Adipose Tissue Biology and the Development of Type 2 Diabetes Mellitus

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1. Dedication

"DER HERR: Hast du mir weiter nichts zu sagen?
Kommst du nur immer anzuclagen?
Ist auf der Erde ewig dir nichts recht?
Die Menschen dauern mich in ihren Jammertagen,
Ich mag sogar die armen selbst nicht plagen.
DER HERR: Kennst du den Faust?
Mephistopheles: Den Doktor?
DER HERR: Meinen Knecht!
Mephistopheles: Fürwahr! er dient Euch auf besondere Weise.
Nicht irdisch ist des Toren Trank noch Speise.
Ihn treibt die Gärung in die Ferne,
Er ist sich seiner Tollheit halb bewusst;
Vom Himmel fordert er die schönsten Sterne
Und von der Erde jede höchste Lust,
Und alle Näh und alle Ferne,
Befriedigt nicht die tiefbewegte Brust."

Dialogue from Faust I (Johann Wolfgang Goethe)

Herewith, I dedicate this thesis to my friend Mohamed, Johann Wolfgang and my Professor Christian. One gave me friendship, one idealism and one allowed me to challenge all of it.
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2. Abstract

As of 2014, the World Health Organization estimated 1.9 billion overweight people, of which 600 million were obese. Yearly, 3.4 million people die from overweight and obesity, when excluding the effects of associated diseases. Overweight is one of the strongest risk factors for development of Type 2 Diabetes Mellitus (T2DM), of which the treatment accounts for 12 percent of worldwide total health expenditure (2015). The individual tragedies that obesity causes are even worse than the estimated worldwide health costs of 673 billion dollars. Since numbers of obesity incidences still rise despite of dietary guidelines being formulated and implemented, further basic research on adipose tissue and diabetes is urgently required.

Scientists continue to search for a causal link between obesity and the development of T2DM. Besides literature reviewing the role of a certain type of adipose tissue in mediating the anti-obesity effect of fibroblast growth factor 21 (FGF21), this thesis is about the adipose tissue organ’s plasticity and its role in the development of T2DM. First, I addressed very basic scientific questions about the adipose organ through the quantification of adipocyte cell numbers and their character by applying a newly developed quantitative polymerase chain reaction based method. Using this tool, we could establish and improve the tamoxifen protocol of Adipoq-tracer and Ucp1-tracer-depleter mouse models.

Secondly, we aimed to prove that induction of adipose tissue specific insulin resistance causes development of T2DM. For this purpose we generated a CreERT2-loxP genetic mouse model of tamoxifen inducible fat-specific Insulin receptor knockout (iFIRKO). Inducing insulin resistance in lean and obese adult mice, we found a temporary development of T2DM that was reversed already 3 weeks after the knockout, but adipose tissue mass remained reduced. iFIRKO mice are long term protected from the obesogenic effect of high fat diet.

Furthermore, we prevented development of hyperphagia and polydipsia in iFIRKO mice with two antioxidants Apocynin and N-acetylcystein (Nac) and strongly reduced hyperglycemia by Apocynin alone. Finally, we could confirm food intake reduction and blood glucose lowering capacity of Apocynin and Nac in the hyperphagic obesity model of Leptin knockout mice. We conclude that insulin resistance in adipose tissue can protect from obesity, but also causes T2DM. This development of T2DM can be targeted downstream of the Leptin Receptor through the use of Apocynin and Nac.
3. Zusammenfassung


Darüber hinaus, konnten wir die Entwicklung von Hyperphagie und Polydipsie in iFIRKO Mäuse durch die Behandlung mit zwei Antioxidanzen Apocynin und N-Acetylcystein (Nac) verhindern und die Hyperglykämie alleine durch Apocynin stark reduzieren. Schliesslich konnten wir den Effekt der Antioxidanzen auf die Futteraufnahme und die Reduktion des Blutzuckerspiegels in einem Maus Modell der Hyperphagie-induzierten Fettleibigkeit der Leptin knockout Maus bestätigen.

Wir schlussfolgern, dass die Induktion von Insulinresistenz im Fettgewebe von Fettleibigkeit schützen
kann, aber auch T2DM verursacht. Diese Entwicklung des T2DM kann durch Apocynin und Nac, dem Leptin Rezeptor nachgeordnet, beeinflusst werden.
4. Introduction

4.1. Adipose tissue and sexual selection

Humans, characterized by Foster and colleagues as eusocial vertebrates [Fos05], underly the evolutionary principle of group selection [Dar59, Now10]. Similar to other species, sexual selection seems to be one of human being’s main biological tasks [Dar71]. Although the perception of beauty is highly subjective, there are records of western cultural trends that were influenced by ideals of society. The volume of adipose tissue is decisive for the evaluation of beauty.

