparison to healthy controls disclosed that also in these samples expression of Dicer was markedly hampered underlining the importance of adipose tissue with regard to exosomal miRNA levels. These results imply that ADicerKO is not only crucial for adipose tissue function itself, but that it also possesses important roles in distinct organs as it can affect protein expression levels in a miRNA mediated manner. Indeed, this was proven in liver samples from ADicerKO mice which exhibited dramatically increased levels of fibroblast growth factor 21 (FGF21). Adipose

tissue transplantation from wildtype (WT) mice was able to not only restore miRNAs in exosomes but was also able to reduce hepatic FGF21 levels underlining the importance of adipose tissue Dicer as a metabolic regulator in liver. Moreover, exosome delivery from WT mice had the same effect as tissue transplantation.

Yet, a critical physiological role of Dicer is not limited to adipose tissue only when it comes to regulating energy metabolism. A liver-specific KO model in mice performed by Sekine et al. 2009, pointed out that a lack of Dicer induces hepatic cell proliferation. Interestingly, also apoptosis was significantly increased in these mice suggesting that hepatocytes with a Dicer KO are more susceptible to undergo programmed cell death. In mutant mice, this led to a severe form of hepatosteatosis and resulted in the spontaneous formation of hepatocellular carcinomas in two thirds of the mice. Strikingly, when analyzing neoplastic tumor samples, it turned out that these exclusively consisted of Dicer deficient cells whereas nonneoplastic areas were reported to be Dicer positive by having escaped Cre-recombinase driven Dicer KO.

In pancreas, the importance of Dicer is reported to play an exceptional role in the formation of beta cell islets [71,72]. A beta cell specific Dicer KO resulted in a huge loss of β-cell mass which was further associated with dramatically reduced secreted as well as total insulin - most likely caused by altered expression of so-called disallowed genes [73]. This eventually caused hampered glucose tolerance and hyperglycemia in mice.

Ablation of Dicer in intestine however is associated with severe implications by causing disorganization of the intestinal tract in mice [74]. This phenotype was characterized by expansion of the crypt zone and reduction of mucus producing goblet cells. The number of tight junctions was significantly downregulated leading to increased intestinal permeability and hence decreased barrier function. Therefore, it is not surprising that gene expression profiling of tissue revealed increased expression of inflammatory factors and elevated amounts of apoptotic cells detected in mutant mice. As a consequence, intestinal Dicer KO in mice caused impaired fat absorption and water retention from food resulting in stunted growth.

In line with findings in liver specific Dicer KO mice, a loss of function approach in cardiac mesenchymal stem cells (C-MSCs) was characterized with increase in proliferation and apoptosis [75]. Metabolically, these cells were identified as being impaired in their cellular respiration and fatty acid oxidation. Additional extracellular flux analysis revealed increased glycolytic activity in these type of Dicer KO cells.

All of these studies performed exclusively pinpoint how crucial Dicer is regarding energy metabolism and what happens when its function is abrogated. Interestingly, the observed phenotypes following a loss of function approach do not seem to be limited to organs directly involved in regulating nutrients utilization. A study in pro-opiomelanocortin (POMC) expressing cells in the brain, deletion of Dicer was associated with hyperphagia and a high risk for developing obesity [76]. Respectively, plasma levels of triglycerides were increased as well as blood glucose levels. This phenotype was characterized with neurodegeneration accompanied by dramatically low levels of POMC and other neuronal proteins involved in satiety regulation such as neuropeptide Y and Agouti-related protein (AGrP) in the hypothalamus. Moreover, stress response was shown to be markedly reduced in these cells. This metabolic phenotype was also confirmed by an additional group [77]. Here, it was observed that expression of UCP1 in BAT is lower than in control mice which was accompanied by a lower body temperature. This indicates that neuronal ablation of Dicer not only affects satiety circuits in the brain but also has crucial systematic effects by functionally impeding BAT.

In summary, the huge variety of Dicer loss of function experiments highlight that this protein holds crucial features regarding energy metabolism in various tissues. With regard to its tissue specific effects, Dicer might be an interesting target for obesity treatment.

In this context, some factors were already identified that can positively regulate Dicer function partially answering some of these questions. Mori et al. 2012 were the first to demonstrate that caloric restriction is an effective method to increase cellular Dicer expression as shown in mice and also in C. elegans. In addition to this, fasting also induced hypothalamic Dicer expression in murine POMC neurons which was identified to be associated with satiety signaling [76].

As revealed by Shan et al. 2008 and additionally by Zhang, Zhang, and Xi 2008, the antibiotic enoxacin - generally used against gastrointestinal and urinary tract infection - induces maturation of miRNAs by facilitating interaction of Dicer and TRBP. To investigate, if this has implications on energy metabolism, Rocha et al. 2020 intraperitoneally injected enoxacin and assessed the metabolic phenotype in mice. They demonstrated that administration of enoxacin has tremendous effects regulating energy homeostasis and were able to identify miR-34-5p as main mediator in this regard. Here, enhanced Dicer expression was measured in adipose tissue of enoxacin treated mice. Moreover, enoxacin induced browning of WAT and elevated expression of brown-marker genes also in BAT as measured by expression levels of UCP1, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1a) and type II iodothyronine deiodinase (DIO2). These effects were additionally confirmed *in vitro*

using human ASC and differentiated 9W cells. Enoxacin treatment was further associated with enhanced metabolic rates and oxygen consumption implying that induced browning has also functional importance. Moreover, improved glucose tolerance as well as insulin sensitivity were observed in treated mice in comparison to control littermates after four weeks of HFD. Enoxacin was able to reverse whitening of BAT following HFD. Notably, in ADicerKO mice none of these effects were observed suggesting that the presence of Dicer in adipose tissue is crucial to metabolically benefit from enoxacin treatment.

Further *in vivo* experiments in mice revealed that aerobic exercise is another possible measure to induce Dicer expression in adipose tissue in an AMPK dependent manner. Interestingly, this was part of a metabolic switch that led to inhibition of glycolysis in adipose tissue in order to have sufficient glucose available for skeletal muscle. This further highlights the metabolic importance of Dicer not only for adipose tissue but also with regard to cross-organ talk and whole-body metabolism. Surprisingly, treatment of sedentary mice with serum obtained from trained littermates was sufficient to induce Dicer expression in WAT indicating that exercise-induced factors contribute to the expression of Dicer in adipose tissue [81].

Strikingly, Guerra et al. 2019 could achieve similar effects by dietary intervention. Here, the sole restriction of sulfur amino acid intake of cysteine and methionine in mice was able to elevate Dicer expression in subcutaneous adipose tissue. This effect was blunted when either of these two amino acids was supplemented. In addition to that, preliminary data studying the impact of nursing in mice revealed reduced body weight, lower body fat and intestinal atrophy in WT pups that were nursed by heterozygous Dicer KO mice [83]. These astonishing findings indicate that there are crucial factors within breastmilk that are only present when Dicer is working properly suggesting that these factors are essential for proper development of mouse pups. Thus, maternal Dicer activity determines metabolic phenotype in offspring.

All these findings pinpoint that Dicer is a very potent metabolic regulator not only in adipose tissue, but also in other organs that are important in regulating energy metabolism such as liver, pancreas and brain. Combined with the different strategies by which Dicer levels can be affected, this makes it an attractive target for future treatment and prevention strategies against overweight and obesity.

1.4 Aim of the study

As outlined, obesity is one of the most prevalent global diseases and is associated with severe comorbidities which not only tremendously harms patients' quality of life but also negatively affects life expectancy and has moreover devastating impact on national social security systems (Blüher 2019; Flegal et al. 2012; Tremmel et al. 2017).

In this regard, several research groups focused on the role of Dicer in AT, as a promising target molecule. Shortly summarized, studies suggested that accurately functional Dicer is positively associated with a healthy metabolic phenotype as it reduces AT inflammation, regulates food intake and enhances oxygen consumption as well as cellular insulin response (Mori et al. 2012, 2014; Reis et al. 2016; Rocha et al. 2020; Schneeberger et al. 2013). Moreover, loss of Dicer in AT is associated with lipodystrophy in humans and mice likewise (Mori et al. 2014; Torriani et al. 2016). This data implies that Dicer carries a protective role in obesity and thereby holds an enormous potential for future treatment options.

Yet, the identification of specific mechanistic events in adipocytes on molecular level remain so far largely unclear. The additional fact that currently available studies have been predominantly performed in mice holds another critical aspect within this topic.

Therefore, our general aim in this study was to elucidate the molecular role of Dicer in preadipocytes and adipocytes in a human setting with the intention to identify its involvement in crucial cellular activities including adipogenic differentiation and metabolic processes. The questions that we aimed to address in this context were:

- Does Dicer hold a key role in adipogenesis?
- Does Dicer regulate the expression of BAT associated genes in mature adipocytes?
- Do human pre-/adipocytes rely on Dicer with regard to their cellular energetic processes?
- To what extent does Dicer affect intracellular levels of mature miRNAs in pre-/adipocytes?

To answer these questions, we first aimed to investigate whether a loss of function impedes the transition of preadipocytes into mature adipocytes after induction of adipogenic differentiation in a human cell strain by examining the cellular morphology as well as the expression of specific marker genes. Next, we sought to elaborate, if reduced Dicer levels impair the expression of the hallmark protein of brown adipocytes UCP1 and other brown adipose tissue or browning related genes. This

included the investigation of potentially altered mitochondrial content as well as the effect on cellular respiration.

An additional subject matter for us to address, was to test if cellular energetic processes depend on appropriate Dicer activity and to delineate the underlying mechanism behind this, if so. As Dicer's main task is embedded in miRNA biogenesis, we were curious to clarify the importance of Dicer on overall miRNA expression in human pre-/adipocytes.

To answer all these questions, we decided to perform a loss of function approach. The model system we used for these experiments were human Simpson Golabi Behmel Syndrome (SGBS) preadipocytes. A second human primary cell strain, namely human multipotent adipose-derived stem cells (hMADS), served as an additional study model.

2. Material and Methods

2.1 Material

2.1.1 Cell strains and bacterial cells

cell type	*supplier*	*order number*
human multipotent adipose-derived stem cells	Ez-Zoubir Amri laboratory	
One Shot™ Stbl3 Chemically Competent E. coli	Thermo Fisher Scientific	#C737303
One Shot™ TOP10 Chemically Competent E. coli	Thermo Fisher Scientific	#C404010
Simpsons Golabi Behmel syndrome preadipocytes	own laboratory	

2.1.2 Cell culture media

name	*base medium*	*additives*	*final concentration*	*pH adjusted to*
0F + 10 % FCS	0F medium	FCS	10 % (v/v)	
0F medium	DMEM/F12	Panthotenat	17 µM	
		Biotin	33 µM	
		Penicilin/Streptomycin	1x	
3FC	0F medium	Cortisol	100 nM	
		Insulin	20 nM	
		T3	0.2 nM	
		Transferrin	0.01 mg/ml	
Glycolytic Rate Assay	XF base medium	Sodium pyruvate solution	1 mM	
medium		GlutaMAX	1x	7.0
LB agar	double-distilled	Bacto™ tryptone	10 g/L	
	water	Bacto™ agar	5 g/L	
		Bacto™ yeast extract	15 g/L	
		NaCl	10 g/L	
LB medium, 5x	double-distilled	Bacto™ tryptone	50 g/L	7.0
	water	Bacto™ yeast extract	25 g/L	
		NaCl	50 g/L	

Mito Stress Assay medium	XF base medium	Glucose solution	10 mM	
		Sodium pyruvate solution	1 mM	
		GlutaMAX	1x	
		BSA	1 % (w/v)	
QuickDiff medium	0F medium	Cortisol	100 nM	
		Dexamethasone	25 nM	
		IBMX	250 µM	
		Insulin	20 nM	
		Rosiglitazone	2 µM	
		T3	0.2 nM	
		Transferrin	0.01 mg/ml	
SOB medium	double-distilled water	Bacto™ tryptone	20 g/L	
		Bacto™ yeast extract	5 g/L	
		$MgCl_2$	10 mM	
		$MgSO_4$	10 mM	
		NaCl	10 mM	
		KCl	2.5 mM	
SOC medium	SOB medium	D-Glucose	20 mM	7.4
XF base medium	double-distilled water	DMEM base	8.3 g/L	
		HEPES	5 mM	

Media were prepared under sterile conditions or filter-sterilized. Additives were directly added to the medium. 3FC, QuickDiff medium as well as Glycolytic Rate and Mito Stress Assay medium were freshly prepared on the day of use. LB medium was kept at room temperature, while SOB and SOC media were frozen by -20 °C. Other media were stored at 4 °C.

2.1.3 microRNA mimics/inhibitors

microRNA	***supplier***	***order number***
hsa-miR-18a-3p mimic	Horizon Discovery	#C-300855-05-0002
hsa-miR-543 inhibitor	Horizon Discovery	#IH-301248-02-0002
negative control mimic	Horizon Discovery	#CN-001000-01-20
negative control inhibitor	Horizon Discovery	#IN-001005-01-20

MicroRNAs mimics, inhibitors and their according controls were resuspended in RNAse-free water yielding a stock concentration of 20 µM, aliquoted and stored at -20°C.

2.1.4 Small interfering RNAs *(siRNAs)*

target	*target sequence (5' - 3')*	*supplier*	*order number*
Dicer1	UAAAGUAGCUGGAAUGAUG	Horizon Discovery	#M-003483-00-0010
	GGAAGAGGCUGACUAUGAA		
	GAAUAUCGAUCCUAUGUUC		
	GAUCCUAUGUUCAAUCUAA		
non-targeting control	UAAGGCUAUGAAGAGAUAC	Horizon Discovery	#D-001206-14-50
	AUGUAUUGGCCUGUAUUAG		
	AUGAACGUGAAUUGCUCAA		
	UGGUUUACAUGUCGACUAA		

SiRNAs were resuspended in RNAse-free water yielding a stock concentration of 20 µM, aliquoted and stored at -20°C.

2.1.5 Plasmids for clustered regularly interspaced short palindromic repeats (CRISPR) gene editing

plasmid	*supplier*	*addgene order number*
pMuLE ENTR U6-sgRNA L1-R5	Ian Frew Laboratory	#1000000060
pMuLE ENTR U6-sgRNA L1-L4	Ian Frew Laboratory	#1000000060
pMuLE ENTR U6-sgRNA R4-R3	Ian Frew Laboratory	#1000000060
pMuLE ENTR CMV-hCas9 L5-L2	Ian Frew Laboratory	#1000000060
pMuLE ENTR CMV-hCas9 L3-L2	Ian Frew Laboratory	#1000000060
pMuSE SB eGFP-P2A-PuroR-DEST	cloned in own laboratory by Jan-Bernd Funcke	-
pMuSE UCP1	cloned in own laboratory by Jan-Bernd Funcke	-
pCMV(CAT)T7-SB100	Zsuzsanna Izsvak Laboratory	#34879

2.1.6 Single-guide RNAs *(sgRNAs)*

target gene	*top strand (5' - 3')*	*bottom strand (3' - 5')*
Dicer1 sgRNA #1	TTCAACGTGGAGCTTACCAG	AAGTTGCACCTCGAATGGTC
Dicer1 sgRNA #2	GTGTTGAGGCCTGACGATGG	CACAACTCCGGACTGCTACC
negative control #1	GTCACCGATCGAGAGCTAG	CAGTGGCTAGCTCTCGATC
negative control #2	ATGGCGCTTCAGTCGTCGG	TACCGCGAAGTCAGCAGCC
UCP1 sgRNA #1	GGGCGGATGAAACTCTACAG	CCCGCCTACTTTGAGATGTC
UCP1 sgRNA #2	GAATCAAACCTCGCTACACG	CTTAGTTTGGAGCGATGTGC

All sgRNAs were provided by biomers.net GmbH (Ulm, Germany), reconstituted to 100 µM in RNAse-free water and stored at -20°C.

2.1.7 Antibodies

target	*target species*	*host species*	*dilution*	*type*	*clonality*	*supplier*	*order number*
Adiponectin	human	rabbit	1/1,000	primary	polyclonal	GeneTex	#GTX112777
Akt	human	rabbit	1/1,000	primary	polyclonal	Cell signaling	#9272
Dicer	human	mouse	1/1,000	primary	monoclonal	abcam	#ab14601
GLUT1	human	rabbit	1/50,000	primary	monoclonal	abcam	#ab115730
IgG	mouse	goat	1/5,000	secondary	monoclonal	Biorad	#12004159
IgG	mouse	goat	1/5,000	secondary	monoclonal	Biorad	#12005867
IgG	rabbit	goat	1/5,000	secondary	monoclonal	Biorad	#12004162
IgG	rabbit	goat	1/5,000	secondary	monoclonal	Biorad	#12005870
IRS1	human	rabbit	1/1,000	primary	polyclonal	Millipore	#06-248
Leptin	human	rabbit	1/1,000	primary	polyclonal	Biovendor	#RD181001220
OXPHOS protein cocktail	human	mouse	1/1,000	primary	monoclonal	abcam	#ab110411
pAkt (S473)	human	rabbit	1/1,000	primary	polyclonal	Cell signaling	#9271
Perilipin	human	rabbit	1/1,000	primary	polyclonal	abcam	#ab3526
pIRS1 (Tyr608/612)	human	rabbit	1/1,000	primary	polyclonal	Millipore	#09-432
PPARγ	human	rabbit	1/1,000	primary	monoclonal	Cell signaling	#2443
TIMM23	human	rabbit	1/1,000	primary	polyclonal	abcam	#ab116329
UCP1	human	mouse	1/1,000	primary	monoclonal	R&D Systems	#536435
Vinculin	human	mouse	1/1,000	primary	monoclonal	Merck	#MAB3574-C
α-Tubulin	human	mouse	1/2,000	primary	monoclonal	Biorad	#12004166

Primary antibodies were diluted in TBST + 2 % (w/v) BSA + 0.03 % (w/v) NaN3, whereas secondary antibody solutions were prepared in blocking buffer.

2.1.8 qPCR and cloning primer

target gene	*sequence forward primer (5' - 3')*	*sequence reverse primer (5' - 3')*
ACACA	CTGTGGCTTCTCCAGCAGAATTTGTTACTC	GATCTGCCATCTTAATGTATTCTGCATTGGCT
ADIPOQ	GGCCGTGATGGCAGAGAT	CCTTCAGCCCCGGGTACT
AGO1	GACATCCCTAAGATCGACGTGT	CCACTTCCCGGTTGACTCTAC
AGO2	TCCACCTAGACCCGACTTTGG	GTGTTCCACGATTTCCCTGTT
AGO3	ATTGATGAGCAACCAAGACCTC	ACAAACACGGTATTTCCGTCTC
AGO4	AACCAATTCGACTGTTAGCCAA	TCTACGAGGCCGTTTTTCAGG
ATGL	GACGAGCTCATCCAGGCCAATGTCTG	GATGGTGTTCTTAAGCTCATAGAGTGGCAGG
ATP5A1	GTATTGCCCGCGTACATGG	AGGACATACCCTTTAAGCCTGA
CEBPα	GACCCTCAGCCTTGTTTGTACTGTATGCC	TTTGGAAAGCTTGTCATAACTCCGGTCCC
CEBPβ	CCGCCCGTGGTGTTATTTAAAGAAGAAACGTC	GCCCGTAGGAACATCTTTAAGCGATTACTCAG
CEBPγ	CCATCGACTTCAGCGCCTACATCGACTC	CCCGCCTTGTGATTGCTGTTGAAGAGGT
CIDEA	CTCATCAGCAAGACTCTGGATGCCCTCG	TTCCAAGATCATGAAATGCGTGTTGTCTCCC
CITED1	GGCAGCATCACTTCCCGCCAATTTATCC	TCATCTCTTGGTTGGCATCCTCCTTCGCAG
DICER	GATGAACTCAACTTTAGAAGGCGG	AGTGTGGAATCTGAGGTATGGGT
DIO2	CGATGCCTACAAACAGGTGAAATTGGGTGAG	AGCCTCATCAATGTAGACCAGCAGGAAGTCAG
FASN	CTACCTGAGCATAGTGTGGAAGACGCTG	CATCCCACTGGTACACCTTCCCACTCAC
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC
GLUT1	CTGGCATCAACGCTGTCTTCTATTACTCCAC	GGCCACGATGCTCAGATAGGACATCCAG
GLUT4	TTCCAACAGATAGGCTCCGAAG	AAGCACCGCAGAGAACACAG
HPRT	GAGATGGGAGGCCATCACATTGTAGCCCTC	CTCCACCAATTACTTTTATGTCCCCTGTTGACTGGTC
HSL	CTTCTGGAAAGCCTTCTGGAACATCACCGA	CTGAGCTCCTCACTGTCCTGTCCTTCAC
HOXC9	GGCAGCAAGCACAAAGAGGAGAAGG	CGACGGTCCCTGGTTAAATACATATTGAAGAG
LEP	GTTGCAAGGCCCAAGAAGCCCA	CAGTGTCTGGTCCATCTTGGATAAGGTCAGG
M13+	ACGTTGTAAAACGACGGCCAGT	-
NDUFB8	CCGCCAAGAAGTATAATATGCGT	TATCCACACGGTTCCTGTTGT
PLIN	GAAGTTGAAGCTTGAGGAGCGAGGATGG	GGCTTCCTTAGTGCTGGTGTAGGTCTTCTG
PGC1α	CTCAAATATCTGACCACAAACGATGACCCTC	GTTGTTGGTTTGGCTTGTAAGTGTTGTGAC
PPARγ	GATCCAGTGGTTGCAGATTACAA	GAGGGAGTTGGAAGGCTCTTC
SDHA	CATGCTGCCGTGTTCCGTGTGGG	GGACAGGGTGTGCTTCTTCCAGTGCTC
TRBP2	CAGGAGTATGGGACCAGAATAGG	ACCCGGAAGGTGAAATTAGGC
UCP1	GGAAAGAAACAGCACCTAGTTTAGGAAGCA	CGTCAAGCCTTCGGTTGTTGCTATTATTCTG
UQCRC2	TTCAGCAATTTAGGAACCACCC	GGTCACACTTAATTTGCCACCAA

All primers were provided by biomers.net GmbH (Ulm, Germany) and reconstituted to yield 10 pM primer stock solutions. Working solutions were prepared by diluting 20 µl of forward with 20 µl reverse primer stock solution in 360 µl sterile, double-distilled water and stored at -20 °C.

2.1.9 microRNA PCR Primer assays

target microRNA	*supplier*	*GeneGlobe catalogue ID*	*catalogue number*
hsa-miR-18a-3p	Qiagen	#YP00204523	#339306
hsa-miR-99b-3p	Qiagen	#YP00204064	#339306
hsa-miR-324-3p	Qiagen	#YP00204303	#339306
hsa-miR-370-3p	Qiagen	#YP00204011	#339306
hsa-miR-543	Qiagen	#YP00204447	#339306
hsa-miR-4760-3p	Qiagen	#YP02101287	#339306
hsa-miR-4760-5p	Qiagen	#YP02102642	#339306
let-7d-3p	Qiagen	#YP00205627	#339306
sno44 (SNORD44)	Qiagen	#YP00203902	#339306

Each microRNA PCR Primer assay was reconstituted with 220 µl RNAse-free water.

2.1.10 Antibiotics

name	*supplier*	*order number*
Ampicillin	AppiChem	#A0839
Kanamycin	Thermo Fisher Scientific	#11815024
Puromycin	Sigma-Aldrich	#P8833

2.1.11 Restriction enzymes

name	*supplier*	*order number*
BamHI	New England Biolabs	#R0136S
EcoRI-HF	New England Biolabs	#R3101S

2.1.12 Buffers and solutions

name	*base solution*	*additives*	*final composition*	*sterile-filtered*	*pH adjusted to*
2-deoxy-D-glucose solution	KRH	deoxy-D-glucose	100 mM		

Blocking buffer	washing buffer	skim milk powder	5 % (w/v)		
Janus Green solution	PBS^-	Janus Green B	0.3 % (w/v)		
KRH buffer	double-distilled water	$CaCl_2 \cdot 2\ H_2O$	2.5 mM	✓	7.4
		HEPES	10 mM		
		KCl	4.6 mM		
		$MgSO_4 \cdot 7\ H_2O$	10 mM		
		NaCl	130 mM		
		$NaH_2PO_4 \cdot H_2O$	2.5 mM		
		Pyruvat	2.5 mM		
Protein lysis buffer	protein isolation buffer	cOmplete protease inhibitor cocktail	1x		
		DTT	1 mM		
Panthotenat/Biotin	DMEM/F12	Biotin	3.3mM		
		Panthotenat	1.7 mM		
Protein isolation buffer	double-distilled water	EDTA	2 mM		7.5
		glycerol	10 % (v/v)		
		NaCl	150 mM		
		Tris-HCl	10 mM		
		Triton X-100	1 %(v/v)		
TBS, 10x	double-distilled water	NaCl	1.5 M		
		Tris-HCl	200 mM		
Washing buffer/TBST	TBS, 1x	Tween 20	0.1 % (v/v)		

2.1.13 Chemical, reagents and kits

name	***supplier***	***order number***
^{14}C-deoxy-D-glucose, 100 µCi/ml	Perkin Elmer	#NEC495050UC
2-deoxy-D-glucose	Sigma-Aldrich	#D8375
Acetyl-CoA	Sigma-Aldrich	#A2056

Antimycin A	Sigma-Aldrich	#A8674
Bacto™ agar	BD	#214010
Bacto™ tryptone	BD	#211705
Bacto™ yeast extract	BD	#212750
BAY 876	R&D Systems	#6199/5
Biotin	Sigma-Aldrich	#B4639
Bolt™ 4-12% Bis-Tris Plus gels, 1 mm, 10 well	Thermo Fisher Scientific	#NW04120BOX
Bolt™ 4-12% Bis-Tris Plus gels, 1 mm, 15 well	Thermo Fisher Scientific	#NW04125BOX
Bolt™ LDS sample buffer, 4x	Thermo Fisher Scientific	#B0007
Bolt™ MES SDS running buffer, 20x	Thermo Fisher Scientific	#B0002
Bolt™ sample reducing agent, 10x	Thermo Fisher Scientific	#B0009
Bradford protein assay concentrate, 5x	Biorad	#5000006
BSA	Sigma-Aldrich	#A7906
BSA protein assay standard	Thermo Fisher Scientific	#23209
cOmplete protease inhibitor cocktail tablets	Roche	#04693116001
Cortisol	Sigma-Aldrich	#H0888
CutSmart® buffer	New England Biolabs	#B6004
Dexamethasone	Sigma-Aldrich	#D1756
D-Glucose	Thermo Fisher Scientific	#Q100-37
Dibutyryl-cAMP	Sigma-Aldrich	#D0627
Direct-zol™ RNA Miniprep Kit	Zymo Research	#R2052
DMEM	Thermo Fisher Scientific	#41966029
DMEM base	Sigma-Aldrich	#D5030
DMEM/F12 (1:1)	Thermo Fisher Scientific	#31330-038
DMSO	Honeywell	#41641
dNTP mix, 20 mM	Thermo Fisher Scientific	#AB0196
DTNB	Sigma Aldrich	#D218200
DTT solution, 100 mM	Thermo Fisher Scientific	#Y00147
EDTA	Carl Roth	#8043
Enoxacin sodium salt	Merck	#557305-250MG
Ethanol	Honeywell	#32205
FCCP	Sigma-Aldrich	#C2920
Fetal calf serum	Thermo Fisher Scientific	#10270106
FGF-2, recombinant, human	PeproTech	#100-18B
Formaldehyde solution, 37-38 % (w/v)	AppliChem	#131328

Free glycerol reagent	Sigma-Aldrich	#F6428
Gel Loading Dye, Purple, 6x	New England Biolabs	#B7024S
Glucose solution, 1 M	Agilent	#103577-100
GlutaMAX, 100x	Thermo Fisher Scientific	#35050061
Glycerol	Carl Roth	#7530
Glycerol standard solution, ~2.8 mM	Sigma-Aldrich	#G7793
HCl solution, 1 M	Honeywell	#35328
HEPES, 1 M	BioChrome	#L1613
Hexan	Sigma-Aldrich	#296090
Human Leptin Quantikine ELISA Kit	RnD Systems	#DLP00
IBMX	Sigma-Aldrich	#I5879
Insulin	Sigma-Aldrich	#I1507
Isopropanol	VWR	#20842
Janus Green B	Carl Roth	#7697
KCl	Merck	#104935
L-glutamine solution, 200 mM, 100x	Thermo Fisher Scientific	#25030024
Lipofectamine™ 2000 Transfection Reagent	Thermo Fisher Scientific	#11668019
LR Clonase™ II Plus enzyme	Thermo Fisher Scientific	#12538120
MEM Non-Essential Amino Acids Solution (100X)	Thermo Fisher Scientific	#11140035
MgCl	Merck	#105833
$MgSO_4 \cdot 7\ H_2O$	Sigma-Aldrich	#230391
miRCURY LNA RT Kit	Qiagen	#339340
miRCURY LNA SYBR Green PCR Kit	Qiagen	#339346
Monarch™ Plasmid Miniprep Kit	New England Biolabs	#T1010L
Na_2HPO_4	AppliChem	#A3567
NaCl	Merck	#106406
$NAH_2PO_4 \cdot H_2O$	Merck Millipore	#106349
NaN_3	Sigma-Aldrich	#S2002
NaOH pellets	Merck	#106498
NEBuffer 3.1	New England Biolabds	#B7203
Neon™ Transfection System 100µl-Kit	Thermo Fisher Scientific	#MPK10025
Nile Red, 1 mg/ml	Sigma-Aldrich	#N3013
Novex™ NuPAGE™ Tris-Acetate SDS Running Buffer	Thermo Fisher Scientific	#LA0041
NuPAGE™ 3-8% Tris-Acetate gel, 10-Well, 1 mm	Thermo Fisher Scientific	#EA0375BOX
NuPAGE™ 3-8% Tris-Acetate gel, 15-Well, 1 mm	Thermo Fisher Scientific	#EA03785BOX

Oligomycin	Sigma-Aldrich	#04876
Opti-Mem™ Reduced Serum Medium	Thermo Fisher Scientific	#31985047
Oxaloacetate	Sigma-Aldrich	#O4126
Panthotenat	Sigma-Aldrich	#P-5155
PBS^-	Thermo Fisher Scientific	#14190094
Penicillin/streptomycin solution, 100x	Thermo Fisher Scientific	#15140122
peqGold DNA ladder mix	VWR	#25-2040
peqGreen DNA/RNA dye	VWR	#732-3196
PhosSTOP™ Phosphatase Inhibitor Cocktail Tablets	Roche	#04906845001
Precision Plus Protein™ All Blue Prestained Protein Standards	Biorad	#1610373
Proteinase K solution	Thermo Fisher Scientific	#25530049
PureLink™ HiPure Plasmid Midiprep Kit	Thermo Fisher Scientific	#K210005
Random Primers	Thermo Fisher Scientific	#48190011
Rosiglitazone	Cayman	#71740
Rotenone	Sigma-Aldrich	#R8875
ROTISZINT® eco plus	Carl Roth	#0016.3
Seahorse XF calibrant solution	Agilent	#102601-100
SeeBlue™ Plus2 Pre-stained Protein Standard	Thermo Fisher Scientific	#LC5925
Skim milk powder	Sigma-Aldrich	#70166
Sodium pyruvate solution, 100 mM, 100x	Thermo Fisher Scientific	#11360039
SsoAdvanced Universal SYBR® Green Supermix	Biorad	#1725274
SuperScript™ II Reverse Transcriptase	Thermo Fisher Scientific	#18064071
T4 Polynucleotide Kinase	New England Biolaba	#M0201S
TE buffer	Thermo Fisher Scientific	#12090015
Trans-Blot® Turbo™ RTA Midi Nitrocellulose Transfer Kit	Biorad	#1704271
Trans-Blot® Turbo™ RTA Mini Nitrocellulose Transfer Kit	Biorad	#1704270
Transferrin	Sigma-Aldrich	#T2252
Triglyceride reagent	Sigma-Aldrich	#T2449
Triiodothyronine (T3)	Sigma-Aldrich	#T6397
Tris base	Sigma-Aldrich	#T1503
Tris HCl	Sigma-Aldrich	# 10812846001
Triton X-100	Serva	#37240
Trypsin/EDTA solution, 0.05 %/0.02 %	Merck	#L2143
Tween 20	AppliChem	#A1389

2.1.14 General material

equipment	*supplier*	*order number*
cell culture dishes, 10 cm	Sarstedt	#83.3902
cell culture dishes, 6 cm	Sarstedt	#83.3901
cell culture flasks, 175 cm²	Sarstedt	#83.3912.002
cell culture plates, 12 well	Corning	#353043
cell scrapers	Sarstedt	#83.1830
cryotubes	Sarstedt	#72.379.992
glass pipettes	VWR	#612-1701
pipette tips, 1250 µl	Sarstedt	#70.1186
pipette tips, 20 µl	Sarstedt	#70.1116
pipette tips, 200 µl	Sarstedt	#70.760.002
plate sealing films	Biorad	#MSB1001
reaction tubes, 0.5 ml	Sarstedt	#72.704.400
reaction tubes, 1.5 ml	Sarstedt	#72.706.400
reaction tubes, 2 ml	Sarstedt	#72.695.400
Seahorse XF96 Cell Culture Microplates	Agilent	#102601-100
Seahorse XF96 Flux Plates	Agilent	#102601-100
serological pipettes, 1 ml	Corning	#357521
serological pipettes, 10 ml	Corning	#4488
serological pipettes, 25 ml	Corning	#4489
serological pipettes, 5 ml	Corning	#4487
stepper pipette tips, 0.5 ml	Eppendorf	#0030089634
stepper pipette tips, 0.5 ml, non-sterile	Eppendorf	#0030089421
stepper pipette tips, 10 ml, non-sterile	Eppendorf	#0030089820
stepper pipette tips, 5 ml	Eppendorf	#0030089669
stepper pipette tips, 5 ml, non-sterile	Eppendorf	#0030089456
sterile filter units, 0.2 µM, 250 ml	Thermo Fisher Scientific	#568-0020
sterile filter units, 0.2 µM, 500 ml	Thermo Fisher Scientific	#569-0020
sterile filters for syringes, 0.2 µM	GE Healthcare	#10462200
syringes, 1 ml	BD	#303172
syringes, 20 ml	BD	#300629
syringes, 50 ml	BD	#300863
tubes for mammalian cell culture, 15 ml	Sarstedt	#62.554.502
tubes for mammalian cell culture, 50 ml	Sarstedt	#62.547.254

2.1.15 Devices

instrument	*manufacturer*
Bolt™ Mini Gel Tank electrophoresis chamber	Thermo Fisher Scientific
BZ-9000E fluorescence microscope	Keyence
CFX Connect Real-Time PCR Detection System	Biorad
Concentrator 5301	Eppendorf
DMi1 light microscope	Leica
Infinite® 200 Pro M Plex microplate reader	Tecan Group Ltd.
NanoDrop™ 2000 spectrophotometer	Thermo Fisher Scientific
Neon™ Transfection System	Thermo Fisher Scientific
Chemidoc™ MP Imaging System	Biorad
Seahorse XFe96 analyzer	Agilent
Mini-Sub Cell GT Cell electrophoresis chamber	Biorad
Multifuge X3FR	Thermo Fisher Scientific
Mikro 220R	Hettich
peqStar 2X Universal Gradient thermocycler	VWR
Trans-Blot Turbo™ Transfer System	Biorad

2.1.16 Software

software	*version*	*supplier*
CFX Manager	3.1.1517.0823	Biorad
Excel	15.0.5431.1000	Microsoft
i-control™ Microplate Reader Software	2.0	Tecan Group Ltd.
Inkscape	0.92.3	GNU General Public License
Image Lab™	6.0.1	Biorad
NanoDrop 2000/2000c	1.4.2	Thermo Fisher Scientific
Prism	8.4.3	GraphPad
SnapGene	4.1.4	GSL Biotech
Wave	2.6.0.31	Agilent
Word	15.0.5431.1000	Microsoft

2.2 Methods

Standard procedures will be mentioned here and apply for all following experiments in this chapter. If not indicated differently, all cells were incubated in 100 % (v/v) saturated water vapor, at 37 °C and 5 % (v/v) carbon-dioxide concentration. Cell media and solutions were pre-warmed in water bath at 37 °C, before every cell culture procedure. Centrifugation was performed at room temperature. And experiments were performed at least in three independent approaches.

2.2.1 Sterile cell culture and adipogenic differentiation of SGBS and hMADS cells

Thawing

Cells stored in liquid nitrogen were thawed by transferring the cell suspension into a 50 ml falcon tube and by slowly adding 10 ml 0F medium + 10 % (v/v) fetal calf serum (FCS). Cells were centrifuged at 200 x gravity (x g) for 10 minutes. Cell pellet were resuspended in 0F medium + 10 % FCS, seeded into a T175 flask and cultivated until subconfluent.

Cell expansion

Cell media were aspirated and cells washed twice with PBS^-. Trypsin/EDTA was used to detach cells while incubated for five minutes and 0F + 10 % FCS to suspend and collect all cells. After 10 minutes of centrifugation at 200 x g, the number of cells was determined using a Neubauer hemocytometer, cells diluted in 0F + 10 % FCS and seeded at the required density into the appropriate cell culture vessel as outlined in Table 1. From this point on, seeding procedure was performed every seven days for SGBS cells, and every three to four days for hMADS cells. For culturing of hMADS cells, cell culture medium was supplemented with FGF-2 at a final concentration of 2.5 ng/ml.

Table 1 - Cell seeding densities

container type	***number of cells seeded SGBS/hMADS***	***medium volume***
T175 flask	*1.5 x 10^5 / 3-4 x 10^5*	*24 ml*
6-cm cell culture dish	*1 x 10^5 for both cell types*	*5 ml*
12-well cell culture plate	*2 x 10^4 per well / 2.25 x 10^4 per well*	*1 ml/well*
Seahorse XF96 cell culture microplate	*2.5 x 10^3 per well for both cell types*	*100 µl/well*

Adipogenic differentiation

Differentiation of preadipocytes was initiated by aspirating the present 0F + 10 % FCS, washing once with PBS^- and adding the appropriate amount of QuickDiff medium – three days post-seeding. After another four days, the QuickDiff medium was replaced by 3FC medium and cells further cultivated for ten more days reaching day 14 of adipogenic differentiation.

Enoxacin treatment during adipogenic differentiation

To test the effect of enoxacin on adipogenic differentiation, human SGBS and hMADS cells were subjected from day 0 until day 14 of adipogenic differentiation with 25, 50 or 100 µM enoxacin diluted in either QuickDiff or 3FC medium. 0.2 % DMSO treated cells served as corresponding controls.

2.2.2 Differentiation rate

To determine the percentage of mature adipocytes following differentiation, mature adipocytes, carrying at least five clearly visible lipid droplets, and preadipocytes were counted in randomly a minimum of three chosen spots within the according cell vessel using the graticule plate within the microscope's oculus. To calculate the total amount of cells in a certain container, the number of cells counted was multiplied by the surface area in mm^2 considering the applied magnification. The number of undifferentiated cells and the total number of all cells counted were used to calculate the mean percentage of differentiation into mature adipocytes after 14 days of differentiation in SGBS and hMADS cells.

2.2.3 Triglyceride content

To evaluate the amount of accumulated lipids, cell medium was aspirated, and triglycerides extracted by thoroughly washing the cells two times with 500 µl hexan/isopropanol (3:2). Triglycerides were harvested individually for each well resulting in three technical replicates per construct. The collected samples were transferred into 1.5 ml tubes and centrifuged for 30 minutes at 30 °C in a vacuum concentrator for evaporation of the organic solvent. Next, samples were either stored in the freezer at -20 °C or subsequently processed for quantification. For further processing, samples were resolved in 100 µl 100 % isopropanol and thoroughly vortexed. The triglyceride standard was prepared by serially diluting glycerol 1:2 in isopropanol resulting in a concentration of 1400, 6700, 350, 175, 87.5 and 43.75

µM triglycerides as well as one standard only containing 100 % isopropanol as zero value. 10 µl of each standard and 2 µl of each sample were pipetted into a well in a 96-well plate. 200 µl triglyceride working solution – consisting of four parts free glycerol reagents and one part triglyceride reagent – were added to each well and incubated for 15 minutes at room temperature while gently shaking at ~80 rpm. In the next step, the extent of the colorimetric reaction was measured via an Infinite® 200 Pro M Plex microplate reader at a wavelength of 550 nm while using the i-control™ Microplate Reader Software. The standard curve created in GraphPad PRISM was employed to calculate the concentrations and to quantify the triglyceride content in each sample. All replicates were measured in duplicates and values were averaged.

2.2.4 RNA isolation, preparation and reverse transcription

To harvest RNA for expression analysis, cell media were aspirated and three wells per condition pooled using 500 µl TRIzol™ reagent with the Direct-zol™ RNA Miniprep kit according to the manufacturer's manual.

To isolate RNA, harvested samples were transferred into labelled 1.5 ml reaction tubes and 500 µl 100 % ethanol were added, samples thoroughly vortexed, pipetted into spin columns and centrifuged at 13,800 x g for one minute. Flowthroughs were discarded, spin columns washed with 400 µl pre-wash buffer and centrifuged at 13,800 x g another minute. This step was repeated once. 700 µl washing buffer were pipetted and spin columns again centrifuged at 13,800 x g for one minute. To eliminate any traces of solutions, centrifugation was repeated after placing spin columns into new collection tubes. Finally, RNA was eluted with 40 µl RNAse-free water by centrifuging at 13,800 x g one minute while spin columns were moved into new 1.5 ml reaction tubes. To avoid degradation, eluted RNA samples were immediately kept on ice. The purity and the concentration of the samples collected were tested in a NanoDrop™ 2000 spectrophotometer at 260 and 280 nm.

Reverse transcription of mRNAs

To assess mRNA expression levels, RNA samples were transcribed into complementary DNA by reverse transcription. For this, reaction mixes were adjusted to 11 µl with RNAse-free water, 1 µl Random Primer (3 µg/ml) were added to a total volume of 12 µl and samples shortly vortexed.

While RNA samples were denaturated at 85 °C for 3 minutes in a thermocycler, a master mix was prepared by mixing 5 µl 5x first strand buffer with 2.5 µl DDT (0.1 M), 1.5 µl sterile water, 1 µl oligonucleotides (10 µM) and 1 µl SuperScript™ (200 units) for each sample.
11 µl master mix was added to each sample and samples shortly vortexed and centrifuged. Reverse transcription was carried out at 25 °C for 15 minutes, 42 °C for 50 minutes, following 70 °C for 15 minutes in a thermocycler. After completion, the samples were diluted in sterile, double-distilled water to obtain an end concentration of 50 ng/µl and stored at 4 °C for short-term respectively at -20 °C for long-term preservation.

Reverse transcription of miRNAs

For reverse transcription of miRNAs, the miRCURY LNA RT Kit from Qiagen was used. RNA samples were diluted to 100 ng up to 5 µl of RNAse-free water. 5 µl of a prepared master mix containing 2 µl of 5x miRCURY RT reaction buffer, 2 µl RNAse-free water and 1 µl 10x miRCURY RT Enzyme Mix were added to each sample. Samples were briefly vortexed and centrifuged. Reverse transcription was carried out 42 °C for 60 minutes followed by an inactivation step performed at 95 °C for five minutes. After completion, the samples were diluted in RNAse-free water yielding an end concentration of 0.33 ng/µl and stored at 4 °C for short-term respectively at -20 °C for long-term preservation.

2.2.5 Quantitative real-time polymerase chain reaction

Assessment of relative mRNA expression

To relatively quantify mRNA expression level, cDNA was amplified and measured via qRT-PCR. First, master mixes containing 5 µl 2x Sso advanced SYBR Super Mix, 3 µl sterile water and 1 µl of a primer working solution (5 µM) per well and target were prepared. 9 µl of the appropriate master mix were dispensed into a 96-well PCR plate, 1 µl cDNA (5 ng/µl) added, the plate sealed using adhesive seals and shortly centrifuged. All samples were analyzed in duplicates for each target. The qRT-PCR was carried out according to the following protocol:

1x	*95 °C*	*for 30 seconds*
40x	*95 °C*	*for 5 seconds*
	and 60 °C	*for 30 seconds*
1x	*65 °C*	*for 5 seconds*
1x	*95 °C*	

Assessment of relative miRNA expression

The miRCURY LNA SYBR Green PCR Kit was used to relatively quantify miRNA expression levels. For this, 7 µl master mix containing 5 µl 2x miRCURY LNA SYBR, 1 µl miRCURY LNA miRNA PCR Assays and 1µl RNAse-free water were added to each well. Next, 3 ml of the according sample was added, plates sealed and briefly centrifuged. All samples were analyzed in duplicates for each target. The miRCURY qRT-PCR was carried out according to the following protocol:

1x	*95 °C*	*for 2 minutes*
40x	*95 °C*	*for 10 seconds*
	and 56 °C	*for 60 seconds*
1x	*65 °C*	*for 5 seconds*
1x	*95 °C*	

2.2.6 Western blotting

While working on ice, cell medium was aspirated from cell culture dishes and cells washed once with 10 ml ice-cold PBS^-. 80 µl lysis buffer were added onto the cells, cells collected by scrapping, transferred into a 1.5 ml Eppendorf tube, thoroughly vortexed and kept on ice for 20 minutes. After incubation, samples were centrifuged at 17,000 x g and 4 °C for 30 minutes. The interphase between cell debris and lipids was carefully pipetted into a 2 ml reaction tube and stored at -20 °C.

Protein concentration of cell lysates was quantified by pipetting 1 µl of each lysate in duplicates into a well of a 96-well plate. 200 µl Bradford Protein Assay Dye Reagent (1x) was added, samples incubated for 5 minutes at room temperature while gently shaking at ~80 rpm and extinction measured at 550 nm in an Infinite® 200 Pro M Plex microplate reader using the i-control™ Microplate Reader Software. To generate a standard curve, 10 µl of diluted BSA solutions with defined concentrations were analyzed in the exact same way. The standard curve created in GraphPad Prism was employed to calculate sample concentrations. All samples were measured in duplicates and values were averaged.

To separate proteins via sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), 25µg protein was diluted in double-distilled water. Buffer (4x) and reducing agent (10x) were added resulting in an onefold concentration of both solutions within each sample. Following preparation, samples were incubated for 10 minutes at 70 °C. 10 µg protein for each sample was loaded onto NuPAGE Novex 4-12 % Bis-Tris gels placed in a Bolt™ Mini Gel Tank electrophoresis chamber filled with NuPAGE MES SDS

running buffer (1x). Electrophoresis was performed at 200 V and 100 W for 35 minutes respectively for 45 minutes when specifically analyzing mitochondrial complexes of the respiratory chain.
For detection of large proteins with a molecular weight > 100 kDa, protein samples were loaded onto NuPAGE™ 3-8% Tris-Acetate gels and run with Novex™ NuPAGE™ Tris-Acetate SDS Running Buffer for 60 minutes at 150 V.

Following SDS-PAGE, proteins were transferred onto Trans-Blot® Turbo™ RTA nitrocellulose membranes by Western Blotting at 25 V and 1.3 mA using the Trans-Blot Turbo™ Transfer System for 7 minutes while protein transfer from NuPAGE™ 3-8% Tris-Acetate was carried out for 10 minutes.

Next, nitrocellulose membranes were blocked for 1 hour in blocking buffer, following washing procedure of three times for 10 minutes in TBST. Finally, membranes were incubated in primary antibody solution overnight at 4 °C or for 1 hour at room temperature. All primary antibodies were diluted in TBST + BSA + NaN_3 as described in 2.1.7.

Following incubation, the primary antibody solutions were removed and stored at 4 °C for later reuse. The membranes were washed three times for 10 minutes in TBST, incubated for 1 hour at room temperature in diluted secondary antibody solution reactive against the according species of the primary antibody (see 2.1.7). Again, membranes were washed three times for 10 minutes in TBST. For semi-quantitative analysis, blot images were visualized using the Chemidoc™ MP Imaging System and densitometrically evaluated with the Image Lab™ software. After analysis, membranes were washed once for 10 minutes in TBST, air-dried at room temperature overnight and stored for reuse on room temperature.

Assessment of GLUT1 protein levels following enoxacin treatment

To evaluate the effect of enoxacin treatment on glucose transporter 1 (GLUT1) protein level, SGBS preadipocytes were stimulated with 25 µM enoxacin for 48 hours. Corresponding control cells were treated with 0.2% DMSO instead. Next, proteins were harvested and analyzed as described in the previous section.