Throughout the history beauty ideals have drastically changed. While during Italian renaissance (1400-1700), women with ample bosoms, rounded stomach and full hips represented the beauty ideal, the roaring twenties (1920s) of the 20th century, changed beauty ideals of western societies long lasting. In the 1920s flat-chested women with a downplayed waist, short bob hairstyle and a boyish figure were trending. After the intermittence phase of Hollywood’s golden age (1930s-1950s) and their curvy women with hourglass figure, large breasts and a slim waist, the post-modern beauty ideal was strongly influenced by thin women with long slim legs and adolescent physique in the swinging sixties (1960s) and the extremely thin heroin chic women of the 90s (1990s) [Gar80]. The post-modern beauty ideal can be described by healthy skinniness, flat stomachs, large breasts and thigh gap. Male beauty ideals are less well studied, but similar trends towards reduced body fat have been reported. Both gay and heterosexual men want to be muscular and thin, and their self-esteem correlates with both factors [Tig07]. This trend of increasing muscularity and decrease in fat mass was found in a systematic study of the Playgirl male beauty magazine editions from 1973 to 1997 [Lei01]. While the muscularity is male specific, the trend for reducing body fat mass is common between male and female beauty ideals, as has been indicated by a study of Playboy magazine centerfold models and Miss America Pageant contestants from 1959 to 1978 and follow up studies between 1979 and 1998 [Gar80, Wis92]. One might say as an expression of post-modernity as such, this beauty ideal is barely reached in western society as exemplified by the United States of America [Mar95].
4.2. Overweight, Obesity and T2DM

It is said about the Spartan lawmaker Lycurgus (700 BC), that he recognized overweight as a health threat, which he counteracted by decreeing gymnastic exercises for overweight men until they would become lean again [Sch26]. The body mass index (BMI) describes the ratio between weight and height\(^2\), its unit is kilogram over square meters and more than 90 % of its variation can be explained by body fat [Que69, Key72, Gar85]. In 2014, the world health organization estimated 1.9 billion people to be overweight worldwide (BMI \(\geq 25\) kg/m\(^2\)), which represented 39 % of women and 38 % of men aged above 18 years. The estimated 600 million obese (BMI \(\geq 30\) kg/m\(^2\)) humans represent 14.9 % of the female and 10.8 % of the male population. In these incredible times, most of the world population lives in countries, the WHO concludes, in which overweight or obesity kill more people than underweight [Org13].

In 2008, the Obesity Society supported the classification of obesity as a disease and thereafter the American Medical Association Council officially ended a long controversy about the disease state [Hes01, A.13]. Linguistically it is questionable to say that it can kill humans, although the hypothesis that obesity can cause further deterioration of a human being’s health that leads to his death is widely accepted. Having an ideal BMI between 20 and 24.9 kg/m\(^2\) is associated with the lowest risk of death in non-smoking adults [Gon10].

For 2015, the Global Burden of Disease 2015 Obesity Collaborators identified BMI as a risk factor for chronic diseases including cardiovascular disease, diabetes mellitus, chronic kidney disease, cancer, and an array of musculo-skeletal disorders [Afs17a]. Epidemiological studies found a strong correlation between increase in BMI and the development of Type 2 Diabetes Mellitus (T2DM) and other diseases [Col16]. T2DM is diagnosed, when glycated hemoglobin (HbA1c) concentration exceeds 6.5 percent of all hemoglobin, or glucose concentration after a fasting period of 8 hours exceeds 7 mmol/L, or the blood glucose concentration 2 hours after oral 75 g glucose uptake exceeds 11.1 mmol/L [Ass17].

In 2005, overall 58 million humans died. Cardiovascular diseases and diabetes caused the death of 18.6 million (32 % of total) humans [Abe07]. In 2010, excluding the effect of its associated diseases, overweight and obesity alone were estimated to cause 3.8 % of disability-adjusted life-years (DALYs) and the death of 3.4 million people worldwide, which is proportional to 3.9 % of years of lost live [Ng14]. Although human tragedy that comes from diseases cannot be counted in money, there are some estimates of their impact on economical life in developing and developed countries. Between 2006 and 2015, Abegunde et al. estimated in 23 developing countries the cost of not reducing the risk for BMI correlated chronic diseases as cardiovascular diseases and diabetes to 84 billion dollars [Abe07].

The International Diabetes Federation (IDF) regularly publishes world wide estimates for diabetes [Atl15]. In its most recent report, they estimate 415 million people to be diabetic and forecast that in 2040 this number could increase further to 642 million. People of the world were expected to spend 12 percent of their total health expenditure on diabetes in 2015, which translated into 673 billion dollars. In developed countries the cost of Diabetes are higher than in middle and low income countries. The American Diabetes Association published in 2013 a report about the costs of Diabetes in the USA of
2012. The total estimated cost of 245 billion dollar for diagnosed diabetes, consists of 176 billion dollar direct medical costs and 69 billion dollar estimated reduced productivity cost [Ass13]. It was estimated that, one fifth of the total US-American health care spending can be attributed to people with diagnosed diabetes.

Foreseeing this trend in 1980, the US departments of Agriculture and Health and Human Service created a federal nutritional policy and initiated a nutritional education campaign. The courageous attempt to tackle the problem of overweight by defining "Dietary Guidelines for Americans" in 1980. These nutritional guidelines, which are revised every 5 years, layed the foundation for international WHO nutrition policy [Org92], but could not prevent today’s obesity pandemic [Rot04]. Between 1980 and 2015, the worldwide prevalence of overweight increased from 29.8 % and 28.8 % to 38 % and 36.9 % in women and men (≥20 years), accordingly [Ng14].