2.2.7 Citrate synthase activity

To assess altered mitochondrial biogenesis, citrate synthase activity was measured, as a marker for mitochondrial mass. For evaluating citrate synthase activity in protein preparations, samples were diluted to 5 µg/10 µl in sterile water. 10 µl sample were pipetted into a 96-well plate, reaction medium

prepared according to the manufacturer's protocol and 180 µl added onto each sample (see Table 2). The citrate synthase activity was indirectly assessed by measuring increase of 2-nitro-5-benzoic acid (TNB) over time. When citrate synthase transforms oxaloactetate and acetyl-CoA into citrate and CoA-SH, the latter reacts with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) by which TNB is formed. TNB specifically absorbs at 412 nm which can be evaluated. Following pipetting, extinction at 37 °C was measured with an Infinite® 200 Pro M Plex microplate reader using the i-control™ Microplate Reader Software. All samples were measured in triplicates and values calculating as described as follows:

1. **Values were corrected for the background extinction:**

   $\Delta E/min_{activity\ slope} - \Delta E/min_{background\ slope} = corrected\ \Delta E/min$

2. **According to Lambert's Law, the corrected ΔE/min determined was utilized to calculate the activity:**

   $Extinction\ E = \varepsilon \cdot b \cdot c$ ε = molar extinction coefficient [L/mol · cm]

   b = path length [cm]

   c = concentration [mol/L]

   In this case, ε = 13.6 and b = 0.625. Hence:

   $\frac{corrected\ \Delta E/min}{13.6 \cdot 0.625}$ = concentration/min = activity [µmol/min]

3. **To calculate the activity per µg protein:**

   $\frac{activity}{amount\ of\ protein\ used}$ = specific activity [µmol/min/µg protein]

Table 2 - Pipetting scheme for citrate synthase reaction medium

reagent	***volume per sample***
DTNB [5 mM]	*4 µL*
acetyl-CoA [10 mM]	*6 µL*
10% (v/v) Triton X-100	*2 µL*
Tris HCl [1 M], pH 8.1	*19 µL*
double-distilled water	*149 µL*

2.2.8 Extracellular flux analysis

For performing the Mito Stress assay, SGBS cells were seeded and subjected to adipogenic differentiation for 14 days as described in section 2.2.1. On the day before measurement, 80 µl of the 3FC medium was aspirated, cells washed once with 100 µl 0F and 100 µl 0F added for further cultivation overnight. Additionally, a Seahorse XF96 FluxPlate was prepared by dispensing 200 µl XF calibrant solution per well and incubated overnight in a CO_2-free incubator at 37 °C. The following day, 100 µl medium were aspirated, adipocytes washed once with 100 µl XF assay medium and then incubated in 175 µl freshly prepared Mito Stress Assay medium for one hour in a CO_2-free incubator at 37 °C. Meanwhile, ports on the FluxPlate were loaded with 25 µl of the appropriate agent and the FluxPlate placed in the analyzer for calibration. For this, dibutyryl-cyclic AMP (cAMP), oligomycin, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) and a stock solution of antimycin A/rotenone (AA/Rot) were reconstituted and diluted in XF assay medium to yield a final well concentration of 0.5 mM, 2 µM, 4 µM respectively of 1 µM. For each inhibitor, the optimal concentration was previously titrated. Following calibration and cell incubation, oxygen consumption as well as extracellular acidification rate were measured in a Seahorse XFe96 analyzer.

Mito Stress Assay

In the Mito Stress Assay, the number of measurements was comprised of 19 cycles each consisting of 3 minutes mixing and 3 minutes assessment intervals. After three basal cycles, cAMP or XF assay medium (controls) were automatically injected and metabolic parameters determined in eight cycles. Then oligomycin was added to inhibit adenosine triphosphate (ATP) synthase and to assess respiration linked to ATP production. After five additional measurements, FCCP was injected to fully uncouple ATP synthesis from electron transport chain and thereby to induce maximal respiration. Following another three cycles, AA/Rot was added to completely suppress oxidative phosphorylation identifying non-mitochondrial respiration rates. Then, oxygen consumption and extracellular acidification rates were determined in four additional cycles (for further details see **Figure 17**).

Normalization for lipid content

To normalize for well-to-well differences in adipocytes number, assay values were normalized for lipid content by Nile Red lipid staining. Accordingly, medium was completely aspirated from each well and cells fixed for 10 minutes at room temperature with 100 µl of 4 % (w/v) formaldehyde solution.

Following this, formaldehyde was removed, adipocytes stained with 100 µl Nile Red diluted by 1000-fold in PBS^- + 10 % (v/v) DMSO and fluorescence emission measured at 600 nm in an Infinite® 200 Pro M Plex microplate reader using the i-control™ Microplate Reader Software.

Glycolytic Stress Assay

For Glycolytic Rate Assay experiments in preadipocytes, 80 µl cell media of siRNA transfected SGBS and hMADS cells was aspirated, cells washed once with 100 µl 0F and 100 µl 0F added for further cultivation overnight. A Flux Plate was prepared as described for the Mito Stress Assay. On the following day, preadipocytes were glucose starved for 1 hour in 175 µl Glycolytic Rate Assay medium. In experiments, where the GLUT1 inhibitor BAY 876 was used, either DMSO or BAY 876 were included at a final concentration of 0.1 % or 100 nM to the medium. While incubating, each Flux Plate port was loaded with 25 µl compound yielding a final well concentration of 10 mM glucose solution, 0.5 µM AA/Rot, 50 µM 2-DG solution and Glycolytic Rate Assay medium following injection.
The number of measurements in this approach was comprised of 15 cycles each consisting of 3 minutes mixing and 3 minutes assessment intervals. After three basal cycles, glucose solution was automatically injected by which the basal glycolytic activity could be determined. Next, AA/Rot was added to fully suppress oxidative phosphorylation enabling to identify compensatory glycolytic activity. After three measurement cycles, 2-DG was added to abrogate glucose metabolism. At a final step, Glycolytic Rate Assay medium was injected, and measurements prolonged for three additional cycles. The obtained data from oxygen consumption and extracellular acidification rates were used to calculate the proton efflux rate as indicated by the manufacturer.

Normalization for cell density

For preadipocyte normalization, the total number of cells within each well was evaluated by staining cells in 50 µl Janus Green B solution for 30 minutes while shaking at 100 rpm. Next, plates were rinsed with tap water and dried. Incorporated Janus Green B was released by cell lysis in 100 µl 0.5 M HCl solution for 10 minutes on a shaker set to 100 rpm and measured emission after excitation at 630 nm in an Infinite® 200 Pro M Plex microplate reader using the i-control™ Microplate Reader Software.

2.2.9 Glucose uptake

To assess glucose uptake of SGBS and hMADS cells, medium of adipocytes at day 13 respectively preadipocytes at day 0 of differentiation was aspirated and cells were carefully washed once with pre-warmed PBS^-. 1 ml pre-warmed 0F medium was added and cells were incubated overnight to initiate glucose uptake experiments.

On the following day, 0F medium was aspirated, cells cautiously washed once with warm KRH containing freshly added BSA at a final concentration of 0.05 % (w/v) and the cells starved for glucose for 3 hours in 1 ml Krebs-Ringer buffer (KRH) + 0.05 % BSA in the incubator.

Glucose working solution was prepared using 13 µl 2-deoxy-D-glucose (100 mM) and 26 µl radioactively labelled ^{14}C-deoxy-D-glucose (100 µCi/ml) and diluted in 611 µl KRH (refers to the volume needed for one plate). 50 µl glucose working solution were added into each well (~ 0.2 µCi/well), incubated for exactly 15 minutes and reaction stopped by aspiration, carefully washing cells twice with 3 ml cold PBS^- and adding 1 ml of 0.1 M NaOH. Cells were suspended in the NaOH solution and each suspension transferred into a pre-labelled scintillation tube containing 8 ml ROTISZINT® eco plus. Harvesting of cells was repeated with additional 1 ml NaOH, scintillation tubes sealed and analyzed in a beta-counter.

Insulin stimulated glucose uptake

For insulin induced glucose uptake, insulin was diluted in KRH + 0.05 % BSA. Three wells were stimulated with 100 µl insulin yielding 0.1, 1 and 100 nM and precisely incubated for 15 minutes in the incubator.

Assessment of glucose uptake following GLUT1 inhibition via BAY 876

For glucose uptake experiments in preadipocytes using the GLUT1 inhibitor BAY 876, cells were pre-incubated for 1 hour in either 0.1 % DMSO or 100 nM BAY 876 during KRH starvation in glucose uptake experiments.

Assessment of glucose uptake following miRNA mimic and inhibitor transfection

Glucose uptake assays in miR-18a-3p mimic transfected preadipocytes were started at day 0 of adipogenic differentiation, whereas experiments in miR-543 inhibitor transfected cells were initiated 48 hours post transfection before conducting glucose uptake experiments (for details see 2.2.13).

Assessment of glucose uptake following Enoxacin treatment

To assess the effect of enoxacin treatment on glucose uptake, SGBS preadipocytes were stimulated with 25 µM enoxacin for 48 hours before conducting glucose uptake experiments. Corresponding control cells were treated with 0.2% DMSO instead.

2.2.10 Insulin sensitivity

To check insulin-dependent pathway activation in the SGBS cell model, insulin dependent phosphorylation of Akt and insulin receptor substrate (IRS1) were analyzed. Therefore, SGBS cells were cultivated in 6 cm dishes. At day 13 of differentiation, the present medium was aspirated and cells washed once with 5 ml pre-warmed PBS^-. 5 ml 0F medium was added and cells incubated overnight.
On the next day, insulin was diluted in 0F medium and cells were stimulated with 100 µl of the appropriate dilution resulting in an insulin end concentration of 1 and 100 nM. The incubation time was precisely 15 minutes. Vehicle control cells served as a reference.

From this point on, the experiments were performed analogous to the Western Blot procedure as described in 2.2.6 - except for the fact that PhosSTOP™ phosphatase inhibitor cocktail was added to the regularly used lysis buffer.

2.2.11 Lipogenesis assay

SGBS cells were seeded and subjected to adipogenic differentiation for 14 days as described in section 2.2.1. Next, cells were carefully washed once with pre-warmed PBS^-. 1 ml pre-warmed 0F medium was added and cells incubated overnight.

On the following day, 0F medium was aspirated, cells cautiously washed once with 3 ml warm KRH buffer containing freshly added BSA at a final concentration of 0.05 % (w/v) and another 1 ml KRH + 0.05 % BSA in the incubator. For insulin sensitivity experiments, insulin was diluted in KRH + 0.05 % BSA. For each condition, three wells were stimulated with 100 µl pre-diluted insulin solution yielding 0.1, 1 or 100 nM and precisely incubated for 15 minutes in the incubator. Next, radioactively labelled glucose was diluted in KRH buffer and 10 µl of this dilution added into each well resulting in a final concentration of 0.1 µCi/ml and incubated for exactly 24 hours. Reaction was stopped by aspirating media, carefully washing cells twice with 3 ml cold PBS^- and adding 1 ml of 0.1 M NaOH. Cells were

suspended in the NaOH solution and each suspension transferred into a pre-labelled scintillation tube containing 8 ml ROTISZINT® eco plus. Harvesting of cells was repeated with additional 1 ml NaOH, scintillation tubes sealed and analyzed.

2.2.12 Leptin secretion

To quantify secretion of leptin in mature human SGBS adipocytes, supernatants were collected at day 14 of differentiation and analyzed using the Human Leptin Quantikine ELISA Kit from RnD Systems.

According to the manufacturer's protocol, 100 µl assay diluent were initially added to each well in a 96 well plate. Next, 100 µl of standard, control and sample were added and incubated for two hours at room temperature. Following incubation, each well was washed in a total of 4 times. 200 µl of leptin antibody conjugate were pipetted into each well and incubated for one hour at room temperature. Conjugate solution was then aspirated and wells washed for four times with. Next, 200 µl substrate solution was added and incubate for 30 minutes at room temperature while protected from light. In a final step, reaction was stopped using 50 µL stop solution per well and extinction was measured at a wavelength of 450 nm in an Infinite® 200 Pro M Plex microplate reader and quantified using the i-control™ Microplate Reader Software.

2.2.13 siRNA and miRNA mimic/inhibitor transfection

In order to perform siRNA and miRNA mimic/inhibitor transfection, cells were seeded at 10 % higher density. After overnight culturing, for each well to transfect, 100 µl transfection solution per 1 ml cell culture medium was prepared by adding 1 µl of 20µM siRNA to 49 µl Opti-Mem™ and incubating for five minutes at room temperature before mixing with 2.5 µl Lipofectamine™ 2000 in 47.5 µl Opti-Mem™. After additional 20 minutes on room temperature, transfection solutions were gently dropped onto the cells yielding a final concentration of 20 nM

In contrast to the general transfection procedure, miR-543 inhibitor transfected cells and their corresponding controls were seeded at the usual cell densities as displayed in Table 1 and cultured for three days. Next, cells were transfected with 20 nM miR-543 inhibitor or non-targeting inhibitor control. Glucose uptake experiments as well as RNA harvesting of these cells were performed 72 hours post transfection procedure.

2.2.14 MultiSite Gateway Cloning for CRISPR-Cas9 mediated gene knockout

To generate loss of function cells, a CRISPR-Cas9 based method was used - as the state of the art for genetic modifications. Originated from bacteria, the endonuclease Cas9 catalyzes the enzymatic degradation of nucleic acids. In this process, guide RNAs serve as reference to recognize specific target DNA [84]. In molecular biology, this feature is exploited to produce genetically modified organisms by creating particular single guide RNAs (sgRNAs) in order to knockout a certain gene of interest [85]. The design of specific sgRNAs and their complementary strand can be accomplished by using the online available CRISPick tool of the Broad Institute which provides an algorism based method for potential sgRNA sequences with high target specificity and low off target effects [86,87]. These sgRNAs are then inserted into a plasmid ENTRY vector. A commonly used tool for this type of genetic engineering is the multiple lentiviral expression (MuLE) system, which allows recombination of several ENRTY vectors simultaneously by making use of the LR Gateway® cloning strategy [88,89]. In this particular approach, the recombination was performed in the additional presence of a multiple sleeping beauty expression (MuSE) vector. The recombined final MuSE vector contains inverted tandem repeats (ITR) that flank the sequence of interest which includes the sgRNA, the Cas9 gene as well as several resistance and reporter gene which are all expressed under their own specific promoter. These ITR however serve as recognition sites for the sleeping beauty (SB) transposase and allow fast and robust integration of the entire sequence of interest into the host genome in a cut and paste mechanism [90,91].

Phosphorylation and annealing

Provided sgRNAs were reconstituted to 100 µM in sterile, RNAse-free water. For annealing and phosphorylation of sgRNA pairs, 1 µl of each of the two matching sgRNA strands (top and bottom, 100 µM) were mixed with 1 µl 10x T4 DNA Ligase Reaction buffer, 0.5 µl T4 Polynucleotide Kinase (PNK) (10 U/µl) and 6.5 µl RNAse-free water and incubated in a thermocycler as followed:

37°C	*for 30 minutes*
95°C	*for 5 minutes*
95°C to 8°C	*by cooling down with 5°C/minute*

SgRNA ligation into pMuLE ENTR vector

Resulting sgRNA duplexes were diluted 1:200 in RNAse-free water yielding a final concentration of 50 nM. Next, these sgRNA duplexes were ligated into an entry vector (see Table 3). This was achieved by

adding 1 µl of 50 nM sgRNA duplex to 50 ng entry vector plasmid, 5 µl 2x Quick Ligase Reaction buffer and 1 µl Quick Ligase to a total volume of 11 µl in sterile water – while working on ice. The according solution was carefully mixed by gently snapping the tube, then briefly centrifuged, and incubated at room temperature for ten minutes.

TOP10 E.coli transformation with ligated pMuLE ENTR vector

Generated sgRNA containing entry vector plasmids were amplified in chemically competent One Shot™ TOP10 E. coli. For this, 7 µl of entry vector plasmid solution were added to the bacterial cells and these subsequently incubated for 30 minutes on ice. Upon incubation, bacteria were heat-shocked for 45 seconds at 42°C, kept on ice for two minutes before adding 250 µl SOC medium and incubating at 37 °C for one hour in an orbital shaker at 225 revolutions per minute (rpm). After this, bacteria cell suspensions were plated on kanamycin containing (50 µg/ml) agar plates and incubated at 37 °C for 12-18 hours. Overnight formed bacterial colonies were picked and inoculated in 5 ml LB medium at 37 °C for 8-16 hours while shaking at 225 rpm. Antibiotic selection for successfully transformed cells was achieved by adding 50 µg/ml kanamycin. On the following day, 500 µl of the liquid bacterial cultures were used for kryopreservation in 250 µl 100 % glycerol while the residual volume was peletted by centrifuging at 4000 x g for ten minutes. Plasmid DNA was isolated using the Monarch™ Plasmid Miniprep Kit according to the manufacturer's protocol and checked for purity and concentration using the NanoDrop™ 2000 spectrophotometer.

Controlling for plasmid integrity

250 ng plasmid DNA were digested at 37 °C for one hour using 0.5 µl of EcoRI-high fidelity restriction enzyme and 2.5 µl CutSmart® buffer in a final volume of 25 µl in sterile water. For undigested controls, 100 ng plasmid DNA was diluted ad 10 µl in sterile water. Loading buffer (6x) was added to plasmid DNA samples and entry vector integrity checked by applying samples to an 1 % agarose gel at 120 V, 400 mA and 100 W for 30 minutes and visualizing digested DNA using a Chemidoc™ MP Imaging System. For additional verification, chosen plasmid DNA samples (750 ng plasmid plus 2 µl 10 µM M13+ forward primer ad 17 µl in sterile water) were sent for Sanger sequencing to Eurofins (Ebersberg, Germany).

LR reaction

Initial entry vector plasmids are part of the MuLE system introduced by the Ian Frew Laboratory. These so-called pMuLE vectors hold L- and R-sites (also known as attL and attR sites) which enable their use for MultiSite Gateway Cloning and allow recombination with multiple constructs. Generated sgRNA containing empty vector plasmids can be linked with an additional entry vector which includes the Cas9 and cloned into a destination vector by making use of the LR Gateway Cloning technology. Final cloning constructs from this LR recombination are termed MuSE plasmids (pMuSE) as the destination vectors carry ITR that are recognized by the engineered SB transposase which permits robust host genome integration.

For pMuSE cloning, all pMuLE entry vectors applied were diluted to 10 fmol/µl ad 10 µl in TE buffer. LR reaction was performed by merging 1 µl of each pMuLE vector with 1 µl (=20 fmol/µl) destination vector and 2 µl 5x LR Clonase™ II Plus enzyme in a total of 10 µl in TE buffer (for details see Table 3). This plasmid DNA solution was incubated for recombination at 25 °C for 24 hours. Following incubation, 1 µl 10x Proteinase K was added to inactivate enzymatic activity at 37 °C for 10 minutes.

Stbl3 E.coli transformation with ligated pMuSE vector and plasmid digest

Chemically competent One Shot™ Stbl3 E. coli were then transformed with 7 µl of the LR reaction solution and final pMuSE vectors isolated as described previously for One Shot™ TOP10 E. coli transformation. Antibiotic selection was achieved using 100 µg/ml ampicillin. To verify successful LR recombination, 500 ng of pMuSE DNA was pipetted into 2.5 µl NEBuffer™ 3.1 and 0.5 µl BamHI restriction enzyme in a total of 25 µl in sterile water and digested at 37 °C for two hours. Plasmid integrity was analyzed as mentioned above.

Table 3 - MuliSite Gateway cloning strategy

knockout approach	***sgRNA***	***entry vector for ligation***	***entry vector for LR recombination***
one sgRNA in final plasmid	*Dicer1/UCP1 #1/#2*	*pMuLE ENTR U6-sgRNA L1-R5*	*pMuLE ENTR CMV-hCas9 L5-L2*
two sgRNAs in final plasmid	*Dicer1 sgRNA #1* *Dicer1 sgRNA #2*	*pMuLE ENTR U6-sgRNA L1-L4* *pMuLE ENTR U6-sgRNA R4-R3*	*pMuLE ENTR CMV-hCas9 L3-L2*

Glycerol stocks from final pMuSE constructs with proven correct integrity were used for inoculation in 100 ml LB medium and 100 µg/ml ampicillin at 37 °C for 8-16 hours while shaking at 225 rpm. The

PureLink™ HiPure Plasmid Midiprep Kit was applied for plasmid DNA isolation as described in the manufacturer's protocol.

2.2.15 Cell electroporation

Introduction of gene knockouts were conducted by co-transfecting human SGBS cells with the cloned pMuSE vector and an additional SB transposase carrying vector (pCMV(CAT)T7-SB100) using the Neon™ Transfection System combined with the according kit and applied as described in the manufacturer's manual.

In particular, 10.5 µg of the final pMuSE construct were added to 500 ng SB transposase vector pCMV(CAT)T7-SB100 in a total of 110 µl in R buffer. For each construct, 1.1 x 10^6 human SGBS cells were pelleted in a 1.5 ml reaction tube at 300 x g for five minutes and resuspended in the prepared transfection solution. 100 µl of that solution were taken up using the Neon™ Transfection System Pipette. Next, electroporation was performed with 1400 V, three pulses and a pulse width of 10 ms. A mock control electroporated in the absence of plasmid DNA was used as transfection control. Cell suspensions were then added to 10 ml 0F + 10 % FCS without antibiotics in a 10 cm cell culture dishes and kept in the incubator overnight. On the following day, cell media were replaced with regular antibiotic containing 0F + 10 % FCS. Additional puromycin at a final concentration of 5 µg/ml was added for antibiotic selection. This process was carried out until mock control cells were entirely dead, while selection media were changed every three days. Following the antibiotic selection process, cells were further cultivated as described in 2.2.1.

2.2.16 miRNA sequencing

RNA samples from SGBS preadipocytes were harvested (as in 2.2.4) 48 hours after siRNA transfection (see 2.2.13) and sent for miRNA sequencing to the Core Facility Genomics of the Ulm University. Here, RNA purity was analyzed in a Nanodrop 1000 and RNA integrity assessed by Tape Station analysis. 200ng of total RNA, measured by Qubit® analysis, was used for library construction applying TruSeq RNA Sample Preparation Kit v2. Size distribution of the libraries was controlled on a DNA1000 ScreenTape® using a Tape Station 4200. Next, multiplexed libraries were sequenced on an Illumina NextSeq550 using a NextSeq 500/550 High Output Kit v2.5 (75 Cycles) to generate ~30 million of single end 75 base pair reads per library.

Bioinformatic analysis was conducted by the Core Unit Bioinformatics of the Ulm University according to the small RNA-seq pipeline approach version v1.1.0 which includes multiple steps of quality control,

normalization, alignment and annotation [92]. The obtained miRNA sequences were mapped against the Genome Reference Consortium Human Build 38 (hg38).

2.2.17 Statistical analysis

Statistical analysis was performed in GraphPad Prism using a significance level of 0.05. Graphs display mean +/- SEM. Further details can be abstracted from the following chapter.

3. Results

To investigate the molecular role of Dicer in human adipose tissue cells, Simpson Golabi Behmel Syndrom (SGBS) cells served as primary model system, while human multipotent adipose-derived stem (hMADS) cells were used to confirm key findings. Both cell strains are primary preadipocyte cells of human origin that are capable to differentiate into mature adipocyte [14,93]. Knockout (KO) and knockdown (KD) approaches were performed for functional characterization of Dicer on the cellular and metabolic phenotype of pre-/adipocytes.

Cells treated with siRNA are referred to as siNT (for non-targeting siRNA/control transfected cells), control cells or only controls. Likewise, Dicer targeting siRNA transfected cells are mentioned as siDicer, Dicer KD or Dicer deficient cells respectively.

3.1 Dicer is well expressed in pre-/adipocytes of human SGBS as well as in hMADS cells

As shown in Figure 8, SGBS cells change their morphological shape and incorporate lipids upon induction of adipogenic differentiation. This phenotype was already visible at day four and became even more prominent with increasing differentiation time (**Figure 8**A). After 14 days, 93 % of SGBS cells were differentiated into mature adipocytes (**Figure 8**C). Data obtained from triglyceride samples revealed that adipocytes had higher lipid content as compared to the initial preadipocyte state (Figure 8B). These findings were in line with expression levels of common pan-adipocyte marker genes, namely peroxisome proliferator-activated receptor gamma (PPARγ), adiponectin (ADIPOQ), and perilipin1 (PLIN). Here, mRNA expression levels of all three genes were barely detectable in the preadipocyte state but increased significantly during adipogenesis for ADIPOQ, PLIN and in PPARγ expression - relative to the housekeeping control hypoxanthine-guanine phosphoribosyltransferase (HPRT) (**Figure 8**D-F). Similarly, protein amounts of perilipin and PPARγ were assessed in Western Blot analysis and confirmed the expression pattern observed on mRNA level within these two chosen targets (**Figure 8**H).

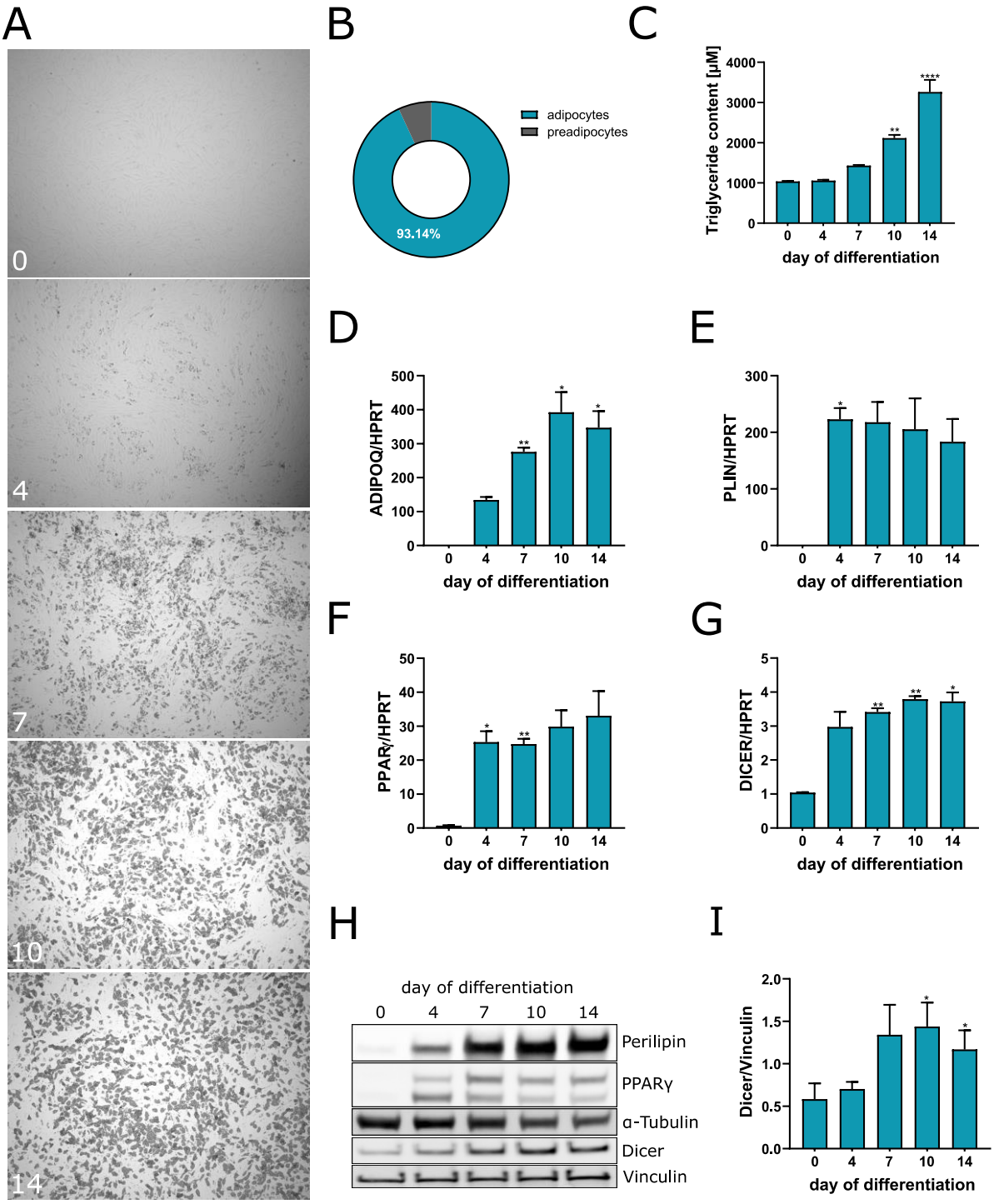


Figure 8 - Expression of Dicer during adipogenic differentiation of SGBS cells. *Human SGBS preadipocytes were cultured and differentiated for adipocyte maturation for 14 days.* ***(A)*** *Micrograph images were taken at day 0, 4, 7, 10 and 14 of adipogenic differentiation. To assess the differentiation efficiency* ***(B)*** *differentiation rates and* ***(C)*** *intracellular lipid content were determined.* ***(D-F)*** *mRNA and* ***(H)*** *protein levels of common pan-adipocyte marker genes ADIPOQ, PLIN and PPARγ served as additional differentiation controls. Moreover, Dicer* ***(G)*** *mRNA and* ***(H+I)*** *protein expression were checked in SGBS cells during preadipocyte-adipocyte-transition. n = at least three independent experiments; one representative blot/image; mean + SEM. One-way ANOVA. *p-value < 0.05, **p-value < 0.01. HPRT respectively α-Tubulin and Vinculin were used as reference genes.*

The expression of Dicer was substantially altered by initiation of adipogenic differentiation being more prominent in adipocytes (**Figure 8**G). The mRNA expression of Dicer was enhanced already during early adipogenesis at day four and plateaued in the late phase of day ten and 14 reaching 3-4-fold expression in contrast to preadipocytes. Similarly, Dicer protein levels were more abundant in the adipocyte state being almost two times higher than prior to induction of adipogenesis (**Figure 8**H+I).

In order to verify these results in a different model system, experiments were performed using hMADS cells. Like in SGBS cells, hMADS cells successfully underwent adipogenic differentiation as depicted in (**Figure 9**A). Microphotographs clearly illustrate the transition of preadipocytes into mature, lipid accumulating adipocytes. Assessing the expression of Dicer on mRNA and protein level revealed enhanced levels in adipocytes compared to preadipocytes in hMADS cells reaching higher expression on mRNA and 1.9-fold increased levels for protein (**Figure 9**B-D).

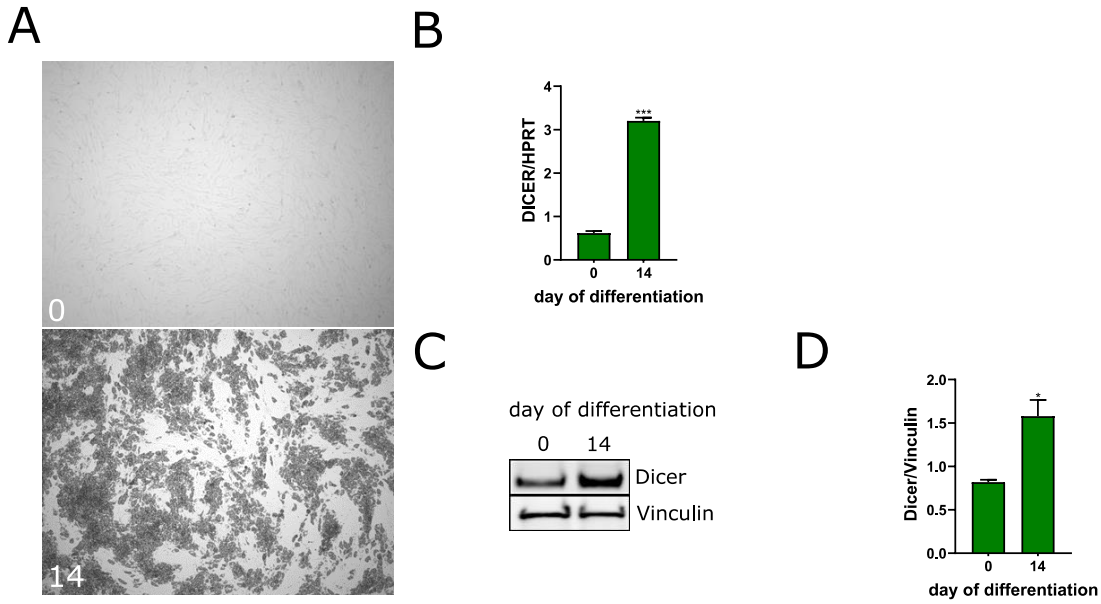


Figure 9 - Dicer is well expressed in hMADS pre-/adipocytes. *hMADS cells were cultured and differentiated for adipocyte maturation for 14 days. Microphotographs of day 0 and day 14 are illustrated **(A)**. Dicer **(B)** mRNA and **(C+D)** protein levels were assessed in preadipocytes (day 0) and adipocytes (day 14). n = at least three independent experiments; one representative blot/image; mean + SEM. One-way ANOVA. *p-value < 0.05, ***p-value < 0.001. HPRT respectively Vinculin were used as reference genes.*

3.2 CRISPR-Cas9-mediated Dicer KO is not sufficient to impair Dicer expression in human SGBS cells

To investigate the molecular function of Dicer in human pre-/adipocytes, a CRISPR-Cas9 approach was performed in SGBS cells to introduce a loss of function of Dicer. For this, two different sgRNAs were cloned into two separate MuSE destination vectors by making use of the LR Gateway® cloning system. Co-transfection of the MuSE vector with an additional SB transposase carrying plasmid enabled robust host genome integration of the desired sequence of interest. Subsequently, transfected cells were antibiotically selected.

Genetically modified SGBS preadipocytes strains from bulk culture with an introduced Dicer-KO were first checked for Dicer expression. As shown in **Figure 10**B, there were no differences when comparing the two KO constructs with the EV control cells on Dicer mRNA level. Analysis of harvested preadipocyte protein however, revealed a significant downregulation of Dicer by 35 % in KO cell strains #1 and by 42 % in KO cell strains #2 (**Figure 10**C+D).

Next, cells were differentiated for 14 days. As depicted on the images in **Figure 10**E, no differences were observed in cellular morphology when comparing the two different KO cell strain adipocytes with the corresponding control cells. There were no visible hints on altered adipogenesis, cell numbers or adverse lipid phenotypes. Moreover, neither differentiation rates were different in all three cell models, nor were levels of accumulated lipids altered between EV and KO cells (**Figure 10**F+G). Expression of ADIPOQ, glucose transporter 4 (GLUT4) and PPARγ - which served as adipogenic marker genes - were comparable in all three cell strains (**Figure 10**H).

Assessment of RNA and protein samples of mature adipocytes revealed that Dicer expression was not hampered upon CRISPR-Cas9-mediated Dicer KO in neither of the two genetically modified strains (**Figure 10**I-K).

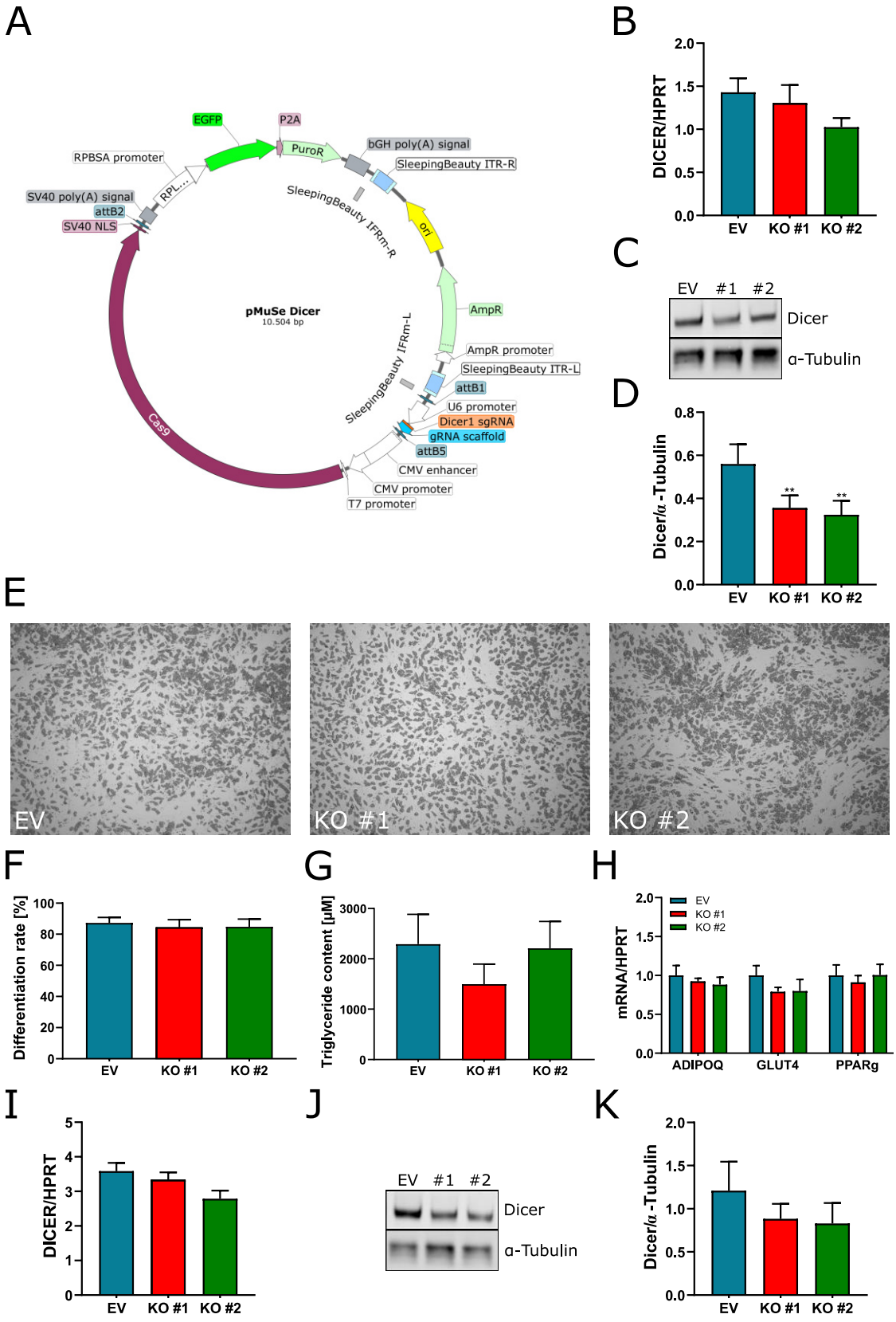

A
EGFP
P2A
PuroR
bGH poly(A) signal
SleepingBeauty ITR-R
RPBSA promoter
RPL...
SV40 poly(A) signal
attB2
SV40 NLS
SleepingBeauty IFRm-R
ori
pMuSe Dicer
10.504 bp
AmpR
AmpR promoter
SleepingBeauty ITR-L
attB1
SleepingBeauty IFRm-L
U6 promoter
Dicer1 sgRNA
gRNA scaffold
attB5
Cas9
CMV enhancer
CMV promoter
T7 promoter
B
DICER/HPRT
2.0
1.5
1.0
0.5
0.0
EV
KO #1
KO #2
C
EV #1 #2
Dicer
α-Tubulin
D
Dicer/α-Tubulin
0.8
0.6
0.4
0.2
0.0
**
**
EV
KO #1
KO #2
E
EV
KO #1
KO #2
F
Differentiation rate [%]
100
80
60
40
20
0
EV
KO #1
KO #2
G
Triglyceride content [µM]
3000
2000
1000
0
EV
KO #1
KO #2
H
mRNA/HPRT
2.0
1.5
1.0
0.5
0.0
EV
KO #1
KO #2
ADIPOQ
GLUT4
PPARg
I
DICER/HPRT
5
4
3
2
1
0
EV
KO #1
KO #2
J
EV #1 #2
Dicer
α-Tubulin
K
Dicer/α-Tubulin
2.0
1.5
1.0
0.5
0.0
EV
KO #1
KO #2

Figure 10 - CRISPR-Cas9 mediated KO of Dicer in human SGBS cells. *Human SGBS preadipocytes were genetically modified by knocking out Dicer1 gene exploiting the CRISPR-Cas9 system. SGBS preadipocytes were transfected with pMuSE-Dicer plasmid, antibiotically selected and controlled for Dicer expression. Two different strains were created (KO #1 and KO #2) as a result of two different Dicer targeting sgRNAs used.* ***(A)*** *Final DNA plasmid construct for CRISPR-Cas9-mediated Dicer KO displaying important regions and elements including Cas9 protein being essential for introducing gene KO using an inserted sgRNA. Additional puromycin and ampicillin resistant genes were embedded for antibiotic selection and EGFP for identifying successfully transfected cells. Each element was under the control of a specific promoter to enable stable and independent expression. Dicer KO was evaluated in preadipocytes investigating harvested* ***(B)*** *RNA and* ***(C+D)*** *protein.* ***(E)*** *Micrograph images were taken following differentiation at day 14 of adipogenesis. The effect on adipocyte differentiation was assessed by determining* ***(F)*** *differentiation rates,* ***(G)*** *triglyceride content and quantifying* ***(H)*** *marker gene expression on qPCR. To check for stable KO in adipocytes, Dicer* ***(I)*** *mRNA and* ***(J+K)*** *protein was evaluated. n = at least three independent experiments; one representative blot/image; mean + SEM. One-way ANOVA. **p-value < 0.01. HPRT respectively α-Tubulin were used as reference genes.*

To achieve a more pronounced effect on Dicer downregulation, SGBS cells were transfected with a new CRISPR-Cas9 vector containing both sgRNAs at the same time. In these cells, Dicer was not affected on mRNA level in preadipocytes, whereas relative protein values declined to 52 % relative to the control cell expression (**Figure 11**B-D).

Cells were then checked for phenotypical alterations after induction of adipogenic differentiation. Here, no changes were observed when comparing EV and Dicer KO adipocytes. There was neither a sign of morphologically distinct features of Dicer KO cells (**Figure 11**E), nor were differentiation rates or triglyceride content affected (**Figure 11**F+G).

Next, downregulation of Dicer upon CRISPR-Cas9-mediated KO with two sgRNAs was tested in mature adipocytes to ensure stable knockdown following the process of adipogenesis. As in **Figure 11**H depicted, comparable to what was observed in the preadipocyte state, there was no effect on Dicer mRNA expression when comparing KO cells and their according controls in mature SGBS adipocytes. Dicer protein amount, however, was halved in Dicer deficient cells (**Figure 11**I+J).

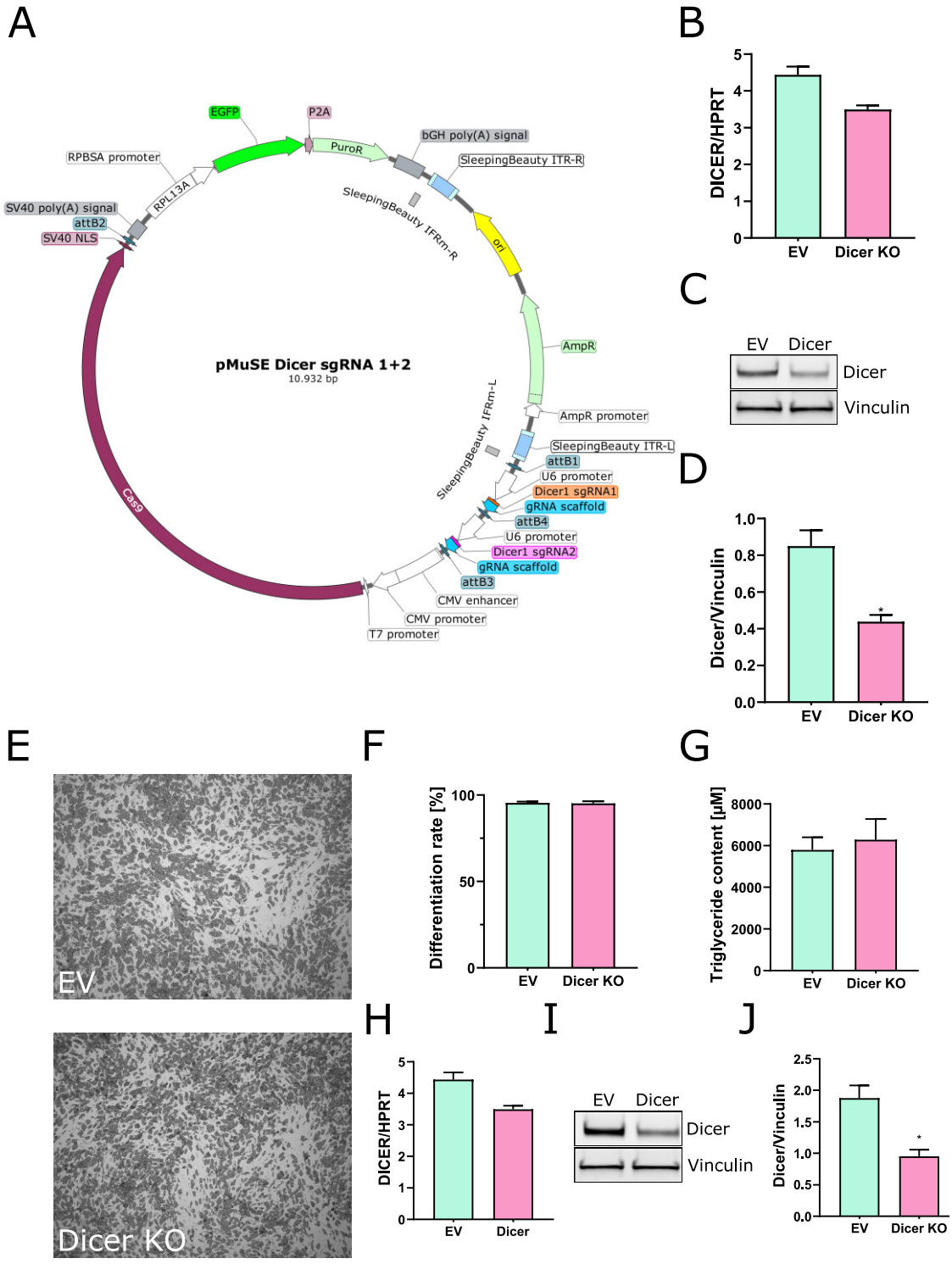


Figure 11 - Effect of CRISPR-Cas9 mediated KO approach with two sgRNAs targeting Dicer on human SGBS cells. *Human SGBS preadipocytes were genetically modified by knocking out Dicer1 gene exploiting the CRISPR-Cas9 system. SGBS preadipocytes were transfected with pMuSE-Dicer plasmid, antibiotically selected and controlled for Dicer expression. Two different Dicer targeting sgRNAs were merged into one plasmid.* ***(A)*** *Final DNA plasmid construct for CRISPR-Cas9-mediated Dicer KO displaying important regions and elements including Cas9 protein being essential for introducing gene KO using an*

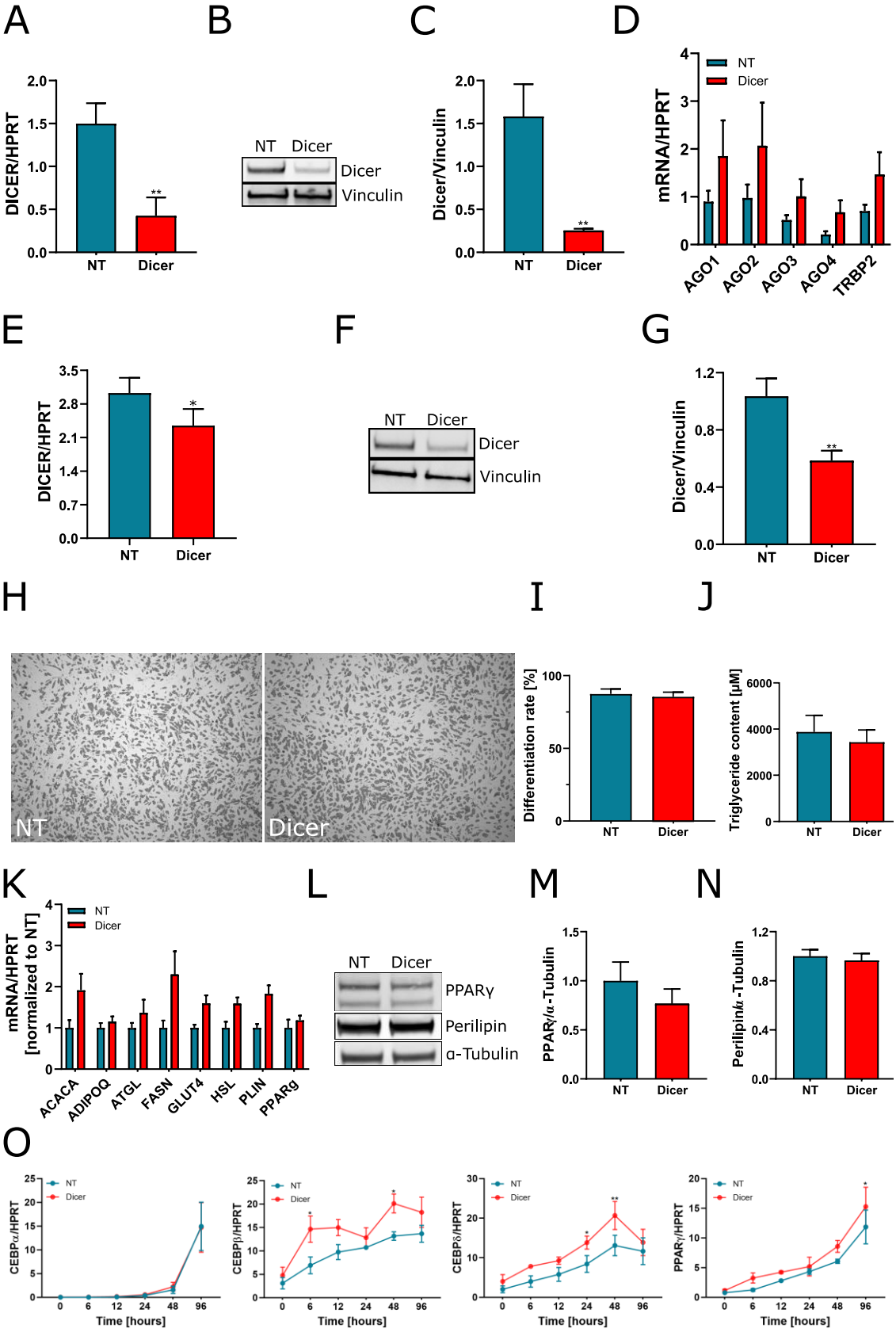
*inserted sgRNA. Additional puromycin and ampicillin resistant genes were embedded for antibiotic selection and EGFP for identifying successfully transfected cells. Each element was under the control of a specific promoter to enable stable and independent expression. Dicer KO was evaluated in preadipocytes investigating harvested **(B)** RNA and **(C+D)** protein. **(E)** Micrograph images were taken following differentiation at day 14 of adipogenesis. The effect on adipocyte maturation was assessed by determining **(F)** differentiation rates and **(G)** triglyceride content. To check for stable KO in adipocytes, Dicer **(H)** mRNA and **(I+J)** protein was evaluated. n = at least three independent experiments; one representative blot/image; mean + SEM. Students' t-test. *p-value < 0.05. HPRT respectively Vinculin were used as reference genes.*

3.3 siRNA mediated Dicer KD does not affect adipogenic differentiation of human SGBS cells

Since the previous approach using a CRISPR-Cas9 system could not be used to adequately address the impact of Dicer loss of function on adipogenic differentiation capacity in human SGBS cells, the aim was to test whether siRNA approach is more efficient.