4.3. Energy Homeostasis and Obesity Development

In healthy humans one can observe a strict regulation of energy homeostasis, which is the homeostatic regulation of food intake and energy expenditure [Sch04, Rya12]. Assuming that energy homeostasis would work the following question seems reasonable: How can overweight and obesity develop? Gaining weight by just increasing the caloric intake is counteracted by negative feedback from adipose tissue [Ken53, SIM68, Dér92, Hag99]. Each individual has certain weight boundaries that the central nervous system defends by adjusting energy expenditure and regulating food intake [Kee86, Far11]. Energy homeostasis describes the process of matching energy intake to energy expenditure. Energy intake is tightly controlled by an orchestrated action of many secreted factors in the periphery that act on neuronal networks and through neuronal networks themselves. This topic is reviewed by Morton et al. [Mor14], but for this thesis it seems necessary to point out the combined role of Leptin and Insulin on suppressing food intake.

The role of secreted factors in the regulation of energy homeostasis was first proven by parabiosis studies [Her59, Col73, Par77, Har84]. The most potent secreted factor that regulates food intake was found to be Leptin, which is secreted by the Adipose Tissue [Zha94], suppresses the orexigenic NPY and stimulates the anorexic POMC neurons [Bas99, Ste95]. Disrupting the brain’s leptin signal induces hyperphagia and weight gain [Coh01]. Mutations in Leptin receptor happen very rarely in humans, but a few rare cases that developed severe obesity have been reported [Mon97]. More commonly is the mutation of the Melanocortin Receptor 4 (MC4R), which was found to be present in around 6 % of obese patients and is supposed to be the most common monogenic obesity factor [Far03]. The mutation of the Leptin receptor or other more common monogenic factors cannot explain the high incidence rate of obesity. Overweight and obesity is accompanied by a raise of the body fat level that the organism defends by adjusting its energy homeostasic set value [Sim73, Dia92, Lei95]. What are the molecular factors that mediate adjustment of the energy homeostatic set value? Leptin resistance is defined as a state that renders the body unresponsive to the anorexic effect of exogenous Leptin. While
circulating Leptin levels increase in obesity models of humans and rodents, Leptin shows a reduced effect on neuronal circuits that regulate food intake [Mye10]. Similar to Leptin, Insulin concentration increases with development of overweight. Not as potent as Leptin, Insulin can reduce food intake via similar neuronal circuits [Woo79]. Insulin inhibits AGRP neurons [Spa00], synergizes with Leptin signal in POMC neurons [Hil10] and agonizes the effect of satiety signaling molecule Cholecystokinin (CCK) [Fig86].

4.4. Inflammation and development of T2DM

Chronic hyperphagia induces fat accumulation that exceeds healthy levels. This leads to overweight, which precedes the diagnosis of T2DM in humans [Loo01]. One common hypothesis links obesity with development of T2DM by excessive fat accumulation triggered inflammation in the adipose tissue, which in turn reduces insulin sensitivity [Hot93, Kah06]. Pro-inflammatory factors as IL-1 and TNFa induce lipolysis, while reducing de novo-fatty acid synthesis in adipocytes and their expression is increased in obese adipose tissue [Pat86, Pri86, Hot95, Moo01, Nis09]. The regulation of insulin receptor signaling by inflammatory factors is supported, by the finding that TNF-a regulates peripheral glucose uptake [Hot93]. Lipid overloaded adipose tissue secretes Monocyte Chemoattractant Protein-1 (MCP-1) and IL-6, which recruits macrophages and thereby worsen its inflammation [Wei03, Xu03]. The key regulator of inflammation Nuclear Factor-kB (NF-kB) is activated in obese adipose tissue and the knockout of NF-kB activator IkB kinase b (IKK-b) in myeloid cells was shown to rescue high fat diet fed mice from peripheral insulin insensitivity [Ark05]. The involvement of NF-kB signaling in development of obesity and insulin insensitivity was further confirmed by the protection of IkB kinase e (IKKe) knockout mice and the improvement of obesity-related metabolic disfunctions in mice treated with inhibitor of TBK1 and IKKe [Chi09, Rei13]. IKK-b phosphorylates inhibitory serine sites of insulin receptor substrate 1 (IRS1) and 2 that decreases positive tyrosine phosphorylation and therefore sensitivity of insulin receptor [BH09]. Another route of interference with insulin signaling has been shown for TNF-a signaling via IKK-b and the suppression of Tuberous Sclerosis 1 (TSC1) [Lee08]. Besides, locally released saturated free fatty acids that are increased in concentration in between the meals can activate TLR4 in adipocytes [Shi06]. There is evidence that inflammation induces a major negative regulator of insulin sensitivity protein-tyrosine phosphatase 1B (PTP1B) by upregulating its expression [Zab08]. Last but not least, genetic deletion of Fas receptor (FasR, CD95) also in adipose tissue was shown to play a role in mediating adipose tissue inflammation and protect from diet-induced insulin insensitivity [Wue10]. Unfortunately most genetic mouse models that aim at suppressing adipocyte inflammation by genetic deletions have been performed with unspecific driver lines (aP2/FABP4-Cre) that also express Cre in many other tissues besides adipose tissue (e.g. macrophages) [Sab08, Wue10].
4.5. New hypothesis in fight against T2DM and Antioxidants