SGBS preadipocytes were transfected with siRNA targeting Dicer. A knockdown by 72 % was detected on the mRNA levels (**Figure 12**A) and 85 % on the protein level after 48 h (**Figure 12**B+C). Of note, the expression of genes involved in miRNA biogenesis did not significantly change (**Figure 12**D). Similarly, Dicer was diminished by 23 % on mRNA level (**Figure 12**E) respectively by 44 % on protein level (**Figure 12**F+G) in the mature adipocyte state.

To identify, if Dicer downregulation affects adiopogenic potential of human SGSB cells, the cellular phenotype of mature adipocyte at day 14 of adipogenesis was investigated. Microphotographs of these cells depicted no morphological alterations between siDicer and siNT cells (**Figure 12**H) and the rate of adipogenic differentiation (**Figure 12**I) as well as the triglyceride content were comparable (**Figure 12**J). Furthermore, the expression of various genes associated with mature adipocytes were analyzed including PPARγ, ADIPOQ, GLUT4, acetyl-CoA carboxylase (ACACA), fatty acid synthase (FASN), PLIN, adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL) was comparable between siNT versus Dicer deficient cells (**Figure 12**K). Likewise, PPARγ and PLIN were equally expressed on the protein level (**Figure 12**L-N).


A
DICER/HPRT
NT
Dicer
B
NT Dicer
Dicer
Vinculin
C
Dicer/Vinculin
D
mRNA/HPRT
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AGO1
AGO2
AGO3
AGO4
TRBP2
E
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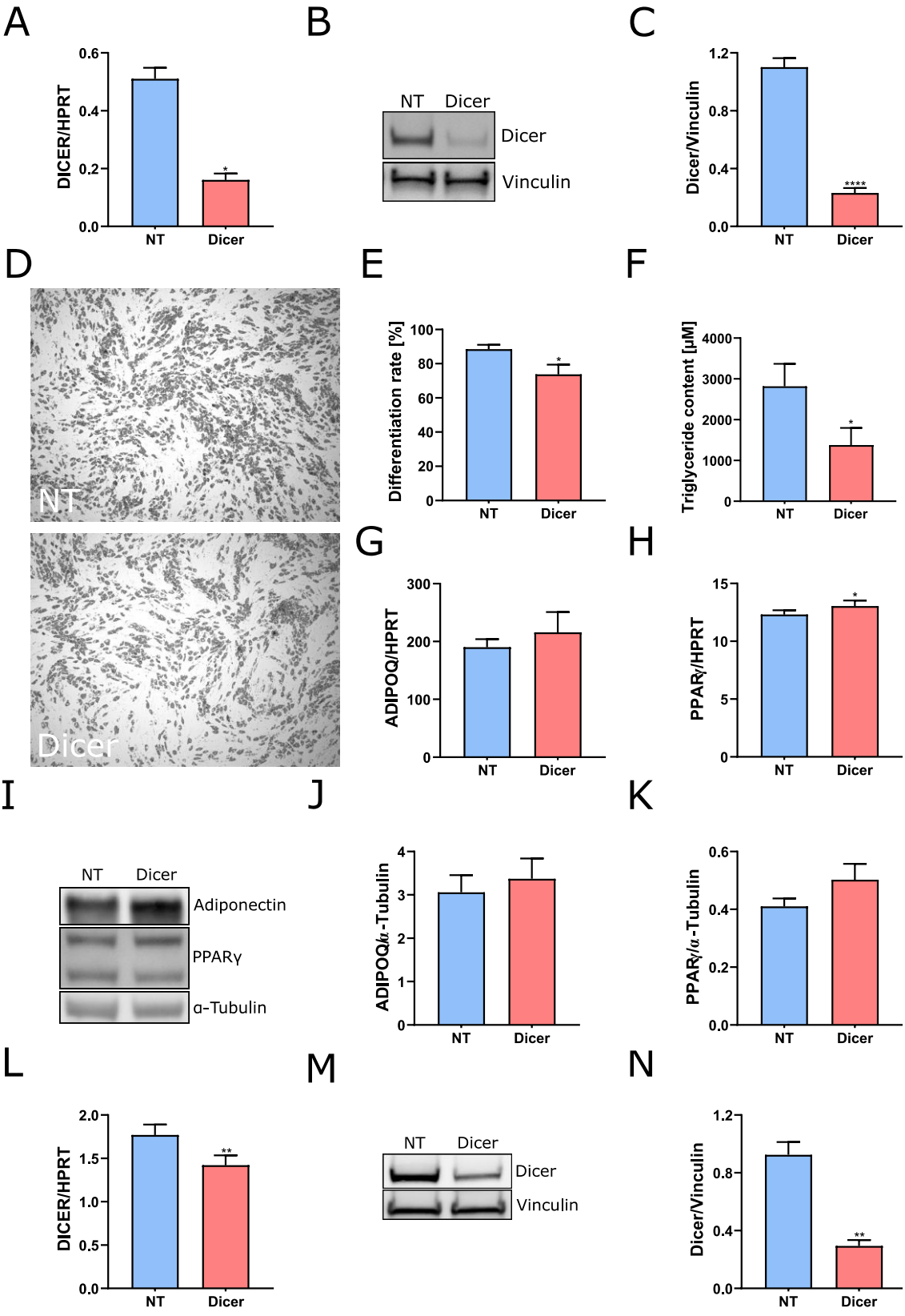
Dicer
Vinculin
G
Dicer/Vinculin
H
NT
Dicer
I
Differentiation rate [%]
J
Triglyceride content [µM]
K
mRNA/HPRT [normalized to NT]
ACACA
ADIPOQ
ATGL
FASN
GLUT4
HSL
PLIN
PPARg
L
NT Dicer
PPARγ
Perilipin
α-Tubulin
M
PPARγ/α-Tubulin
N
Perilipinα-Tubulin
O
CEBPα/HPRT
CEBPβ/HPRT
CEBPδ/HPRT
PPARγ/HPRT
Time [hours]

Figure 12 - siRNA induced knockdown leads to a pronounced downregulation of Dicer in human SGBS pre-/adipocytes. *Human SGBS preadipocytes were transfected with 20nM of Dicer-targeting (Dicer) or non-targeting (NT) control siRNA following the day of seeding.* ***(A)*** *mRNA and* ***(B+C)*** *protein levels of Dicer were measured to monitor knockdown efficiency in the preadipocyte state.* ***(D)*** *The effect of Dicer deficiency on miRNA processing associated genes was checked via qPCR.* ***(E)*** *Following adipogenic maturation process, adipocyte micrographs were taken at day 14.* ***(F)*** *Differentiation rates as well as* ***(G)*** *lipid content were used for assessing the degree of preadipocytes-adipocyte-transition, while relatively quantifying* ***(H)*** *RNA and* ***(I-K)*** *protein levels of adipocyte marker genes served as additional references. Dicer* ***(L)*** *mRNA and* ***(M+N)*** *protein was analyzed to confirm stable knockdown in mature adipocytes at the final stage of differentiation.* ***(O)*** *To determine differences in the early phase of differentiation, adipogenic marker genes CEBPα, CEBPβ, CEBPδ and PPARγ were analyzed on qPCR. n = at least three independent experiments; one representative blot/image; mean + SEM. One-way ANOVA/Students' t-test. *p-value < 0.01, **p-value < 0.01. HPRT respectively α-Tubulin and Vinculin were used as reference genes.*

Since siRNA mediated KD was more prominent in the preadipocyte state, mRNA of known early adipogenic marker genes were assessed within the first four days after induction of differentiation to rule out that a lack of Dicer in the early phase of adipogenesis rather delays the differentiation process instead of completely abolishing it. As depicted in **Figure 12**O, relative quantification of CCAAT/enhancer-binding protein alpha and delta (CEBPα and CEBPδ) disclosed no alterations between siNT and siDicer cells, whereas on the other hand CCAAT/enhancer-binding protein beta (CEBPβ) and PPARγ expression were significantly higher in Dicer KD cells (**Figure 12**O).

To verify the findings observed in Dicer deficient human adipocytes on adipogenesis in an additional model system, hMADS cells were used to repeat siRNA transfection experiments. While Dicer mRNA was reduced to 31 %, protein levels were diminished by 80 % compared to siNT preadipocytes (**Figure 13**A-C). Investigating siRNA transfected hMADS adipocytes under the microscope, suggested less mature adipocytes as a result of a Dicer KD (**Figure 13**D). Consistent with this, differentiation rates and the amount of accumulated triglycerides within mature adipocytes were markedly lower upon Dicer KD in hMADS cells (**Figure 13**E+F). Further analysis of Dicer mRNA and protein in mature adipocytes confirmed a stable downregulation of 22 and 69 % after differentiation (**Figure 13**L-N). Further examination of adipogenic marker genes ADIPOQ and PPARγ showed that both targets were equally expressed, since neither mRNA nor protein content was altered (**Figure 13**G-K). On the contrary, PPARγ mRNA levels were significantly increased in siDicer hMADS adipocytes.


A
DICER/HPRT
0.6
0.4
0.2
0.0
*
NT
Dicer
B
NT
Dicer
Dicer
Vinculin
C
Dicer/Vinculin
1.2
0.8
0.4
0.0
****
NT
Dicer
D
NT
Dicer
E
Differentiation rate [%]
100
80
60
40
20
0
*
NT
Dicer
F
Triglyceride content [µM]
4000
3000
2000
1000
0
*
NT
Dicer
G
ADIPOQ/HPRT
300
200
100
0
NT
Dicer
H
PPARγ/HPRT
15
10
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Adiponectin
PPARγ
α-Tubulin
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4
3
2
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NT
Dicer
K
PPARγ/α-Tubulin
0.6
0.4
0.2
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NT
Dicer
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DICER/HPRT
2.0
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Vinculin
N
Dicer/Vinculin
1.2
0.8
0.4
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**
NT
Dicer

Figure 13 - Dicer siRNA efficiently downregulates Dicer levels in hMADS cells. *hMADS preadipocytes were transfected with 20 nM of Dicer-targeting (Dicer) or non-targeting (NT) control siRNA following the day of seeding.* ***(A)*** *mRNA and* ***(B+C)*** *protein levels of Dicer were measured to monitor knockdown efficiency in the preadipocyte state.* ***(D)*** *Following adipogenic maturation process, adipocyte micrographs were taken at day 14.* ***(E)*** *Differentiation rates as well as* ***(F)*** *lipid content were used for assessing the degree of preadipocytes-adipocyte-transition, while relatively quantifying* ***(G+H)*** *RNA and* ***(I-K)*** *protein levels of adipocyte marker genes ADIPOQ and PPARγ served as additional references. Moreover, Dicer* ***(L)*** *RNA and* ***(M+N)*** *proteins were analyzed to confirm stable knockdown in mature adipocytes at the final stage of differentiation. n = at least three independent experiments; one representative blot/image; mean + SEM. Students' t-test. *p-value < 0.01, **p-value < 0.01. HPRT respectively α-Tubulin and Vinculin were used as reference genes.*

3.4 Enoxacin treatment impairs adipocyte differentiation in both SGBS and hMADS cells

The aim was to identify whether an increase in Dicer activity can affect adipocyte differentiation. For this, a known enhancer of Dicer mediated processing of miRNA and commercially available compound, named enoxacin, was utilized to treat human SGBS cells during differentiation. From day 0 until day 14, human SGBS cells were treated with different doses of enoxacin, while DMSO treated cells served as controls.

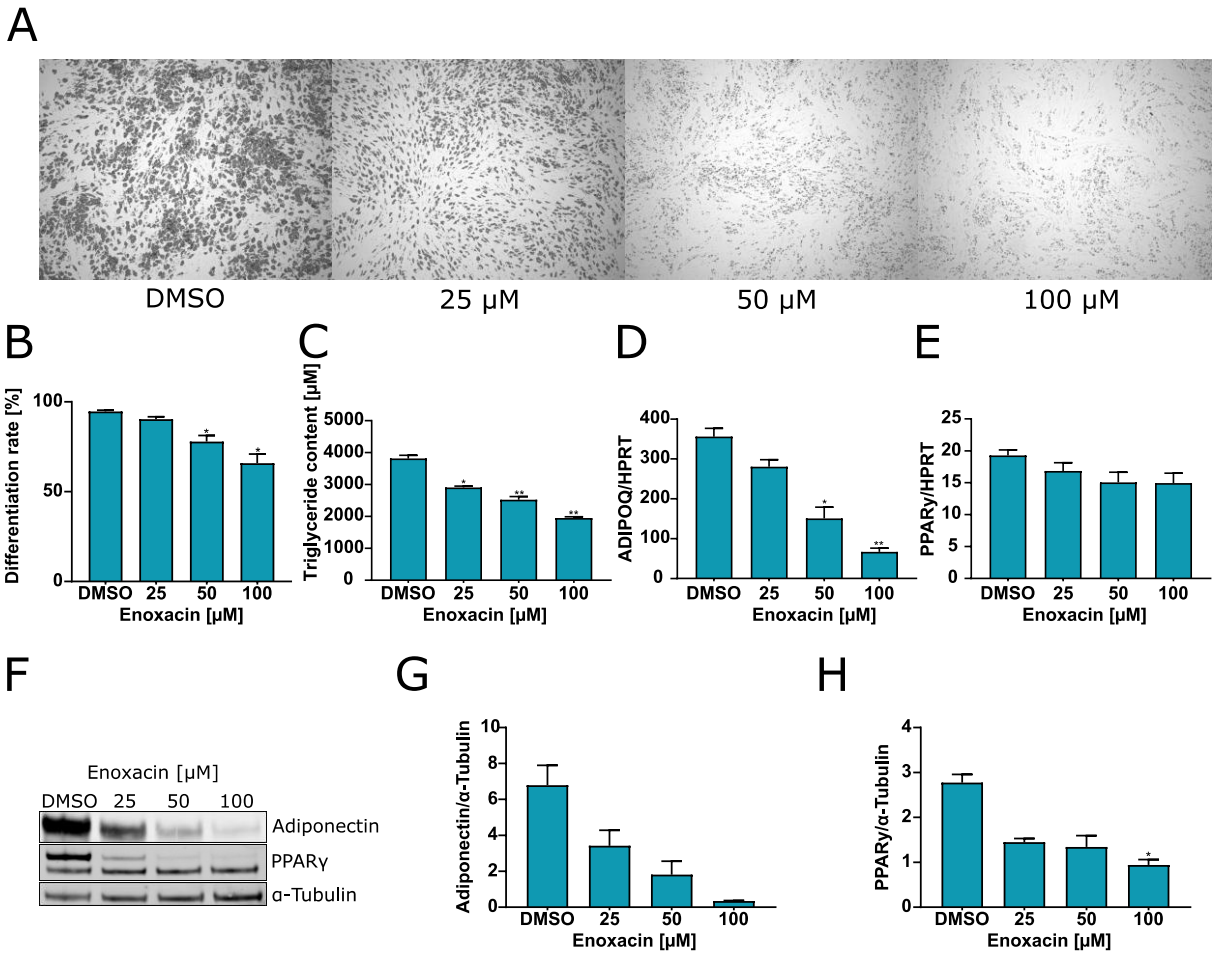

Figure 14 - Enoxacin treatment causes reduced adipogenesis of human SGBS cells. *SGBS preadipocytes were treated with 25, 50 or 100 µM from day 0 until day 14 of adipogenesis. DMSO treatment served as corresponding control.* ***(A)*** *Microphotographs of mature adipocytes under different treatment conditions. Moreover, multiple analyses were performed to assess the extent of transdifferentiation of preadipocytes into mature adipocytes determining* ***(B)*** *differentiation rates,* ***(C)*** *triglyceride content and expression of adipogenic marker genes ADIPOQ and PPARγ on* ***(D+E)*** *mRNA and* ***(F-H)*** *protein level. n = at least three independent experiments; one representative blot/image; mean + SEM. One-way ANOVA. *p-value < 0.01, **p-value < 0.01. HPRT respectively α-Tubulin was used as reference gene.*

As depicted in **Figure 14**A, microphotographs indicated that enoxacin impairs adipogenic differentiation of human SGBS cells in a dose-dependent manner. In line with this observation, differentiation rates were markedly reduced in cells that were treated with 50 and 100 µM enoxacin (**Figure 14**B). Moreover, the level of triglycerides was significantly lower in all concentrations upon treatment – ranging from 24 to 49 % decrease in triglyceride content (**Figure 14**C). Relative quantification of the adipocyte marker gene ADIPOQ was in line with these findings (**Figure 14**D). Expressed mRNA of ADIPOQ was drastically reduced by 58 % in 50 µM and 82 % in 100 µM treated cells. On the same hand, PPARγ mRNA appeared to be unaffected, while relative protein amounts of ADIPOQ and PPARγ tended to be impaired in a dose dependent manner. Yet, except for PPARγ protein levels in 100 µM enoxacin treated adipocytes, Western Blot results obtained did not reach statistical significance (**Figure 14**F-H).

All experiments were also performed in hMADS cells and led to comparable results as previously seen in SGBS cells. Upon treatment with increasing doses of enoxacin a decrease in the rate of adipogenic differentiation (**Figure 15**A, B) and triglyceride content (**Figure 15**C). Examining marker gene expression revealed that relative mRNA content of ADIPOQ and PPARγ remained unaffected (**Figure 15**D+E). In contrast, 25 µM was associated with increased ADIPOQ mRNA level. Further analysis of adipocyte protein, however, showed a clear reduction of both marker genes in every dosage applied – except for ADIPOQ protein amounts of 25 µM enoxacin treated cells (**Figure 15**F-H).

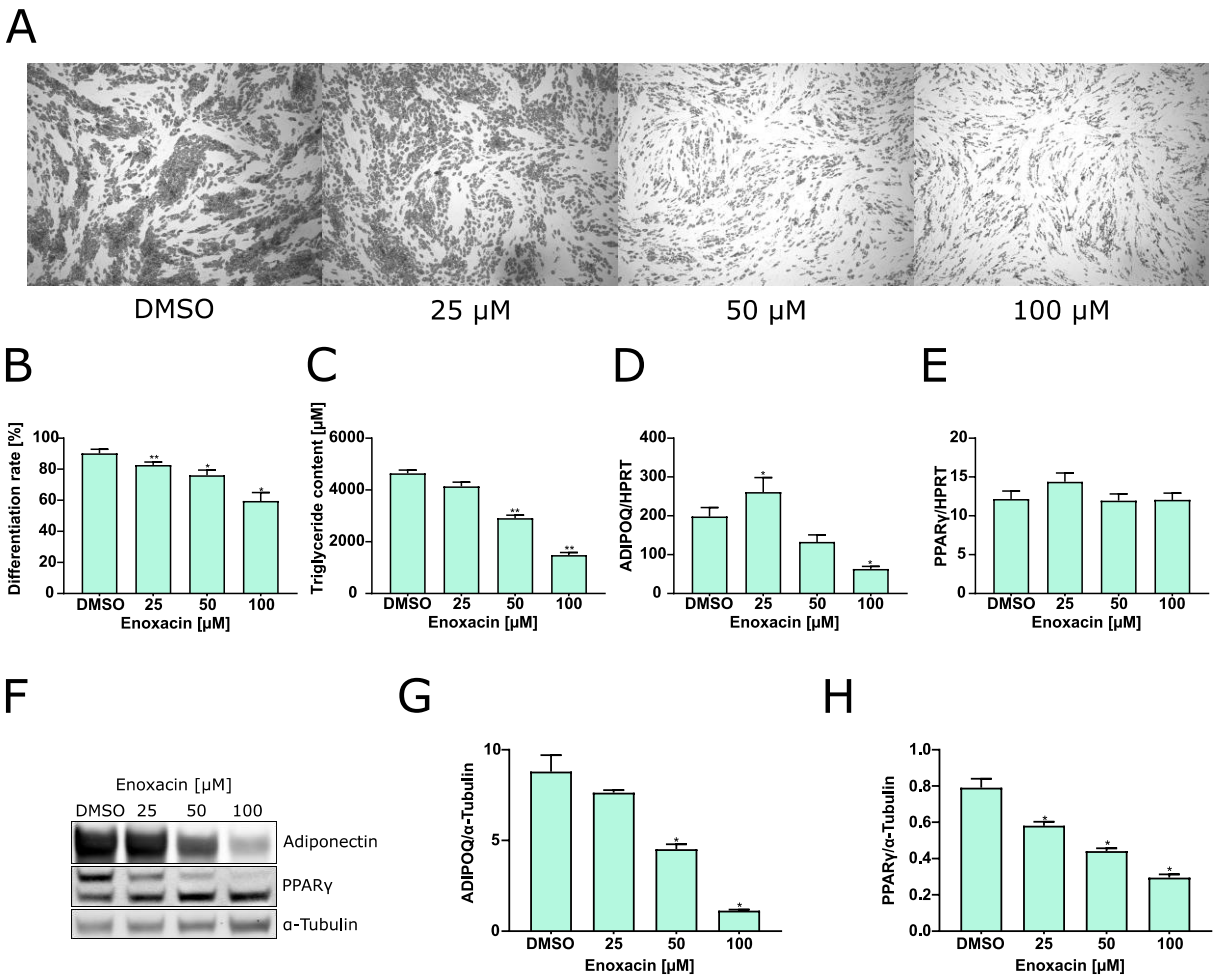

Figure 15 - Enoxacin impairs transition of hMADS cells into mature adipocytes. *hMADS preadipocytes were treated with 25, 50 or 100 µM from day 0 until day 14 of adipogenesis. DMSO treatment served as corresponding control.* ***(A)*** *Microphotographs of mature adipocytes under different treatment conditions. Moreover, multiple analyses were performed to assess the extent of transdifferentiation of preadipocytes into mature adipocytes determining* ***(B)*** *differentiation rates,* ***(C)*** *triglyceride content and expression of adipogenic marker genes ADIPOQ and PPARγ on* ***(D+E)*** *mRNA and* ***(F-H)*** *protein level. n = at least three independent experiments; one representative blot/image; mean + SEM. One-way ANOVA. *p-value < 0.01, **p-value < 0.01. HPRT respectively α-Tubulin was used as reference gene.*

3.5 Dicer KD mediates a preferentially white phenotype but has no functional relevance on cellular respiration of human SGBS cells

SGBS cells are of WAT origin, yet they express the brown adipocyte marker UCP1 when adipogenic differentiation is induced in the presence of a PPARg agonist [94]. Therefore, the aim was to examine whether the absence of Dicer affects UCP1 expression and features related to brown adipocyte function.

As shown in **Figure 16**A, mRNA expression of UCP1 was downregulated by ~79 %, while other BAT and WAT browning associated genes, namely PGC1a, cell death activator (CIDEA), DIO2, were unaffected

when comparing siNT and siDicer adipocytes (**Figure 16**B+C). Likewise, UCP1 protein was significantly decreased by 47 % on the protein level. On the contrary, leptin - a postulated primarily white adipocyte marker – was measured more than threefold higher on mRNA (**Figure 16**A), while protein amount nearly doubled (~1.8) (**Figure 16**B+D). In collected cell culture supernatants secreted leptin was increased by 61 % as a result of siRNA mediated Dicer KD. In other WAT associated genes such as Cbp/p300-interacting transactivator 1 (CITED1) and homeobox protein C9 (HOXC9) there were no changes when analyzing their mRNA expression (**Figure 16**A).