Insulin insensitivity of adipose tissue reduces lipid uptake and induces lipolysis, which increases concentration of free fatty acid (FFA) in blood. Developmentally, disruption of insulin signaling cascade in adipose tissue leads to lipoatrophy and diabetes development [Geo04, Sof16]. The observation that body weight reduction strongly correlates with time of T2DM diagnosis is in line with the supposed insulin insensitivity of adipose tissue [Loo01]. The hypothesis that insulin resistance is a physiological defense mechanism against metabolic stress, questions the dogma that insulin resistance has to be counteracted in obese T2DM patients [Sal12, Nol15]. It is very controversial whether T2DM might be the failure of a defense mechanism against cardiovascular problems. As the Action to Control Cardiovascular Risk in Diabetes (ACCORD) study showed, intensive glycemic control in T2DM patients was associated with greater weight gain and increased mortality rate [DSG08, Nol13]. Since oxidative stress has been proposed to trigger Insulin resistance [Hou06b, And09, Bas09], the initiation of a cellular antioxidant response, could have beneficial effects on stressed vasculature [Hoe09]. Muscle specific over-expression of catalase, which protects the cell from reactive oxygen species (ROS) by catalyzing the conversion of H2O2 into H2O and O2, protected against HFD-induced insulin insensitivity [And09]. Although there is evidence for positive health effects of antioxidants (e.g. Nac [Ful02], Vitamin C [Hir00], Vitamin E [Cab93] and lipoic acid [Gol02]) for T2DM patients [For01], in 2007 the American Diabetes Association questioned the usefulness of antioxidants for treatment of T2DM [Ass07], because larger interventional trials with antioxidant food supplements could not confirm the proposed health benefit [Che06, Cze06, GP95, Liu06].

4.6. Central regulation of Obesity and T2DM

There is strong evidence for a brain-centered glucoregulatory system (BCGS) [Sch13b]. Inflammation of hypothalamus might be the consequence of lipid accumulation [Pos09]. Some authors propose the hypothalamus to be the starting site of obesity development, because in mice and rats on obesity-inducing high fat diet one can show inflammatory signaling first in the hypothalamus already after a few days [Tha12]. Within one week of HFD feeding neuronal injuries become evident in the Arcuate Nucleus [Tha12], while inflammation in the periphery was reported to appear later. With further development of obesity the hypothalamus injury worsens. Reducing hypothalamic inflammation by intracerebroventricular (icv) injection of immuno-neutralizing antibodies against TLR4 or TNF impacted peripheral insulin resistance as one of the most important symptoms of obesity and T2DM [Mil12]. After the report that fibroblast growth factor (FGF) 21 acutely lowers glucose and sensitizes insulin resistant mice by acting on liver and adipose tissue [Xu09b], FGF21 was further shown to act centrally to increase energy expenditure and to promote weight loss [Owe14]. An insulin-independent glucose disposal enhancing effect of FGF19 that leads to blood glucose lowering of obese mice was also confirmed to be centrally mediated by icv injections. The strongest evidence for the central control of whole body glucose regulation however comes from a study of Scarlett et al., in which they show that
a single central injection of FGF1 can revert hyperglycemia in genetically obese mice (ob/ob and db/db) for weeks [Sca16].

### 4.7. Treatment strategies against Obesity and T2DM

A very ancient method to reduce food intake and prevent overweight is fasting. Patients with increased hunger show low compliance to this ancient method. Every year, the American Diabetes Association (ADA) publishes the standards of medical care in diabetes, which consist of the most current evidence-based recommendations for diagnosis and treatment of adults and children with all forms of diabetes [Ass17]. According to the standards of medical care in diabetes, a patient should be actively included in the development of a diabetes management plan. This plan also includes lifestyle management and psychosocial care beside the prescription of drugs to reach the goal of glycemic targets of an HbA1c of smaller than 7 percent. As part of the pharmacologic therapy for T2DM, it is recommended to initially treat patients with metformin. Metformin increases peripheral insulin sensitivity, but also reduces food intake and mildly reduces body weight in obese and diabetic patients [Lee98]. Treatment with Insulin should be considered when HbA1c exceed 10 percent and or blood glucose concentrations exceed 16.6 mmol/L. In case non insulin monotherapy at a maximal tolerable dose fails to achieve the HbA1c target, the ADA recommends the addition of GLP-1 receptor agonists as a second oral drug. For patients with cardiovascular diseases and long-standing failure of glycemic target, empagliflozin or liraglutide are recommended, because they have been shown to reduce cardiovascular mortality. The ADA estimates that 33-49 percent of patients do not meet their tragets of controlling hyperglycemia, blood pressure, or cholesterol, beside the advances in diabetes care schemes [Ass17].

The most effective [Por95], but also most coercive treatment of obesity and diabetes is Bariatric surgery [Vet09]. The term Bariatric surgery unites many different surgery types including laparoscopic adjustable gastric banding (LAGB), vertical banded gastroplasty (VBG), biliopancreatic diversion (BPD) and Roux-en-Y gastric bypass (RYGB). A chronic mean loss of excess weight was reported to vary between the different surgery types, but is around 61 percent across all procedures [Buc04,Sha06,Vet09]. In a randomized control trial, Bariatric surgery resolved diabetes in 73 percent of cases [Dix08].
5. Aim of this work

This thesis can be divided into three parts. These parts represent manuscripts that are already published, or have been submitted for publication.

The first part is a mini review on the question of whether FGF21 needs brown adipose tissue to mediate its therapeutic effect.

The second part of this thesis is an accounting of adipose tissue and its plasticity. We applied in vivo genetic tracing methods in mice in order to quantify all adipocytes, brown or brite/beige adipocytes.

The final part of this thesis aimed at understanding the physiological implication of malfunctioning adipose tissue in disease development. The theory that explains how obesity causes Type 2 Diabetes Mellitus (T2DM) is still in need of additional evidence. As extensively discussed in the introduction section, obesity has been linked to adipose tissue inflammation, which in turn was shown to induce insulin insensitivity. Based on these assumptions, I tried in this thesis to give causal evidence of how obesity could cause T2DM. We developed a genetic mouse model that would allow us to induce insulin resistance only in adipose tissue. Finally, we aimed at interfering with the development of T2DM by antioxidant treatment in this mouse model.
6. FGF21, energy expenditure and weight loss - How much brown fat do you need?

6.1. Contributions
Leon G. Straub and Christian Wolfrum
LGS and CW wrote and revised the manuscript

6.2. Abstract
Background: Fibroblast growth factor 21 (FGF21) belongs to the large family of fibroblast growth factors (FGFs). Even though FGF signaling has been mainly implicated in developmental processes, recent studies have demonstrated that FGF21 is an important regulator of whole body energy expenditure and metabolism, in obesity.

Scope of review: Given the fact that obesity has developed epidemic proportions, not just in industrialized countries, FGF21 has emerged as a novel therapeutic avenue to treat obesity as well as associated metabolic disorders. While the metabolic effects of FGF21 are undisputed, the mechanisms by which FGF21 regulate weight loss have not yet been fully resolved. Until recently it was believed that FGF21 induces brown fat activity, thereby enhancing energy expenditure, which concomitantly leads to weight loss. Novel studies have challenged this concept as they could demonstrate that a part of the FGF21 mediated effects are retained in a mouse model of impaired brown adipose tissue function.

Major conclusions: The review illustrates the recent advances in FGF21 research and discusses the role of FGF21 in the regulation of energy expenditure linked to brown fat activity.

6.3. Introduction
Higher energy intake than expenditure causes excessive accumulation of fat and leads to the development of obesity and associated metabolic disorders, which has reached epidemic proportions not only in developed countries but worldwide [Pre05]. Furthermore, obesity can lead to the development of various co-morbidities, like type 2 diabetes, cardiovascular diseases, neurodegenerative disorders and
some types of cancers, that severely impact life quality and expectancy [Mus99, Cal03]. While treatments for the obesity related co-morbidities are available, albeit with varying success, no therapeutic avenues, with the exception of highly invasive bariatric surgery, exist to treat obesity directly. Several approaches that target the central nervous system to reduce food intake have failed in this context due to massive side effects [Li11].

As it can both store and burn calories, adipose tissue has gained renewed attention recently as a possible target for the treatment of obesity due to its Janus-faced character. In this context, adipose tissue can be functionally divided into two main depots, namely white and brown adipose tissue. White adipose tissue is characterized by the presence of large unilocular adipocytes whose main function is the storage of energy and the release of fatty acids during fasting and starvation. Brown adipose tissue is characterized by the presence of smaller cells with multilocular lipid droplets. In contrast to that of white adipose tissue, the primary function of brown adipose tissue is to produce heat, a feature which is achieved through the function of uncoupling protein 1 (UCP1). UCP1 effects a proton leak from the mitochondrial intermembrane space to the mitochondrial matrix, effectively short circuiting the electron transport chain [Can04]. Thus, as its name suggests, UCP1 uncouples oxidation from phosphorylation, driving a futile cycle that produces heat. In addition, to classical brown adipocytes, which are mainly localized to the interscapular depot, other brown like adipocytes, termed either beige or brite adipocytes, can be found in predominantly white adipose tissue depots [Bar14, Ros14]. These cells have received substantial attention in recent years due to the fact that their appearance is dependent on induction. The origin of these cells, however, remains a matter of debate [Ros13, Wan13, Lee15].

During cold exposure the sympathetic system activates brown fat via secretion of catecholamines that stimulate β3-adrenergic receptors on the adipocyte surface. In rats, brown adipose tissue has been shown to be responsible for over 60 percent of the excess heat production during cold stimulation [Fos79]; also in newborn humans, the role of brown fat in protecting against hypothermia has been appreciated for a long time [Hea72, Lea89]. In addition, several independent groups recently demonstrated that brown fat is also activated by cold and contributes to heat production through non-shivering thermogenesis in adult humans [ML09, Lan13, Sai09, Vir09, Cyp09]. Yoneshiro et al. showed in 2013 that repeated cold exposure leads to recruitment of brown fat in humans and increases cold-induced gains of energy expenditure [Yon13]. Further corroborating a functional role for brown fat in the adult human was gained from a study by Cypess et al., which demonstrated that β3-adrenergic receptor agonists can not only increase glucose uptake of brown fat but also increase resting metabolic rate by approximately 13% [Cyp15].

Brown fat activation is achieved by a variety of factors, including but not limited to natriuretic peptide A, β3-adrenergic receptor agonists and other circulating factors such as fibroblast growth factor 21 (FGF21). Until recently, the energy expenditure enhancing and weight loss effects of FGF21 were thought to be mediated mainly by brown fat thermogenesis, because FGF21 has been demonstrated to directly stimulate thermogenic gene expression and to induce browning of white fat. Thus, FGF21 seemed to be a suitable candidate for both the recruitment of brown cells in the white fat, as well as for the activation of endogenous brown fat.
6.4. Identification of FGF21

FGF21 is a member of the prominent fibroblast growth factor (FGF) superfamily, which currently encompasses 22 members [Kha09].