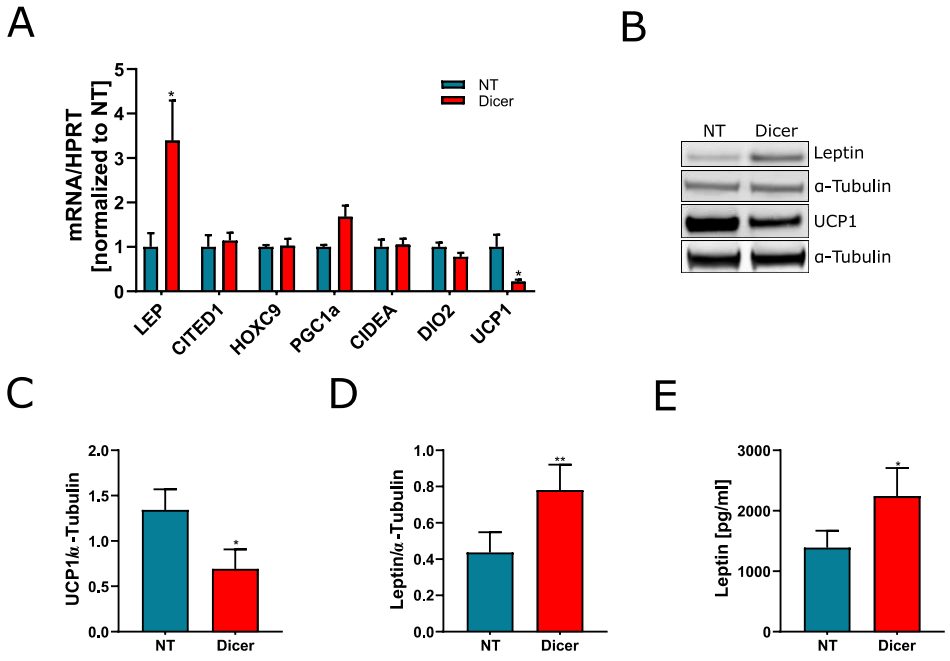

Figure 16 - Dicer KD favors a preferentially white adipocyte phenotype. (A) *WAT and BAT associated marker gene expression of LEP, CITED1, HOXC9, PGC1a, CIDEA, DIO2 and UCP1 was examined in mature SGBS adipocytes via qPCR.* ***(B)*** *Protein level of LEP and UCP1 were assessed in Western Blot experiments and* ***(C+D)*** *relatively quantified.* ***(E)*** *An ELISA-based method was performed to quantify leptin secretion in collected adipocyte supernatants. n = at least three independent experiments; one representative blot; mean + SEM. Students' t-test. *p-value < 0.01, **p-value < 0.01. HPRT respectively α-Tubulin was used as reference gene.*

To examine whether the strong downregulation of UCP1 causes functional metabolic alterations, cellular respiration was measured in an extracellular flux analyzer. The sequential injection of different chemical compounds allows the dissection of different respiratory parameters **Figure 17**A) Compared to NT controls, cellular respiration of Dicer deficient cells remained unchanged as reflected by basal, ATP linked, proton leak as well as cAMP rate respiration. These findings are illustrated in **Figure 17**B+C.

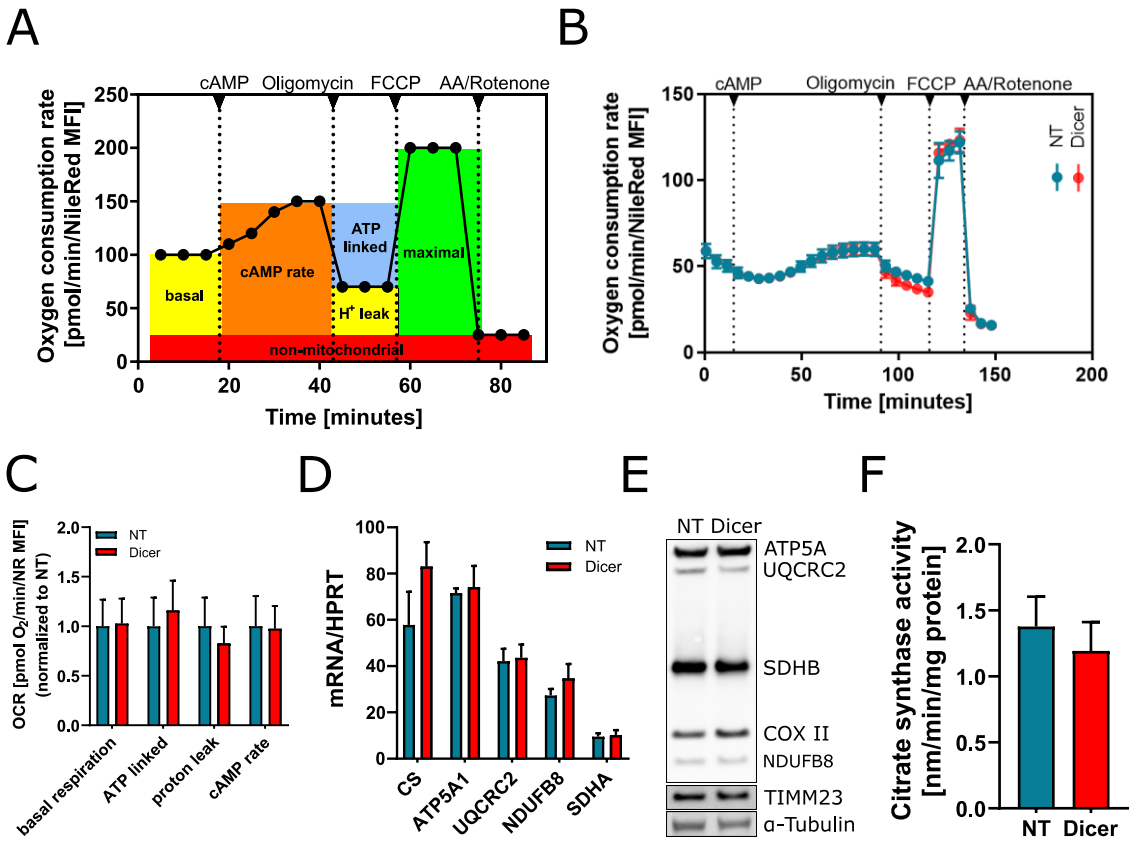

Figure 17 - Dicer knockdown has no effect on cellular respiration of SGBS adipocytes. *Oxygen consumption and cellular mitochondrial load were determined in mature SGBS adipocytes.* ***(A)*** *Exemplary scheme of different cellular oxygen consuming processes. Consecutive injection of dibutyryl-cAMP (cAMP, 500 nM), oligomycin (2 µM), FCCP (4 µM) and Antimycin A/Rotenone (AA/Rotenone, 1.5 µM) allows the assessment of different respiratory parameters.* ***(B)*** *Cellular respiration was evaluated using a plate-based respirometer in mature SGBS adipocytes.* ***(C)*** *Basal, ATP linked, proton leak, acute response and cAMP rate respiration were calculated from the respective oxygen consumption rates (OCR). Raw OCR values were normalized for lipid content using NileRed staining (NileRed MFI).* ***(D)*** *qPCR analysis was used for determining mitochondrial gene expression.* ***(E)*** *Proteins from mature adipocytes at day 14 were harvested and checked for expression of key mitochondrial genes of the electron transport chain complexes I-V and TIMM23 in Western Blot, while* ***(F)*** *citrate synthase activity was measured using adiopcyte protein samples as additional readout for mitochondrial content. n = at least three independent experiments; one representative blot/OCR plot; mean + SEM. HPRT respectively α-Tubulin was used as reference gene.*

The mRNA expression of mitochondrial markers such as citrate synthase (CS), ATP synthase F1 subunit alpha (ATP5A1), cytochrome b-c1 complex subunit 2 (UQCRC2), NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8 (NDUFB8) and succinate dehydrogenase subunit alpha (SDHA) was comparable between siDicer and control cells. Likewise, subunits of electron transport chain complexes I-V (ATP5A, UQCRC2, succinate dehydrogenase subunit beta (SDHB), cytochrome c oxidase subunit 2 (COXII) and NDUFB8) and mitochondrial import inner membrane translocase subunit Tim23 (TIMM23) were equally expressed on protein level (**Figure 17**D+E). Furthermore, there was no difference in mitochondrial CS activity (**Figure 17**F).

3.6 UCP1 KO in human SGSB cells only affects proton leak related oxygen consumption

To correctly classify the importance of UCP1 deficiency regarding oxygen consumption, a UCP1 KO was performed in human SGBS cells using CRISPR-Cas9. As depicted in **Figure 18**B, the KO approach resulted in a complete loss of UCP1 protein in both sgRNAs used (UCP1 (1) and UCP1 (2)) compared to the two control cell types (EV1 and EV2) in the mature adipocyte state. While only the particularly UCP1 associated proton leak respiration was slightly reduced by 27 % in UCP1 KO cells, other respiratory parameter remained unchanged (see **Figure 18**C).

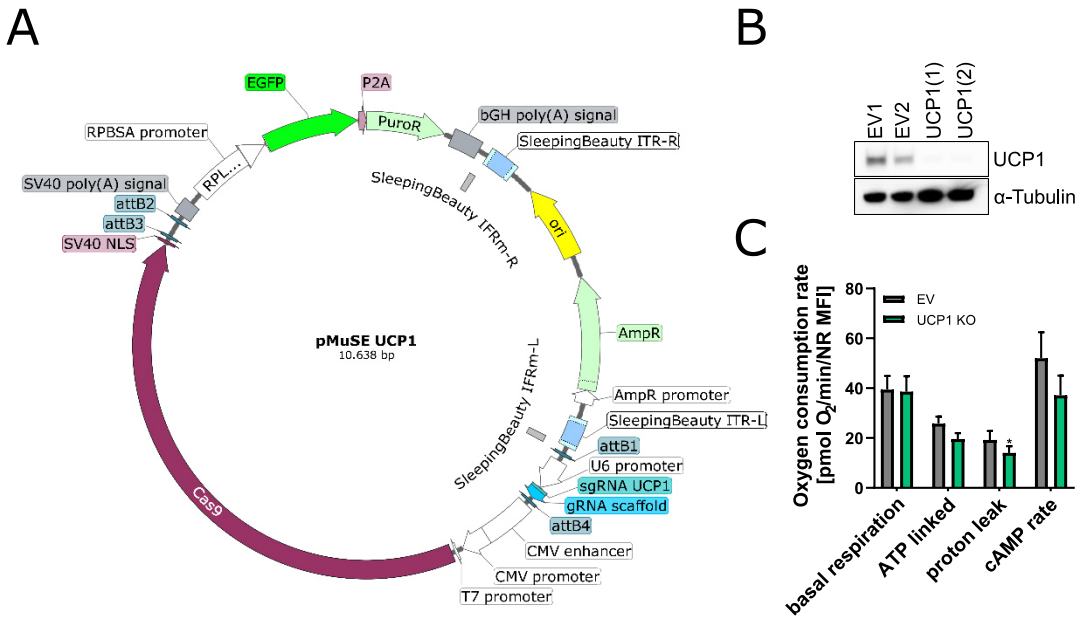

Figure 18 - CRISPR-Cas9 mediated UCP1 knockout only slightly affects cellular oxygen consumption in SGBS adipocytes. (A) *Final DNA plasmid construct for CRISPR-Cas9-mediated UCP1 KO displaying important regions and elements including Cas9 protein being essential for introducing gene KO using an inserted sgRNA. Additional puromycin and ampicillin resistant genes were embedded for antibiotic selection and EGFP for identifying successfully transfected cells. Each element was under the control of a specific promoter to enable stable and independent expression.* ***(B)*** *Oxygen consumption rates were assessed using a plate-based extracellular flux analyzer.* ***(C)*** *Western blot was performed to verify efficient UCP1 KO in SGBS adipocytes. n = at least three independent experiments; mean + SEM. α-Tubulin was used as reference gene. According to Tews D et al. 2019. "Elevated UCP1 levels are sufficient to improve glucose uptake in human white adipocytes". Redox Biol. 101286. doi:10.1016/j.redox.2019.101286.*

3.7 Dicer KD impairs glucose uptake in human SGBS adipocytes

To assess a potential involvement of Dicer in cellular metabolic processes, siDicer adipocytes were functionally characterized regarding their behavior in glucose consumption.

siDicer adipocytes displayed significantly decreased glucose incorporation of radioactively labeled glucose in the basal, unstimulated state by 34 % in comparison to siNT controls (**Figure 19**A). Next, cells were stimulated with different insulin concentration prior to their exposure to glucose. In this experiment, increasing glucose uptake values were observed with higher insulin concentration in both, controls as well as in siDicer cells (**Figure 19**B). Comparison of the two groups showed that Dicer KD significantly lowered glucose consumption in cells treated with 1 and 100 nM insulin.

As another method to investigate metabolic function and insulin sensitivity, a de novo lipogenesis assay was performed examining long-term incorporation of glucose. Results obtained from this approach were in line with previous glucose uptake assays and depicted an impaired ability of human SGBS adipocytes to absorb glucose when Dicer expression is downregulated (**Figure 19**C). Additional experiments in insulin stimulated adipocytes revealed that also here lipogenesis is significantly lower in siDicer cells stimulated with 10 and 100 nM insulin (**Figure 19**D) suggesting a reduced response to insulin of Dicer deficient adipocytes.

In order to further phenotype insulin sensitivity, protein was harvested from siNT and siDicer adipocytes that were either untreated or stimulated with either 1 or 100 nM insulin to check phosphorylation of two important target protein in insulin-induced signaling pathway, namely Akt and IRS. Whereas insulin had no effect on the general expression levels of Akt and IRS, phosphorylation of both targets positively correlated with insulin concentration (**Figure 19**E). When comparing the ratios between the phosphorylated and the unphosphorylated state of Akt and IRS in siNT and siDicer adipocytes, neither of these two candidates differed between these two groups (**Figure 19**F+G).

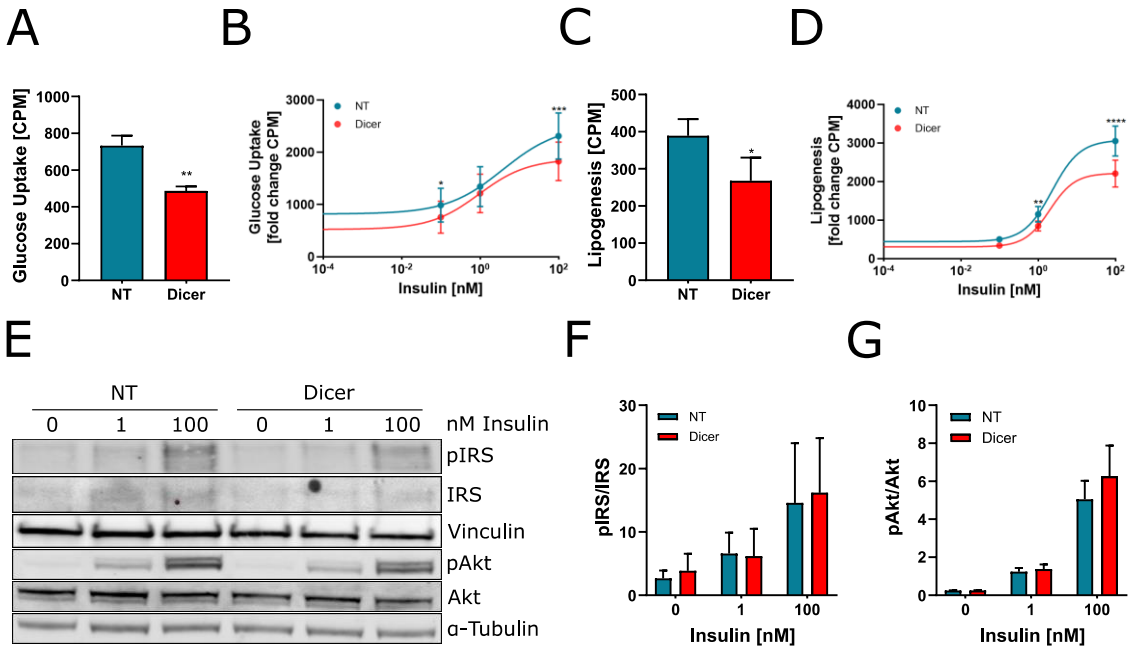

Figure 19 - siRNA knockdown of Dicer impairs glucose consumption of SGBS adipocytes. *Glucose uptake experiments in mature adipocytes were performed under* ***(A)*** *unstimulated and* ***(B)*** *insulin stimulated conditions* by measuring uptake of radioactively labeled ^{14}C-deoxyglucose. ***(C+D)*** *Similarly, long-term incorporation of glucose for 24 hours was assessed in a lipogenesis assay approach.* ***(E)*** *To check for alterations in insulin sensitivity, phosphorylation status of target genes IRS and Akt were relatively quantified and* ***(F+G)*** *densitometrically analyzed. n = at least three independent experiments; one representative blot; mean + SEM. HPRT respectively α-Tubulin or Vinculin was used as reference gene.*

3.8 Effects of Dicer KD on glucose uptake are more prominent in human SGBS preadipocytes

Since the metabolic phenotype of siDicer adipocytes regarding glucose utilization appeared to be more prominent in untreated cells, the aim was to examine whether this metabolic effect is associated with the insulin-independent glucose transporter 1 (GLUT1).

As the expression of GLUT1 is more abundant in preadipocytes compared to mature adipocytes [95,96], SGBS preadipocytes were transfected with either NT or Dicer-targeting siRNA and glucose uptake values measured in siDicer preadipocytes. Obtained data revealed that glucose uptake was significantly reduced by 70 % in Dicer deficient preadipocytes relative to the according control cells (**Figure 20**A). To elucidate this metabolic phenotype in more detail, a Glycolytic Rate Assay was performed using an extracellular flux analyzer. By assessing fluctuation in oxygen rates and the extracellular pH prior and after administration of different compounds, the proton efflux rate was calculated as a measure for the extent of different forms of glycolytic activity. As depicted in **Figure 20**B and C, basal glycolysis rates

were markedly lower in siDicer cells compared to controls. Whereas NT cells iPER levels averagely reached 31 pmol/min/ex 630nm, the capacity of Dicer deficient cells was more than halved to 14.2 pmol/min/ex 630nm. Further inhibition of the electron transport chain by administration of Antimycin A/Rotenone enabled to identify the cellular compensatory capacity resulting in an elevated cellular glycolytic activity to meet the required energy demand. As a consequence, PER values increased in both siNT and siDicer preadipocytes (58.8 in siNT respectively 52.8 pmol/min/ex 630nm in siDicer preadipocytes). Similar to the observations in basal glycolysis, however, compensatory glycolysis of Dicer KD cells was significantly reduced compared relative to siNT controls.

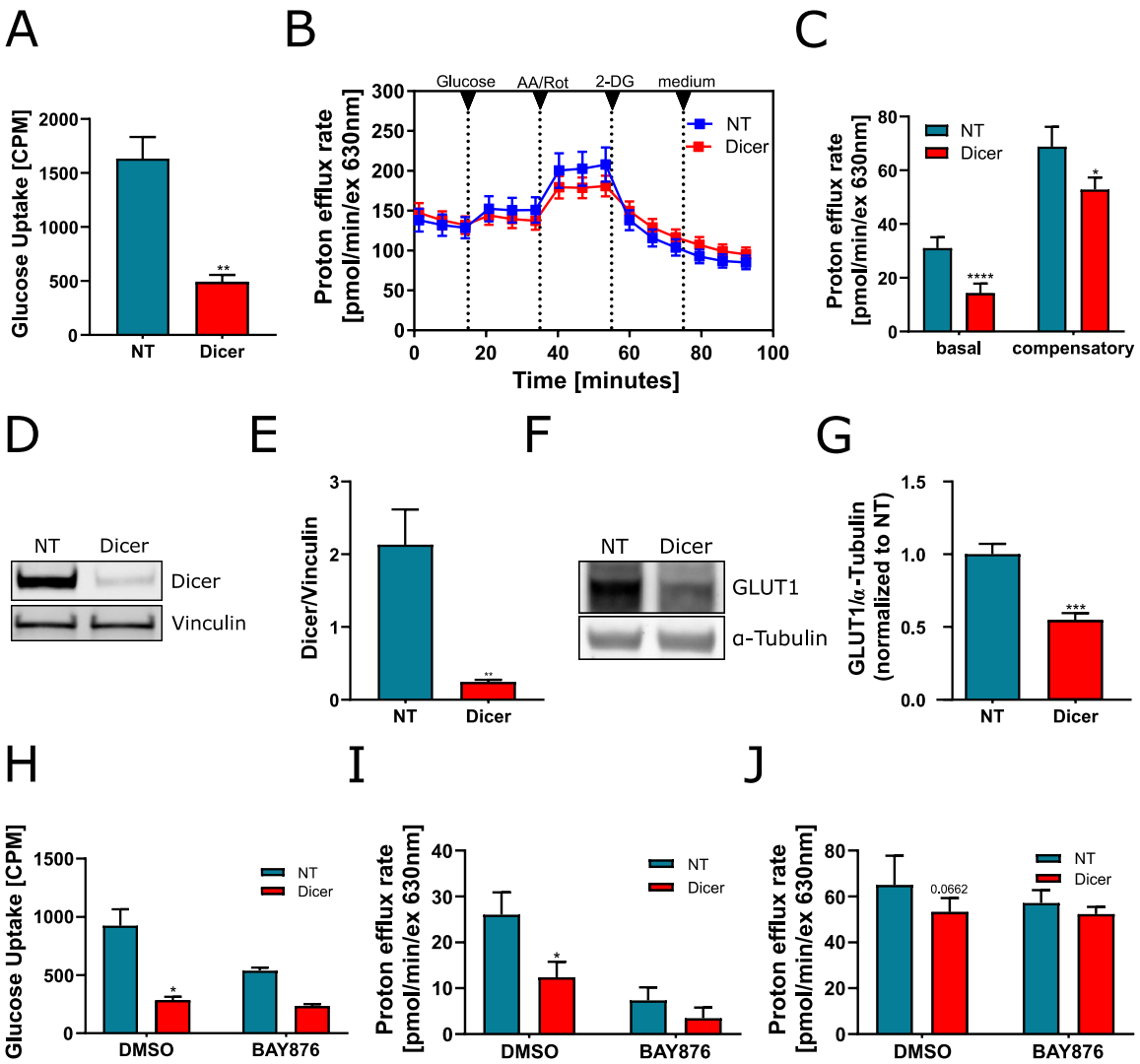

Figure 20 - Impairment of cellular glucose uptake by Dicer KD is more pronounced in SGBS preadipocytes. (A) *Glycolytic activity in SGBS preadipocytes was assessed by measuring uptake of radioactively labeled ^{14}C-deoxyglucose in the basal, unstimulated state.* ***(B)*** *Proton efflux rates (PER) were measured and calculated in a plate-based extracellular flux analyzer. To distinguish between* ***(C)*** *basal and compensatory glycolysis, glucose (10 µM), antimycin A and rotenone (AA/Rot, 500 nM) and*

*2-deoxyglucose (2-DG, 50 µM) were injected sequentially. PER values were normalized to total cell counts using JanusGreen staining (ex 630nm). Cellular abundance of **(D+E)** Dicer and **(F+G)** GLUT1 were quantified on protein level in Western blot experiments. **(H)** Glucose uptake experiments were repeated in pre-treated SGBS preadipocytes - with either 50nM of GLUT1-specific inhibitor BAY 876 or DMSO control (0.1 %) for 2h. Similarly, PER measurements were repeated in order to evaluate **(I)** basal and **(J)** compensatory glycolysis following GLUT1 inhibition. n = at least three independent experiments; one representative OCR plot/blot; mean + SEM. α-Tubulin or Vinculin was used as reference gene.*

To verify Dicer KD and to elucidate the involvement of GLUT1 within this metabolic phenotype, Western Blot experiments were performed. As shown in **Figure 20**D and E, Dicer protein was dramatically inhibited in siDicer preadipocytes with a residual Dicer expression of 11 % compared to siNT controls. Along with this, GLUT1 protein was downregulated by 45% as a result of a Dicer KD (**Figure 20**F+G).

3.9 Chemical inhibition of GLUT1 by BAY 876 abrogates the effects seen on glucose metabolism after siRNA mediated Dicer KD in human preadipocytes

As GLUT1 expression was markedly reduced on protein level of Dicer deficient SGBS preadipocytes, further experiments were performed to specifically address GLUT1 dependent glucose incorporation. To achieve this, previous metabolic assays performed in preadipocytes were repeated while cells were now pre-incubated for one hour with 100 nM of BAY 876, a highly potent and selective GLUT1 inhibitor.

Similar to our previous findings, vehicle control treated siDicer and siNT preadipocytes significantly differed in their basal glucose uptake levels (**Figure 20**H). In particular, uptake values of siNT controls were 3.24-fold higher than in Dicer deficient cells. Preadipocytes which were pre-incubated with BAY 876, however, did not show any alteration between the two groups. Whereas CPM values dropped by 42 % in siNT preadipocytes, BAY 876 treatment had no effect on Dicer deficient SGBS preadipocytes (from 285 to 232).

In additional Glycolytic Rate Assay experiments, results confirmed the decreased basal glycolysis in Dicer deficient cells within the DMSO control group. Again, BAY 876 treatment abrogated this metabolic phenotype as it dropped PER levels from 26 to 7.3 in siNT preadipocytes, in contrast to 12 to 3.5 in Dicer KD preadipocytes (**Figure 20**I). When analyzing compensatory glycolysis rates, no differences were detected, neither in the DMSO control nor in the BAY 876 treatment group (**Figure 20**J).

3.10 siRNA mediated Dicer KD in hMADS preadipocytes mimics the metabolic phenotype observed in human SGBS cells

In order to exclude those results observed in cellular metabolic behavior are due to cell strain specific characteristics, glucose uptake and extracellular flux analysis assays were also performed in hMADS cells.