Based on various genetic mutations, FGFs were first implicated in embryonic development, regulating proliferation and differentiation as well as organ morphogenesis. The spectrum has been expanded recently, and it is now accepted that FGF signaling plays an important role in the pathogenesis of several diseases. This is especially true for FGF21, which has emerged as a new therapeutic target for the regulation of whole body metabolism.

FGF21 was first cloned in 2000 as a novel hepatokine with unknown function [Nis00]. Only five years later it was shown by the Lilly Research Laboratories that FGF21 is an important factor regulating glucose uptake [Kha05] as FGF21 knockout mice exhibit a mild weight gain, increased adipocyte size and slightly impaired glucose homeostasis [Bad09]. Furthermore, when challenged with a ketogenic diet, FGF21KO mice develop hepatosteatosis and gain weight concomitant with an impairment in ketogenesis and glucose homeostasis, as well as a downregulation of PGC1α and PGC1β both in liver and white fat. Conversely, systemic injection or transgenic overexpression of FGF21 leads to protection from diet-induced obesity, concomitant with a significant reduction in blood glucose and triglyceride levels [Kha05,Xu09a]. Interestingly, FGF21 overexpression also causes a marked increase in food intake under chow and high fat conditions, suggesting that either energy expenditure and/or nutrient uptake in these animals is induced. Taken together, these facts identify FGF21 as a major regulator of metabolic utilization of energy containing metabolites; therefore FGF21 is considered a novel therapeutic target for the treatment of obesity and its associated metabolic disorders [Bar14,Bee09,Liu15,Yon15,Ito14].

6.5. Regulation of FGF21 expression

FGF21 is expressed in liver, pancreas, fat tissue and muscle [Nis00]. In adult humans, fructose ingestion acutely stimulates circulating FGF21 levels [Dus15] and in liver, FGF21 expression is induced after birth in response to intake of milk during suckling [Hon10] through the action of PPARα. Due to its high expression in brown adipose tissue several studies have addressed the regulation of FGF21 expression and secretion in response to activation of this organ. For example, Hondares et al. showed that higher expression leads to increased secretion by analyzing arteriovenous differences in FGF21 concentration across interscapular brown fat [Hon11]. In contrast, work from Chartoumpekis et al. could not demonstrate an increase in FGF21 serum levels after an acute cold induction at the end of a 12 h starvation period [Cha11]. Keipert et al. compared FGF21 mRNA levels in muscle, heart, liver, subcutaneous white and brown fat from animals at different housing temperatures. Interestingly, at thermoneutrality, only liver seems to express FGF21 at a level comparable to that in activated brown adipose tissue. In Ucp1 genetically ablated mice (UCP1KO), FGF21 expression is induced in brown adipose tissue as well as in circulation [Kei15,Sam15]. This increase only occurs during cold adaptation, suggesting that brown fat communicates with other organs, maybe to compensate for the loss in thermogenic capacity. In
contrast, transgenic overexpression of UCP1 switches on FGF21 production in muscle suggesting that this phenotype is specific for brown fat and might be diametrically opposite in other organs [Kei14].

6.6. FGF21 signaling

FGF21 signaling utilizes the classical intracellular FGFR signaling pathway, but, in contrast to other FGFs, it does not bind directly to the FGFR. Instead, FGF21 mediates its function via the transmembrane receptor beta-Klotho, which is a FGFR co-receptor with a high expression in liver, fat and the central nervous system. Whole body deletion of beta-Klotho leads to a complex phenotype including developmental defects such as growth retardation and a counterintuitive increase in glucose tolerance and insulin sensitivity. In brown adipose tissue of these mice, however, UCP1 levels and body temperature are reduced, suggesting that beta-Klotho deficient mice have less energy expenditure than wild-type mice. Since it was shown that other FGFs such as FGF19 can also signal via beta-Klotho, this finding could be explained by a broader ligand spectrum for this surface receptor [Yan12]. The cell autonomous effect of FGF21 on adipocytes was first reported in 2005 by Kharitonenkov, et al. [Kha05], who demonstrated an FGF21 mediated induction of glucose uptake in mature adipocytes. In 2012, Ding et al. showed that aP2-cre mediated ablation of beta-Klotho leads to blockade of the acute insulin-sensitizing effect [Din12]. However, given the fact that aP2-cre mediated deletion targets multiple cells within the adipose tissue including adipocyte precursors, macrophages and endothelial cells [Sha13, Elm09, Mak01], it is unclear whether this effect is due to FGF21 signaling in adipocytes.

Since brown fat activity is tightly regulated by the sympathetic nervous system it remains unclear whether the systemic effects of FGF21 are due to a cell autonomous regulation of brown fat activity or if this effect is mediated via the CNS. A central effect of FGF21 has been demonstrated by intracerebroventricular (icv) injection of FGF21 [Sar10], which, similar to systemic FGF21 injection, led to increased energy expenditure, food uptake and insulin sensitivity. However, central FGF21 administration failed to reduce body weight and size of adipose tissue, suggesting that FGF21 effects are mediated both peripherally and centrally. Further evidence for the importance of FGF21 in regulating central pathways comes from animal models with hypothalamic- and hindbrain-specific genetic deletion of beta-Klotho in conjunction with an FGF21 overexpression model, which demonstrated that centrally or peripherally administered FGF21 increases sympathetic activation in brown adipose tissue and that this effect can be blunted by icv injection of an FGFR1 inhibitor (PD173074) [Owe14]. In addition to its endocrine function, FGF21 may have paracrine or autocrine functions, as fat-specific knockout of FGF21 inhibits browning of white adipose tissue in adaptive thermogenesis [Kle12].

6.7. FGF21 and brown fat uncoupling - new insights

Recent reports of Samms et al. and Veniant et al. [Sam15, Vén15] investigate the causal link between FGF21 increased browning and the loss of body weight. In accordance with previous results [Kle12],
the study by Veniant et al. demonstrates that FGF21 injection leads to an increase in brown adipose tissue mass concomitant with an increase in UCP1 expression in brown adipose tissue, while the effect in inguinal white adipose tissue is lost at thermoneutrality. Interestingly, the effect on weight loss is retained even when FGF21 is injected into UCP1KO mice, suggesting that the effects of FGF21 on weight loss are independent of brown fat activation. A closer look at the data, however, reveals that this is a simplified assumption. Scrutiny of the energy expenditure data suggests that in both studies the effect of FGF21 is reduced in UCP1KO mice, a finding that is especially evident in the study by Samms et al. in which FGF21 induced energy expenditure seems to be almost completely abolished in UCP1KO mice. Similar evidence can be gained from circulating metabolic parameters. While the effect of FGF21 on FFA and cholesterol are retained in UCP1KO mice, the effect on circulating glucose seems to be reduced or completely blunted. One important point to note is the effect of FGF21 on food intake in the context of Ucp1 ablation. The study by Veniant et al. observes a reduced induction of food intake in UCP1KO while the study by Samms et al. doesn’t observe an induction of food intake by FGF21 in wild type mice and actually reports a reduction of food intake in UCP1KO mice when treated with FGF21; the discrepancy could be due to differences in the dosing paradigm and housing temperature. Taking into account that UCP1KO mice have a reduced food intake upon FGF21 treatment, one would expect them show a reduction in body weight, a finding that is not observed. Therefore, it is possible that this difference is due to the effect of FGF21 on weight loss is mediated through the activation of brown adipose tissue.

In UCP1KO mice both brown fat FGF21 mRNA levels and circulating levels of FGF21 are increased significantly [Kei15]. Since some effects of FGF21 are abolished in UCP1KO animals the question remains how FGF21 reduces body weight independent of UCP1. A more detailed look at the regulation of energy balance is required. First of all, most tissues contribute to the metabolic rate and oxygen consumption of an organism and in the adult state the majority of calories ingested, are finally lost as heat. The contribution of different tissues to mammalian metabolic rate (independent of exercise) mainly depends on the genetic background and the environmental temperature. Based on the reported functions for FGF21 and given the literature of FGF21 and brown fat activation, the main question that arises from the recent studies is whether FGF21 acts as a physiological feedback molecule from thermogenically active tissues to integrate information about the available capacity and, if so, whether this alteration sensitizes other tissues to the action of FGF21.

To answer these questions it is necessary to review the literature on UCP1 mediated and brown fat mediated uncoupling in detail. It is well established that the genetic ablation of UCP1 induces obesity in mice living at thermoneutrality [Fel09]. Furthermore, overexpression of UCP1 under the aP2 and the human skeletal actin promoter protects against development of genetic and diet-induced obesity, respectively [Kop95,Kla05]. These data, which have been reviewed comprehensively [Can10], suggest that UCP1 function is the main driver of brown adipose tissue thermogenesis and that loss of UCP1 leads to a decrease in energy expenditure and concomitant obesity. However, several reports do not fit this paradigm. One example is a study by Liu et al. that demonstrates that loss of UCP1 can protect from diet induced obesity under certain conditions [Liu03]. Based on this controversy, several
studies have addressed the UCP1-independent adrenergic heat production with somewhat controversial results and opinions range from 20 to 50% of brown fat capacity [OO07, Low93] and it has been suggested that these effects could be due to UCP1-independent increases in thermogenesis in white adipose tissue [Gra03, Ukr06]. How these thermogenic processes are regulated is unclear at the moment; however, futile cycling as well as fatty acid mediated uncoupling of the mitochondrial membrane have been suggested as possible mechanisms. For example, in mammalian muscle, sarcolipin was identified as a regulator of non-shivering thermogenesis [Bal12]. Also, She et al. reported increased energy expenditure associated with the activation of a futile protein turnover cycle in peripheral branched-chain amino acid metabolism [She07]. Furthermore, simultaneous activation of lipogenesis and lipolysis as well as prolonged beta3-adrenergic receptor activation can induce futile cycling both in white and brown adipose tissue [Mot14, Kis14].