In line with initial findings in SGSB preadipocytes, Dicer deficient hMADS cells showed significantly decreased glucose uptake values by ~57 % with compared to siNT control group (**Figure 21**A). Further experiments revealed that also basal glycolysis was impaired as a result of Dicer siRNA treatment (**Figure 21**B). Here, Dicer KD in hMADS was sufficient to reduce basal glycolytic activity to 64 % relative to the control cells. Similarly, siNT hMADS preadipocytes had 1.25-fold higher PER levels in compensatory glycolysis relative to siDicer hMADS cells.

To elucidate the involvement of GLUT1 also in hMADS cells, preadipocytes were again pretreated with the GLUT1 specific inhibitor BAY 876. Comparable to the previous results, siNT and siDicer hMADS cells depicted significantly altered CPM values within the DMSO control treated group when metabolic behavior was analyzed in glucose uptake assays (**Figure 21**C). Yet, no differences were detectable when hMADS preadipocytes were treated with BAY 876 in advance.

Although statistically not significant, analysis of the extracellular flux of siDicer hMADS cells tent ($p = 0.0820$) to have a lower basal glycolytic capacity comparing siNT versus siDicer (**Figure 21**D). Treatment with BAY 876 was able to reduce basal glycolytic activity in both groups, while showing no difference between control and Dicer KD preadipocytes. The AA/Rot induced impairment of oxidative phosphorylation resulted in 15 % lower compensation by increased glycolytic activity in siDicer preadipocytes than in siNT (**Figure 21**E). Again, BAY 876 mediated GLUT1 inhibition abolished differences between the cell models.

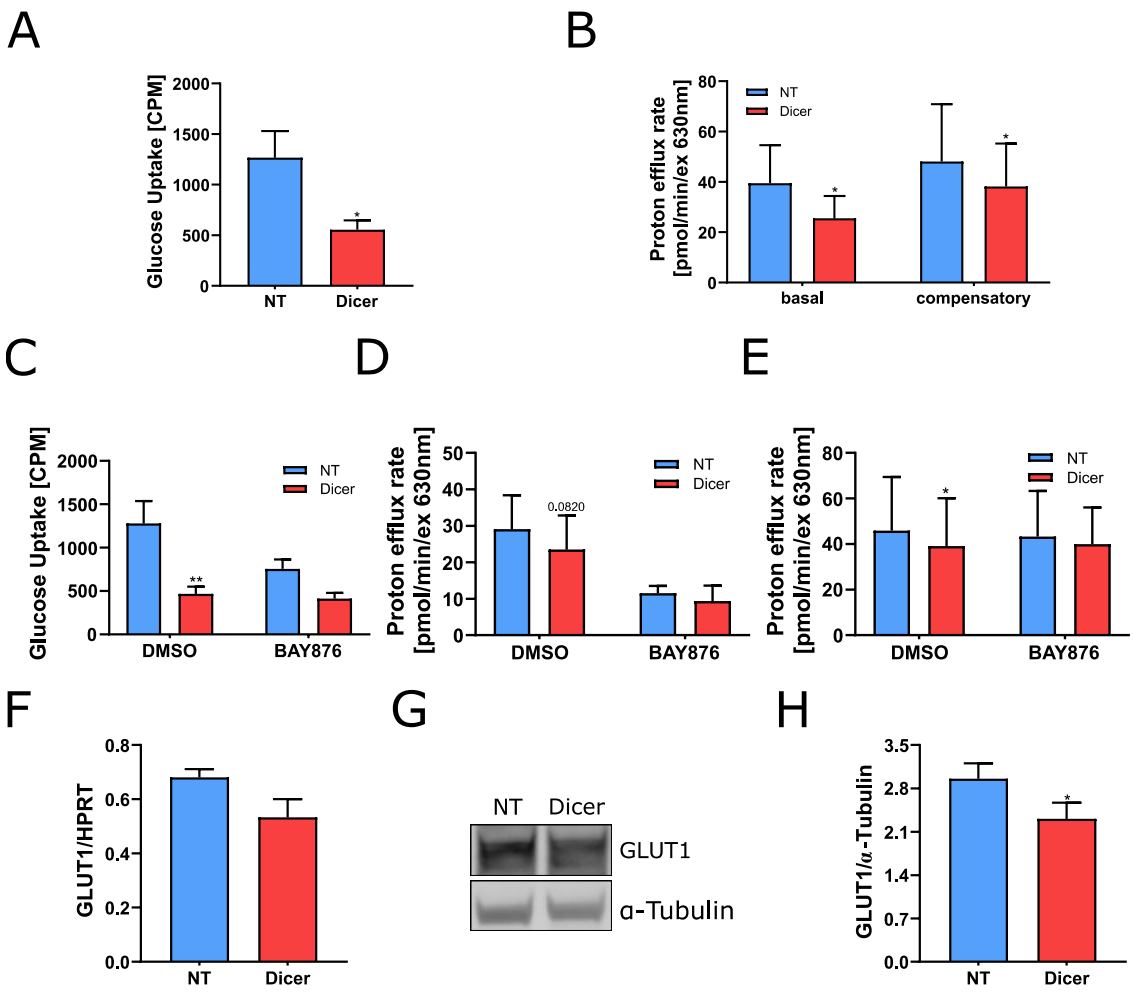

Figure 21 - hMADS preadipocytes display similar metabolic phenotypes compared to SGBS cells following siRNA mediated Dicer knockdown. (A) *Glycolytic activity in hMADS preadipocytes was assessed by measuring uptake of radioactively labeled ^{14}C-deoxyglucose in the basal, unstimulated state.* ***(B)*** *Proton efflux rates (PER) were assessed in a plate-based extracellular flux analyzer and basal and compensatory glycolysis distinguished by sequentially injecting glucose (10 µM), antimycin A and rotenone (AA/Rot, 500 nM) and 2-deoxyglucose (2-DG, 50 µM). PER values were normalized to total cell counts using JanusGreen staining (ex 630nm).* ***(C)*** *Glucose uptake experiments were repeated in pre-treated SGBS preadipocytes - with either 50nM of GLUT1-specific inhibitor BAY 876 or DMSO control (0.1 %) for 2h. Similarly, PER measurements were repeated in order to evaluate* ***(D)*** *basal and* ***(E)*** *compensatory glycolysis following GLUT1 inhibition. GLUT1 levels were quantified on* ***(F)*** *mRNA and on* ***(G+H)*** *protein level in Western blot experiments. n = at least three independent experiments; one representative blot; mean + SEM. HPRT respectively α-Tubulin was used as reference gene.*

3.11 Enoxacin treatment increases GLUT1 mediated glucose uptake in human SGBS preadipocytes

To further pinpoint that Dicer is the main driver causing the observed metabolic phenotype and to thereby exclude that GLUT1 impediment is not a result of siRNA induced off-target effects, human SGBS preadipocytes were treated for 48 hours with the Dicer activity enhancer enoxacin prior to carrying out glucose uptake experiments.

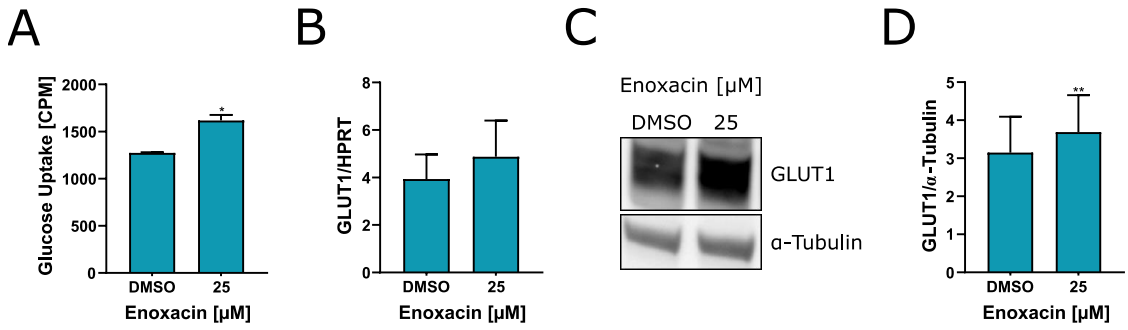

Figure 22 - Enoxacin treatment raises glucose consumption via GLUT1 upregulation. *SGBS preadipocytes were treated with 25 µM enoxacin for 48 hours prior to performing the experiments. DMSO treated cells (0.2 %) served as according controls.* ***(A)*** *Glucose uptake experiments were completed in enoxacin and DMSO treated SGBS preadipocytes. Expression of GLUT1 was checked via* ***(B)*** *qPCR and* ***(C+D)*** *Western blot analysis. n = at least three independent experiments; one representative blot; mean + SEM. HPRT respectively α-Tubulin was used as reference gene.*

SGBS preadipocytes treated with 25 µM enoxacin had elevated cellular glucose uptake by 27 % compared to the according DMSO control (**Figure 22**A). While mRNA expression of GLUT1 did not show changes, that were statistically different, analyzing harvested protein in Western Blot experiments, displayed substantially increased GLUT1 levels which were about 1.2-fold higher than in the control cells (**Figure 22**C+D).

3.12 GLUT1 mediated impairment in glucose metabolism of Dicer deficient human SGBS cells is not regulated by miR-18a-3p, nor by miR-543 solely

In a last step, the goal was to identify miRNAs which were responsible for the metabolic phenotype of impaired glucose uptake in Dicer deficient human SGBS cells. For this approach, a miRNA sequencing of siNT versus siDicer transfected preadipocytes was performed to evaluate differentially expressed miRNAs comparing the two groups.

As a result, 1,126 miRNAs species were detected in SGBS preadipocytes. In order to sort for relevant miRNAs only, thresholds have been set for p-value (< 0.05), fold change (< 0.7 or > 2) and base mean expression value (> 40) leading to the identification of nine differentially expressed miRNAs (**Figure 23**A). Eight of these miRNAs – namely miR-18a-3p, -99b-3p, -324-3p, -370-3p, -543, -4760-3p, 4760-5p and let-7d-3p - were re-analyzed in a miRNA specific qPCR procedure, while there was no commercially available primer for miR-12135. Two miRNAs were confirmed to be significantly altered between siNT and siDicer preadipocytes (**Figure 23**B). MiR-18a-3p was detected 2.6-fold higher in siDicer preadipocytes compared to siNT controls, whereas the expression of miR-543 was reduced by 35 % in Dicer deficient cells.

To elucidate if these two miRNAs might be responsible for the metabolic phenotype in Dicer deficient cells, SGBS preadipocytes were transfected with either synthesized miR-18a-3p mimic or miR-543 inhibitor before glucose uptake experiments were conducted.

Data obtained from these experiments did not show any effect on glucose uptake in miR-18a-3p mimic transfected SGBS cells, although miR-18a-3p expression was considerably increased in mimic treated preadipocytes (relative expression values: 0.0016 NT vs. 292 miR-18a-3p) (**Figure 23**C+D). Likewise, no adverse metabolic effects were observed in miR-543 inhibitor treated preadipocytes when compared to their according NTi control (**Figure 23**E). Also here, successful transfection was confirmed by assessing markedly decreased miR-543 expression levels in inhibitor treated preadipocytes (relative expression values: 3.3 x 10^{-3} in NTi vs. 2.7 x 10^{-5} in miR-543i) (**Figure 23**F).

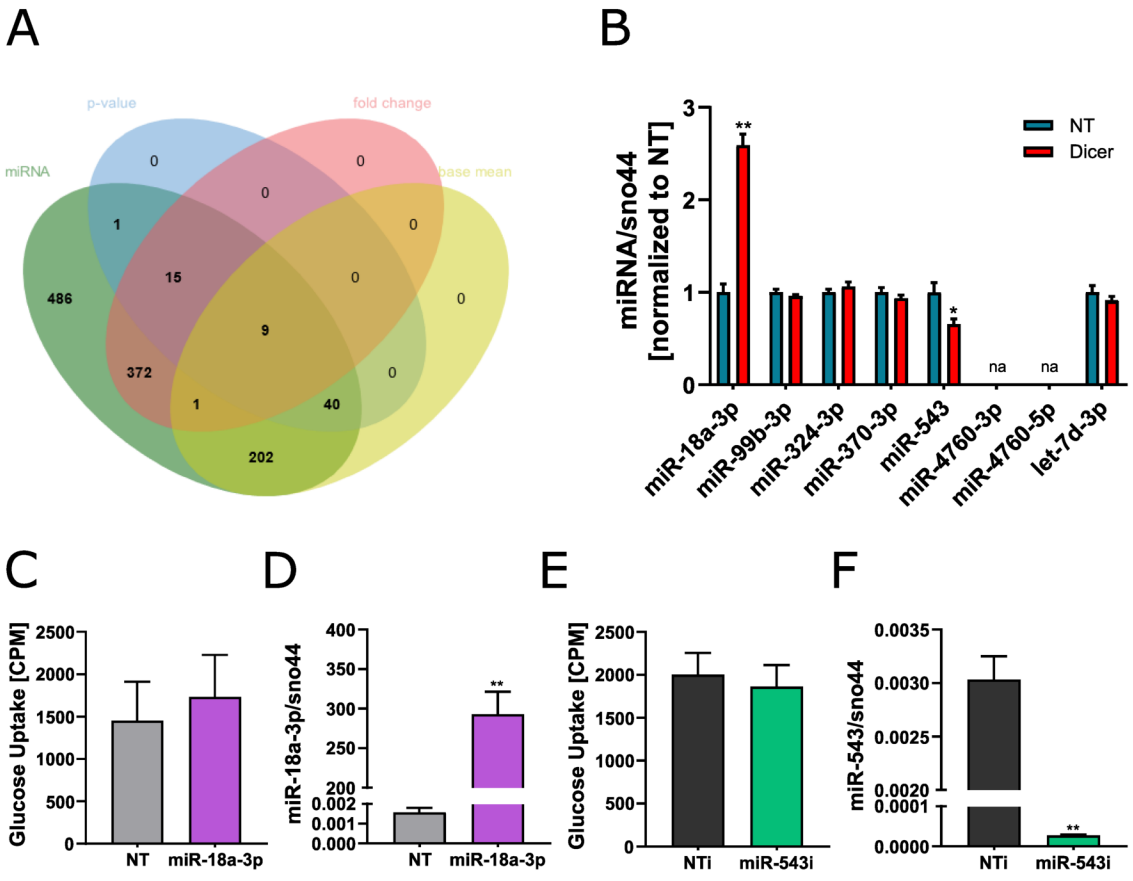

Figure 23 - Identification of target miRNAs in siRNA treated human SGBS preadipocytes. (A) *Venn diagram displaying the strategic approach of identifying potential target miRNAs by discriminating expressed miRNAs for p-value (< 0.05), fold change (< 0.7 respectively > 2) and base mean expression value (> 40).* ***(B)*** *qPCR evaluation of pre-selected target miRNAs. Glucose uptake assays were performed following* ***(C)*** *miR-18a-3p mimic or* ***(E)*** *miR-543 inhibitor transfection.* ***(D+F)*** *Successful transfection was verified by qPCR. n = at least three independent experiments; one representative blot; mean + SEM. HPRT respectively α-Tubulin was used as reference gene.*

4. Discussion

Obesity represents a proceeding threat for human societies especially in high-income countries [2]. In this regard, Dicer is a promising target to investigate in the field of obesity research. Although there is large scientific data available from investigations in mice, there is an enormous lack in adequate evidence for metabolic implications in humans.

Based on this, the aim of this work was to identify Dicer's specific molecular function in the metabolic context in a human system of AT. To address this issue, a Dicer LOF was introduced using human SGBS and hMADS cells.

4.1 Dicer loss-of function in human cell strains has no effect on adipogenic differentiation neither in human SGBS nor in hMADS cells

SGBS preadipocytes are primary cells extracted from subcutaneous adipose tissue of an infant suffering from Simpson Golabi Behmel Syndrome [90,91]. One of the main features of these cells is their high differentiation capacity when exposed to chemically defined medium. Likewise, secretion of adipokines as well as gene expression pattern show results comparable to primary adipocytes. As opposed to usual primary material obtained, proliferation and differentiation can be maintained over several generations without any need for immortalization. Given that physiological as well as metabolic characteristics are more representative for the investigation of human adipocyte function compared to other commonly used cell lines such as 3T3-L1 cells [90,92–94].

Within this work results obtained from SGBS cells demonstrated that this cell strain serves as an adequate model system to analyze the functional relevance of Dicer in human preadipocytes and mature adipocytes at the first place. Not only did SGBS preadipocytes perfectly undergo adipogenic differentiation, which was indicated on multiple level. Even more importantly, expression levels of Dicer were already present in the preadipocyte state which was crucial for further functional studies on Dicer. Results obtained from experiments in hMADS cells were in line with these findings. Thus, these results indicate that Dicer is present and well expressed in SGBS as well as in hMADS cells and that, moreover, the total abundance of Dicer in both cell strains was more prominent in adipocytes compared to preadipocytes indicating that the functional role of Dicer might be more important in the mature, differentiated state. The latter phenomenon is in line with previous findings in mouse 3T3-L1 cells [95].

As we aimed to assess the cellular function of Dicer on molecular level, a LOF of human Dicer was introduced in SGBS bulk culture in a CRISPR-Cas9 mediated approach. As previous studies suggested, we hypothesized that knocking out Dicer will impair preadipocytes-adipocyte transition of human SGBS cells [66,67,95,96].

When analyzing the cellular phenotype in SGBS cells, however, there were interestingly no morphological differences when comparing the EV controls with both Dicer KO cell models in mature adipocytes. Moreover, neither differentiation rates nor lipid content or adipogenic marker gene expression appeared to be affected. Similarly, there was no effect on differentiation of SGBS cells, when both sgRNA – which were used in the first KO approach – were merged into one final CRISPR-Cas9 plasmid. Also here, none of the parameters assessed suggested an inhibitory effect of Dicer on adipogenesis in a human preadipocyte cell strain. Differentiation rates as well as incorporated triglyceride levels remained unaffected comparing EV and Dicer KO adipocytes.
That is, that the CRISPR-Cas9 mediated approach was inefficient to fully knock out Dicer in SGBS cells.

An important question that arises in this context is: why does the KO approach in SGBS cells only cause a mild knockdown rather than completely abolishing Dicer expression?

To address the Dicer LOF in a different approach, SGBS preadipocytes were transfected with Dicer targeting siRNA. As a result, KD efficiency of Dicer achieved 72 % on mRNA and even 80 % on protein level in preadipocytes and was thereby more effective than previous CRISPR-Cas9 KO experiments. Even more importantly, in contrast to the KO apporach this phenotype was stable throughout adipogenic differentiation. Thus, also SGBS adipocytes displayed remarkably reduced Dicer levels. Surprisingly, still none of the adipogenic parameters assessed suggested any effect of Dicer deficiency on differentiation capacity of human SGBS cells nor was there any morphological alteration. Interestingly, Dicer siRNA treated SGBS cells even displayed increased levels of CEBPβ and PPARγ.

Although KD efficiency was relatively high using siRNA, the obtained data in this work was conflicting with previous publications. Mudhasani, Imbalzano, and Jones 2010 were the first research group to show that Dicer impedes adipogenic differentiation. Here, a tamoxifen-induced, conditional Dicer KO was performed in mouse embryonal fibroblast (MEF). These cells, that are capable to differentiate into mature adipocytes, displayed drastic impairment into adipocyte transition upon Dicer ablation. This phenotype, which was proven by Oil Red O lipid staining, was underlined by diminished marker gene expression of PPARγ, GLUT4 and FASN. Similar results were obtained when experiments were re-

performed in primary mouse preadipocytes. In both models, PCR analysis confirmed the total deletion of Dicer tested in genomic DNA. In line with these findings, Mori et al. 2014 reported comparable discoveries in isolated brown adipose preadipocytes. Originated from transduced mice holding a Cre recombinase under an adipocyte specific promotor, lipid stainings revealed markedly lower lipid content compared to cells harvested from control mice. Interestingly, there was no effect on adipocyte marker gene of CEBPα, PPARγ and human adipocyte fatty acid-binding protein (aP2) when analyzed via qPCR.

At this point, it is important to note that Bernstein et al. 2003 reported that a full-body Dicer KO is lethal in mice. Correspondingly, Wienholds et al. 2003 described similar consequences in zebrafish highlighting that adequate Dicer activity is crucial in vertebrates. This could eventually mean that a severe Dicer KO/KD, also in SGSB cells, might have effects on cellular survival impacting proliferation and apoptosis as already earlier reported for other cell types [78–80,98]. However, we did not make such observations during cell culturing.

Yet, although CRISPR-Cas9 technique introduces double-strand brakes in order to generate specific gene KOs, this method is also known to cause loss of heterozygosity as a consequence of monoallelic KO events taking place [99–101]. In this context, this would mean that CRISPR-Cas9 initiated gene deletion might lead to the generation of SGBS preadipocytes which have either a homozygous or a heterozygous Dicer KO. Due to the pivotal role of Dicer in cellular processes, SGBS preadipocytes with a heterozygous loss of function might have improved survival and/or proliferate faster and therefore overgrow SGBS preadipocytes which hold a homozygous deletion. Taking this into account might explain why there could still be Dicer present in genetically modified SGBS cells. The very reason for having the high possibility of simultaneously culturing both, heterozygous together with homozygous Dicer KO SGBS cells, is that transduced SGBS preadipocytes were cloned from bulk cultures as they cannot be grown out of one single cell. Of note, the probability that cells with no genetic modification at all survived is rather unlikely as all cells underwent an antibiotic selection process after being transduced.

An explanation for unexpected differences seen in SGBS cells when comparing with results described from previous studies might therefore be due to the residual Dicer activity in SGBS cells which still might be sufficient to enable proper adipogenic differentiation. A similar phenomenon was previously reported in Dicer deficient MEFs, where a heterozygous Dicer ablation had no effect on preadipocyte-to-adipocyte transition, whereas a biallelic KO evidently impaired adipogenesis [66].

An additional important aspect to consider is that previous studies used murine cell culture models to investigate Dicer's function in adipose tissue [66,67,71]. Since SGBS cells are human primary cells, varying cellular response to diminished Dicer levels might be justified by species differences as cellular characteristics widely differ between mice and human as researcher previously stated [102–104].

This explanation would furthermore describe why Fujimoto et al. 2012 and Rocha et al. 2020 came to similar conclusions as their colleagues when investigating the role of Dicer in adipogenesis using mouse 3T3-L1 respectively C3H10T1/2 mesenchymal cells. Also, this would explain why primary mouse preadipocytes from subcutaneous AT (sAT) lost their differentiation capacity upon Dicer ablation, whilst no effect was observed in SGBS cells [66]. Although SGBS cells can be considered as human counterpart to mouse sAT preadipocytes, sAT of mice reportedly relies on accurate Dicer function as its absence led to the phenotype of lipodystrophy in mice - which was demonstrated in two independent studies [67,71]. This suggests that Dicer activity is vital for especially mouse sAT preadipocytes and might therefore very likely be a critical component in adipogenesis in these particular mouse cells.

Besides all this, it is important to further note that Mudhasani, Imbalzano, and Jones 2010 utilized embryonic fibroblasts to identify the role of Dicer in adipogenic differentiation. Embryonic cells (EC) very much differ from other cell types considering their genetic features and cellular demands [105]. In particular, EC are reported to respond more sensitively to cellular stress, including changes in intrinsic miRNA levels [106]. As a consequence, the absence of appropriately functional Dicer was shown to be associated with severe defects in general differentiation capacity of EC [107]. Thus, differences in adipogenesis observed might be additionally explained by different cellular requirements with regard to Dicer in MEFs compared to SGBS cells.

Another important aspect that is important to note when comparing our findings with BAT preadipocytes is the fact that SGBS preadipocytes are WAT primary cells and thus are not immortalized. Hence, SGBS cells represent a better understanding of the actual in vivo situation. Furthermore, immortalization aims to increase cellular proliferative capacity by manipulating genetic features. As proliferation is a competing mechanism to cell differentiation, additional disruption of this well-balanced system might cause more sensitive reaction to any kind of alteration that is involved in this fundamental cellular process [108].

Supporting data to our findings in SGBS cells comes from Dicer siRNA transfected hMADS preadipocytes. Here, siRNA mediated Dicer reduction led to remarkable KD efficiency in both

undifferentiated preadipocytes and mature adipocytes. Although Villageois et al. 2012 stated in their publication to have observed impaired adipogenesis in hMADS cells upon siRNA induced Dicer LOF according to some unpublished data, there was notably no inhibition in ADIPOQ or PPARγ neither on mRNA nor in protein level when performed in our hands. Yet, differentiation rates were slightly reduced by approximately 15 %, but still reached about 74 %, while triglyceride levels were evidently decreased upon Dicer KD. However, taking into account, that siRNA achieved an 80 % KD in preadiocytes and 70 % in adipocytes rather suggests that - in contrast to the descriptions in the literature - Dicer seems to be no key mediator in human adipogenesis.

Additional experiments in both cell strains further emphasized this observation. Treatment of SGBS and hMADS cells with enoxacin, tremendously compromised adipogenic differentiation in a dose-dependent manner. Enoxacin is a well-characterized fluoroquinolone antibiotic, which was proven to enhance miRNA processing mediated by Dicer by facilitating dsRNAs and TRBP interaction [84,109].

Differentiation rates, lipid content as well as adipogenic marker gene expression dramatically dropped compared to DMSO treated controls. Most importantly, the inhibition of adipogenic differentiation was consistent in both, SGBS and in hMADS cells. Interestingly, no such events were monitored in mouse white subcutaneous preadipoctes [85]. According to this, it appears that a compound-induced Dicer gain of function (GOF) is involved in downregulating human preadipocyte differentiation. However, it is very important to point out, that further investigations are required to verify enhanced miRNA processing in order to underline that the effect observed SGBS and hMADS cells is due to increased Dicer activity and not caused by any type of off-target effects. Experiments in Dicer KO/KD cells would help to clarify whether this effect relies on a Dicer-independent mechanism or not.

Taken together, scientific literature provides evident data implying the importance of Dicer in mouse adipogenesis. This could not be confirmed using the human SGBS and hMADS cell strains. Increasing Dicer activity by enoxacin treatment, yet, rather suggests that a pharmacologically induced GOF of Dicer even impairs differentiation. To the best of our knowledge, we are the first to show that downregulation of Dicer does not inhibit adipogenesis in a human primary cell strain.

Yet, for further clarification of this particular phenotype a more effective KO approach needs to be executed. One possibility to do so, would be to culture single cell clones of transduced Dicer KO cells. Due to the limitation in SGBS proliferation out of one single cell, the use of feeder cells would be necessary in this context. After cultivation of the separated SGBS Dicer KO clones, the KO efficiency

needs to be evaluated which can be done by regular qPCR or Western blot or more precisely by performing either a Dicer enzyme-linked immunosorbent assay (ELISA) or a specific Dicer activity approach according to Davies and Arenz 2008.

Moreover, the use of primary material extracted from fresh human AT could be used for additional LOF experiments helping to exclude any cell strain specific observations. These cells could then further be compared to either murine cell strains or freshly isolated mouse cells in order to delineate potential species differences regarding the importance of Dicer in the adipogenesis phenotype.

4.2 Downregulation of Dicer impairs UCP1 expression but does not affect mitochondrial biogenesis and cellular respiration of human SGBS adipocytes

Dicer is reported to have a pivotal function in the context of BAT biology. As described by Mori et al. 2014, BAT of adipose tissue specific Dicer KO mice was associated with cellular hypertrophy resulting in enlarged adipocytes with unilocular lipid droplets and a severe whitening phenotype characterized by a loss of UCP1 expression. Metabolically, these mice were insulin resistant and prone to develop increased AT inflammation. Additional evidence for the importance of Dicer in BAT arose from rescue experiments performed by Rocha et al. 2020. Here, induction of Dicer activity by enoxacin was able to reverse this phenotype and to induce gene expression of UCP1, CIDEA and DIO2.

According to available data from the current literature, we hypothesized that a Dicer KD negatively affects regulation of BAT associated gene expression and associated metabolic processes in SGBS adipocytes.

In SGBS cells, the silencing of Dicer did not lead to morphological alterations, but instead caused a robust downregulation of UCP1 expression on mRNA and protein level. While Mudhasani et al. 2011 and Mori et al. 2014 reported also impaired levels of PGC1α, CIDEA and elongation of very long chain fatty acids protein 3 (ELOVL3), this was not seen in SGBS cells. Leptin though inversely correlated with UCP1 expression and was thus remarkably increased. This pattern was consistent throughout qPCR, Western blot and ELISA analysis suggesting enhanced leptin secretion in Dicer deficient human SGBS cells. Increased leptin mRNA was also observed in BAT of Dicer KO mice and immortalized brown adipocytes [67].

Further investigations in SGBS adipocytes focused on potential effects on mitochondrial mass and oxidative metabolism associated with lowered UCP1 levels. In Dicer deficient SGBS cells, mitochondrial content was not altered - being measured via qPCR, Western blot and citrate synthase activity assay.

There was also no evidence of Dicer affecting oxygen consumption in mature human SGBS adipocytes. Although UCP1 levels were dramatically reduced, proton leak respiration was unchanged in Dicer deficient adipocytes.

This was contradictory to previously published UCP1 studies in which UCP1 expression correlated with mitochondrial mass as shown in HEK293 and murine cells [17,111,112]. In line with this, UCP1 KO mice show a tremendous loss of mitochondrial proteins was characterized underlining a positive correlation between UCP1 levels and mitochondrial proteins [114]. Findings by Poher et al. 2015 in Lou/C, but interestingly not in Wistar rats came to similar conclusions. Furthermore, UCP1 activity per se is linked with increased cellular oxygen consumption and proton leak respiration [1,111]. Interestingly though, Mori et al. 2014 assessed oxygen consumption in Dicer ablated mice – consequently also lacking UCP1 – using a comprehensive lab monitoring system. Here, they could measure slight induction of oxygen consumption in ADicerKO mice, while further investigations in immortalized BAT adipocytes displayed impaired proton leak respiration serving as a functional readout of the detected UCP1 downregulation.

Likewise, Rocha et al. 2020 elaborated that enoxacin induced increase in Dicer activity not only reversed BAT whitening thereby elevating UCP1 protein, but also enhanced cellular respiration in vivo and in immortalized white adipocytes. Additional loss of function experiments in cardiac mesenchymal stem cells also revealed impaired cellular respiration as a result of a Dicer KO [80].

Yet, there is evidence that human primary adipocytes might react differently to altered cellular levels of UCP1. Previous findings from our laboratory delineated that UCP1 overexpression was not associated with changes in mitochondrial biogenesis in SGBS adipocytes although oxygen consumption was massively increased [1]. On the contrary, as examined in this thesis, most respiratory parameters remained unchanged in UCP1 KO SGBS adipocytes - with one single exception. The absence of UCP1 induced a significant decrease of proton leak linked oxygen consumption of nearly 37 %. However, in Dicer deficient SGBS adipocytes UCP1 protein was reduced by 47 % with no effect on cellular respiration. The data set provided by UCP1 KO SGBS adipocytes suggests that only a complete loss of UCP1 might be potent enough to affect SGBS adipocyte respiration.

Moreover, it is of utmost importance to note that although SGBS cells derive from WAT, long-term treatment with the PPARγ agonist rosiglitazone during differentiation is able to induce UCP1 expression in these cells [115]. Yet, UCP1 is the hallmark protein of BAT [12]. Its cellular significance might therefore be way less relevant in white adipocytes such as SGBS cells and therefore also might not

affect mitochondrial biogenesis in this model system to a significant extent. This is especially important, because inhibition of UCP1 expression caused by Dicer deficiency occurs in a relatively small scale compared to typical loss of function approaches. This could also be the explanation why other BAT associated genes were not affected in contrast to results found in the literature.

In summary, findings in human primary SGBS adipocytes reveal that Dicer deficiency impairs UCP1 expression in a so far undiscovered process, most likely in a miRNA dependent manner, and thereby underline previously published data. Additionally, it did not affect cellular respiration or mitochondrial biogenesis. Yet, further experiments are needed to identify the mechanism of action by which Dicer affects cellular UCP1 levels, to elucidate the differences in WAT versus BAT derived cells and to compare human and mouse cells in this particular context.

4.3 GLUT1 mediated glucose metabolism relies on proper Dicer function in human pre-/adipocytes

ADicerKO mice were shown to exhibit impaired glucose clearance and insulin sensitivity [63]. This is why, we were interested to examine whether this phenotype also occurs in Dicer deficient SGBS cells.

In contrast to unchanged metabolic parameters regarding cellular respiration in siDicer adipocytes, downregulation of Dicer in human SGBS adipocytes led to a decrease in glucose uptake. In line with previous findings in mice lacking Dicer in adipose tissue [63], insulin response was reduced in siDicer adipocytes as shown in two independent metabolic assays. Surprisingly, besides decreased glucose uptake values in insulin stimulated samples, there were no additional signs of insulin pathway activation. Neither phosphorylated IRS nor Akt were increased. This was an unexpected finding as insulin mediated glucose uptake comes along with enhanced phosphorylation of these two target proteins [97]. Yet, differences observed in metabolic assay values were already present in the unstimulated state and, even more importantly, stable throughout the measurements implying that hampered uptake values might rather arise from insulin independent conditions.

Insulin independent glucose uptake is mainly mediated via glucose transporter GLUT1, while its expression is reportedly higher in preadipocytes compared to adipocytes [95,96]. Therefore, experiments were repeated using SGBS preadipocytes instead. Glucose uptake values in SGBS preadipocytes were significantly hampered upon Dicer KD. This phenotype was also confirmed in an additional metabolic assay. Experiments investigating GLUT1 mRNA as well as protein levels further

revealed drastically decreased GLUT1 expression in siDicer preadipocytes underlining a crucial involvement of GLUT1 within this metabolic process.

In addition to our findings in human SGBS preadipocytes, there was supporting evidence when examining the metabolic outcome of a Dicer LOF in hMADS preadipocytes. This dataset further emphasized that Dicer deficiency causes diminished glucose uptake in human preadipocytes. Since GLUT1 protein levels were provenly downregulated in both siDicer SGBS and hMADS preadipocytes, we conclude that Dicer is essential for basal glucose uptake in a GLUT1 dependent manner.

Interestingly, enoxacin mediated stimulation of Dicer activity led to opposite effect by triggering incorporation of glucose in SGBS cells. Assuming that this was due to increased miRNA processing by Dicer – as implied in previous experiments - this might indicate that specific miRNA have a crucial impact on glucose metabolism in human preadipocytes [78,79]. Indeed, there are several miRNAs reported to be associated with cellular glucose uptake [98–100]. Yet, important to note, our experimental approach lacks verification of Dicer activation upon enoxacin treatment. To exclude possible adverse effects, it requires further assessment of Dicer activity in the future, before considering the screening for alterations in miRNA expression to identify possible miRNAs targets responsible for the observed phenotype.

In contrast to our findings, Dicer knockout in cardiac muscle cells increased glycolysis as measured via enhanced basal glycolysis as well as compensatory glycolysis as measured in extracellular flux analysis while assessing oxygen consumption [75]. Yet, it needs to be mentioned that these results were accompanied by impaired oxidative phosphorylation, which was provenly not the case in SGSB cells. As a consequence, the higher demand in glucose might rather compensate for defective cellular respiration in cardiomyocytes. Likewise, similar observations were made in mice carrying an AT specific Dicer KO. Although there were no reports on altered GLUT1 expression [31,63,69], increased lactate levels were described in AT of ADicerKO mice [69]. This might suggest a higher glycolytic activity. Nevertheless, also here this metabolic phenotype was associated with a respiratory impairment as mitochondrial DNA and electron transport chain mRNA marker were markedly reduced.

As UCP1 expression is associated with increased glycolytic flux [101,102], we questioned whether altered UCP1 levels in Dicer deficient SGBS cells could be responsible for shifting energetic balance towards an impaired glycolytic state. At least, this would perfectly be in line with our previous study, where we could show that overexpressing UCP1 in SGBS cells increased basal glucose metabolism

[103]. Moreover, also in this study, treatment with the specific GLUT1 inhibitor BAY 876 indicated a relationship of UCP1 and GLUT1 in this context. However, UCP1 KO did not affect basal glucose uptake in mature SGBS adipocytes, even though UCP1 protein levels were much lower than in Dicer KD cells [103]. An additional fact, that speaks against the UCP1 assumption is that its expression is exclusive to mature adipocytes. Yet, any connection to the metabolic phenotype in Dicer deficient SGBS cells would therefore not explain why this alteration was also present in preadipocytes. Still, this does not exclude that at least in mature adipocytes decreased UCP1 levels might contribute to diminished glucose incorporation in Dicer LOF cells.

In preliminary experiments, mRNA levels of monocarboxylate transporter 1 (MCT1) and monocarboxylate transporter 4 (MCT4), the two main transporters responsible for cellular lactate and pyruvate shuttling, were assessed. From cancer studies, there is evidence that alteration in these two transporters affect cellular glycolysis [104]. However, preliminary data revealed elevated mRNA level of both, MCT1 and MCT4, therefore giving no hint on metabolite accumulation that might cause a negative feedback loop. Yet, this requires further examination including the identification whether mRNA expression levels correspond to MCT1 and MCT4 protein levels. Additionally, lactate levels could be quantified in the supernatants and checked for potential alteration between control and Dicer deficient cells.

Taken together, this work provides evidence that cellular glucose metabolism in human preadipocytes and mature adipocytes greatly depends on properly functional Dicer activity as shown in two different human cell strains. Additional experiments chemically enhancing Dicer mediated miRNA maturation provided additional proof for our findings. Moreover, GLUT1 was identified as a key mediator in this particular process. To the best of our current knowledge, we were the first to describe this type of altered glucose metabolism upon Dicer inhibition via GLUT1.

Yet, it requires further experiments to completely delineate the entire molecular mechanism behind this phenotype. Reportedly, GLUT1 expression is regulated via mammalian target of rapamycin (mTOR) complex 1 in 3T3-L1 adipocytes, while in brown adipocytes mTOR complex 2 was shown to enhance GLUT1 levels [105,106]. In general, mTOR signaling per se is commonly known as a pivotal regulator of crucial cellular processes including proliferation, cell growth and nutrient sensing [107–109]. Thus, it would be absolutely necessary to analyze the mTOR pathway to check for potential alterations induced by siRNA mediated Dicer KD. Since there is a link between chronic hypoxia, GLUT1 expression and

cellular Dicer levels, a potential involvement of hypoxia inducing factor 1 alpha (HIF-1α) should be part of future investigations [108,110,111].

4.4 Neither miR-18a-3p, nor miR-543 affect glucose metabolism in SGBS preadipocytes

While observing the metabolic alterations in Dicer deficient human SGBS preadipocytes, the question arose, how this phenotype is triggered by diminished cellular Dicer levels. Since Dicer is a key enzyme in miRNA biogenesis, the assumption was that altered miRNA levels are the reason for diminished glucose uptake observed in SGBS and hMADS preadipocytes caused by siRNA mediated Dicer KD. In line with this, previous findings suggest that loss of Dicer causes severe impairment in maturation of miRNAs [36,132,133].

In contrast to the literature, among the over a thousand miRNAs which were detected in Dicer deficient SGBS preadipocytes during the sequencing approach, only 65 miRNAs were significantly altered. Even more interestingly, 37 of these miRNAs were in fact upregulated upon Dicer siRNA treatment. This was not what we expected as several research groups reported otherwise [36,132,133]. Moreover, Thomou et al. 2017 described that AT is a key source of miRNAs as a tissue-specific loss of Dicer had devastating effects on mature miRNA levels.

A possible explanation for this observation might be due to the fact that the siRNA mediated KD was not able to completely block Dicer expression in SGBS preadipocytes. As most expressed miRNAs are reportedly cell-type enriched, residual Dicer activity could have been sufficient to enable processing of a majority of the most relevant miRNAs for SGBS preadipocytes [134]. In addition, miRNAs were provenly shown to have a high stability which can reach from several hours to a few days [135,136]. This would be perfectly in line within the time frame of transfection and harvesting of the preadipocyte samples (48 hours). Moreover, it is important to note, that the Dicer mediated miRNA maturation represents the canonical pathway of miRNA biogenesis. Yet, many miRNAs can also be processed in a Dicer independent manner [36,137]. More specifically, Kim et al. 2016 describe different clusters of miRNAs which do not rely on Dicer processing.

Additionally, it should be taken into consideration that given the small sample size of three replicates, the likelihood that possible miRNAs involved in this metabolic phenotype are missed, must be assumed as relatively high – which could at least explain why the number of downregulated miRNAs in SGBS

preadipocytes was generally low compared to what could have been expected according to previously published data.

However, to find a miRNA target which could help to explain the metabolic phenotype that we observed, we further analyzed all differentially regulated miRNAs and sorted for p-value (< 0.05), fold change (> 0.7 or < 2) and base mean expression (> 40) and performed additional qPCR evaluation. As a result, two specific miRNAs, namely miR-18a-3p and miR-543, emerged following this procedure. Whereas miR-18a-3p was found to be significantly upregulated, miR-543 was measured drastically diminished upon Dicer KD. Both miRNAs were previously reported to be involved in cellular glucose metabolism. In particular, decreased levels of miR-18a were measured in serum of T2D patients and negatively correlated with homeostasis model assessment of insulin resistance (HOMA-IR), while its overexpression led to improved insulin sensitivity in 3T3-L1 and C2C12 cells [138]. MiR-543, on the other hand, was downregulated in kidney tissue of mice with diabetic nephropathy as it compensated for high glucose induced tissue damage [139].

Yet, neither miR-18a-3p mimic transfection nor inhibitor transfection of miR-543 were able to reproduce the metabolic phenotype observed in Dicer deficient SGBS preadipocytes. Moreover, preliminary data investigating the combination of both miRNA conditions did also not show any metabolic alteration. All these datasets suggest that it is unlikely that these two miRNAs are responsible for the effects examined in glucose metabolism of Dicer deficient human SGBS preadipocytes – at least not solely. Especially for miR-543 this was rather not what was expected as additional literature provided evidence that it enhances glycolysis in osteosarcoma cells via HIF-1α [140]. As a consequence, the assumption was that a lack of miR-543 would cause impaired glucose uptake and thereby mimic the phenotype investigated in Dicer KD in SGBS preadipocytes.

Besides this, it is absolutely important to question, if not one but rather multiple miRNAs jointly contribute to the impairment in cellular glucose uptake in Dicer deficient preadipocytes. This would be in line with observations made in human endothelial cells [143]. Here, Dicer silencing caused the reduction of two miRNAs (miR-222 and miR-221) that are important for proliferation of this particular cell type. Interestingly though, mimic transfection of both, miR-222 and miR-221, was not sufficient to abolish the antiproliferative effect caused by the absence of Dicer. The authors assume that although miR-222 and -221 are essential for proliferation, these two miRNAs may rather regulate cellular growth of endothelial cells in a cluster with other miRNAs.

Thus, at this point, it remains unclear how impaired Dicer levels affect cellular glucose uptake. Yet, it needs to be kept in mind that pharmacologically enhancing Dicer mediated miRNA processing via enoxacin triggered glycolysis as it upregulated GLUT1 expression. This suggests a potential involvement of miRNAs in preadipocytes' glucose metabolism via GLUT1. However, increased miRNA processing induced by enoxacin still needs to be confirmed in this context. To further underline this, it would be therefore interesting to test, whether a chemical inhibition of Dicer mediated miRNA processing via xanthone derivates - as outlined by Murata et al. 2013 and 2016 - would lead to similar results as compared to siRNA induced loss of Dicer activity. Moreover, the use of enoxacin also in Dicer deficient cells would be a feasible approach to confirm whether enoxacin increases glucose uptake of preadipocytes in a Dicer dependent manner.

Also important to note is that Dicer might as well affect preadipocyte metabolism in a miRNA independent pathway. Dincbas-Renqvist et al. 2009 for example demonstrated that Dicer undergoes protein-protein interaction with 5-lipoxygenase (5-LO), a commonly known inflammatory mediator. Since a proinflammatory environment is associated with hampered glucose metabolism, interaction with 5-LO might contribute to lowered glucose incorporation by promoting inflammation [144]. This interaction raises the question of whether additional, so far unknown Dicer binding partner might have a pivotal function in this setting. Also, other processes in which Dicer is known to be involved such as processing of RNA-related fragments, pre-ribosomal RNA or in DNA damage repair should be part of future investigations as it has not been analyzed in this thesis.

5. Summary and outlook

With regard to the increasing global prevalence of obesity, there is a tremendous need to further delineate metabolic processes to identify potential targets for future treatment options.

This being said, Dicer is a well-studied enzyme in miRNA biogenesis, that was additionally shown to have implications in metabolic processes [63,66,69,80]. Although its role has been intensively examined in AT of mice, its metabolic importance remains rather unclear in the human setting.

This study provides strong evidence that Dicer is a crucial factor in human AT metabolism as its relative absence affected cellular glucose uptake in human pre-/adipocytes to an enormous extent. In particular, Dicer deficient cells revealed remarkably diminished glucose uptake capacity, as shown in two different human model systems. Additional proof was offered by chemical induction of Dicer activity via enoxacin which generated the opposite effects to the LOF approach. Furthermore, the impairment in glucose incorporation was even more prominent in preadipocytes, whereas treatment with BAY 876 was able to vanish metabolic alterations. This led to the identification of GLUT1 as key regulator in this metabolic phenotype.

In contrast to previous scientific reports, we described that Dicer KD does not seem to have a crucial role in preadipocyte-to-adipocyte differentiation, at least not in the human cell model system, as evaluated in SGBS and hMADS cells. Yet, interestingly, and very much like in the murine situation, Dicer deficient cells appeared to favor a preferably white phenotype. That is, cellular UCP1 levels were comparably low, whereas WAT associated leptin abundance increased. However, this phenotype was not demonstrated to have any functional relevance as cellular respiration remained unaffected. This was particularly shown by unchanged mitochondrial content and unaltered oxygen consumption upon siRNA mediated Dicer KD.

The expectation was that intrinsic miRNA levels might be the reason for the cellular phenotypes observed. Surprisingly, miRNA sequencing data only showed few miRNAs to be altered, including miR-543 and miR-18a-3p. Investigation of these two miRNAs in metabolic assays did not lead to the identification of the key mediator miRNA which is responsible for inducing decreased glycolytic activity in human SGBS cells.

In summary, this work underlined the importance of Dicer as a promising target molecule in metabolic processes. Future studies should further focus on identifying how Dicer mediates glucose uptake in pre-/adipocytes in order to create new strategies for obesity treatment.

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Appendix

pMuSE Dicer sgRNA 1 – plasmid sequence – single sgRNA approach

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pMuSE Dicer sgRNA 2 – plasmid sequence – single sgRNA approach

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pMuSE Dicer sgRNA 1+2 – plasmid sequence – double sgRNA approach

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pMuSE non-targeting sgRNA 1 – plasmid sequence

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pMuSE non-targeting sgRNA 2 – plasmid sequence

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pMuSE UCP1 sgRNA 1 – plasmid sequence

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AGCCTGCTGAAGCAGGCTGGTGACGTCGAGGAGAATCCTGGCCCC

Statutory declaration

I hereby declare that I wrote the present dissertation with the topic:

The role of Dicer in adipocyte differentiation and metabolism

independently and used no other aids that those cited. In each individual case, I have clearly identified the source of the passages that are taken word forword or paraphrased from other works. I also hereby declare that I have carried out my scientific work according to the principles of good scientific practice in accordance with the current „Satzung der Universität Ulm zur Sicherung guter wissenschaftlicher Praxis“ (Rules of the University of Ulm for Assuring Good Scientific Practice).

Ulm, 21. January 2023

(Signature)

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EDUCATIONAL BACKGROUND

10/2014 – 04/2018 **Molecular Nutritional Sciences, Master of Science**
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09/2015 – 12/2015 **Nutritional Sciences, Master of Science**
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10/2011 – 10/2014 **Nutritional Sciences, Bachelor of Science**
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10/2010 – 10/2011 **Nutritional Sciences, Bachelor of Science**
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09/2009 – 03/2010 **Ecotrophology, Bachelor of Science**
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09/1999 – 09/2008 **German High School/University Entrance Diploma**
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PROFESSIONAL SCIENTIFIC EXPERIENCE

since 04/2018 **PhD student in Molecular Medicine**
Ulm University Medical Center
Cellular characterization of CRISPR/Cas9 and siRNA mediated loss of function in Dicer protein in human adipose tissue cells including assessment of metabolic dynamics by i.a. extracellular flux analysis, expressional analysis as well as the impact on cellular microRNA abundance

04/2017 – 03/2018 **Master's degree candidate**
Ulm University Medical Center
In-depth metabolic phenotyping of UCP1 overexpressing human adipocytes including sterile cell culture of human pre-/adipocytes, expression analysis via semi-dry Western Blot and qPCR, assessment of oxygen consumption rates and investigation of glucose metabolism and insulin sensitivity

10/2015 – 01/2016 **Research Scientist**
Wisconsin Institute for Medical Research
Successful establishment of orthotopic human multiple myeloma xenografts in NSG mice and ELISA-based quantitative analysis of immunoglobulins in mice sera

03/2015 – 07/2016	**Student Employee** **Institute of Biochemistry and Nutrition, University of Hohenheim** Mentoring of laboratory courses, thesis mentoring and correction of exams and laboratory reports
03/2014 – 10/2014	**Research Intern** **German Cancer Research Center** Successful investigation of differentially expressed microRNAs in colorectal cancer patient tissue and metabolic analysis of urinary samples using H-NMR
09/2013 – 02/2014	**Bachelor's degree candidate** **University of Hohenheim** Introduction of stable expression of intracellular Osteopontin in HEK293 cells

PUBLICATIONS

Tews, D.*, **Pula, T.***, Funcke, J. B., Jastroch, M., Keuper, M., Debatin, K. M., Wabitsch, M., & Fischer-Posovszky, P. (2019). Elevated UCP1 levels are sufficient to improve glucose uptake in human white adipocytes. *Redox biology*, *26*, 101286. https://doi.org/10.1016/j.redox.2019.101286

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