6.8. Conclusions

FGF21 induces weight loss and leads to reduction of some key metabolic parameters in UCP1KO mice. While some of these effects seem to be conserved and thus independent of UCP1, others seem to be blunted. In particular, the FGF21 mediated changes in food intake seem to be dependent on UCP1, suggesting a compensatory central regulation maybe through the induction of circulating FGF21. In line with this notion recent work by Schultz et al. might be important, as it suggests a fixed thermogenic capacity, which is tightly regulated by compensatory effects [Sch13a]. Thus, it is possible that under physiological conditions the effects of FGF21 are mainly mediated by brown adipose tissue through UCP1, while under abnormal conditions such as UCP1 ablation, these effects might be mediated via other tissues. In light of these findings, and although UCP1-dependent thermogenesis is the most potent and possibly the most relevant physiological regulator of non-shivering thermogenesis, other mechanisms that have been proven to play a role in thermogenesis should be considered in the context of FGF21 signaling.
7. An account of the adipose organ

7.1. Contributions

Leon G. Straub, Yael Rachamin, Dianne Dapito and Christian Wolfrum

LGS and CW designed research study
LGS, YR and DD conducted experiments
LGS and YR acquired data
LGS and YR analyzed data
LGS wrote the manuscript
CW, DD and YR revised manuscript

7.2. Abstract

Objective: Potential drawbacks of tamoxifen inducible CreERT2 recombination technology lead us to test and improve the CreERT2 induction protocol. Our improved protocol enabled the quantification of adipocyte numbers and characterization of adipose tissue depots’ plasticity.

Methods: We employed the Adipoq-tracer and Ucp1-tracer-depleter BAC transgenic mouse models, which express CreERT2 either under the adipocyte-specific, or under brown/brite cell-specific promoter elements and measured recombination of flstopfltdRFP transgene sequence at ROSA26-locus by quantitative polymerase chain reaction (PCR). To study the washout time needed for the tamoxifen dose of our choice, we ablated Ucp1-expressing cells with diphtheria toxin.

Results: In subcutaneous white adipose tissue (WAT), increased amounts of tamoxifen do not lead to higher numbers of recombined cells. After ablation of Ucp1 expressing-cells, it took only 2 weeks until the majority of newly formed brown adipocytes were no longer recombined. While brown adipocyte number in brown adipose tissue (BAT) reaches its maximum number within 1 week, the number for Ucp1-expressing cells in subcutaneous WAT continuously increases for at least 3 weeks.

Conclusion: The dosage of Tamoxifen required for efficient recombination can be reduced to 2 mg/mouse and one time gavaging. Tamoxifen washout for targeting newly formed cells with a second tamoxifen
stimulus after a first tamoxifen stimulus with 4 mg/mouse takes 2 weeks. In terms of the number of Ucp1-expressing cells, subcutaneous WAT shows a more long term adaptation to cold than BAT.

7.3. Introduction

Obesity and its associated diseases have reached pandemic scope [Afs17a]. A lack of effective treatment strategies against its causes renders further basic research into the development and plasticity of the adipose organ necessary. Since its development, genetic tracing experiments have greatly advanced our knowledge about the adipose organ. The first generation of adipocyte specific-tracing was performed with Cre-loxP technology [Ste81, Orb92]. Cre is a site-specific recombinase that very efficiently mediates deletion of a sequence in between two loxP-sites in vivo [Ana09, Raj96]. The distinct character of brown and white adipose tissue depots has been supported by results of the first Ucp1-Cre tracer experiments that employed lacZ expression to label Ucp1-positive cells [Mou01].

Furthermore, the tamoxifen-inducible Cre, also called CreERT2, advanced the genetic tool box by also enabling temporal control of genetic recombination [Fei97], which is required to prove the interconversion of adipocytes from the white to brite (or beige) cell type. Indeed, this transgene allowed for the detection of inter-conversion of brite adipocytes into white adipocytes and a subsequent re-establishment of brite identity upon chronic cold exposure intermitted by warm adaptation, thus supporting the idea of adipose tissue as an organ with high plasticity [Ros13].

Pulse chase experiments require the washout of tamoxifen to ensure the absence of labeling in newly-formed cells. Valid criticism has been raised towards the applicability of tamoxifen-inducible CreERT2 as a genetic tool for the study of adipose tissue biology because the washout time of tamoxifen at widely used doses was reported as too long [Ye15]. In addition, the high dosage of tamoxifen and oil was reported to have a toxic effect on the adipose tissue.

Hereby, we contribute to an ongoing debate about applicability of tamoxifen-inducible CreERT2 genetic tool in studying adipose tissue plasticity. Recombination of cells can be studied by the quantification of recombined genomes and we present some new tools for adipocyte recombination. First, we used a transgenic mouse model of adipocyte-specific expression of tamoxifen-inducible CreERT (Adipoq-tracer) to test the tamoxifen dose required to saturate the number of recombined cells in different depots of adipose tissue. Second, we applied the diphtheria toxin depletion function of the previously published Ucp1-tracer-depleter mouse model to determine the washout time of tamoxifen [Buc05, Ros14]. Finally, we applied the quantification of recombined cells in the Ucp1-tracer-depleter mice to define the extent of plasticity within different adipose tissue depots. This quantitative analysis approach might, in the future, help to overcome many problems that are inherent to microscopic approaches. We hope to enable the research community in using their valuable mouse lines more efficiently.
7.4. Materials and methods

7.4.1. Animals

**Transgenic Mouse Strains**

The Ucp1-tracer-depleter mouse line was previously described [Ros13]. Briefly, it is a crossing of the three mouse strains:

Ucp1-GFP (Tg(Ucp1-GFP) 445), Ucp1-CreER (Tg(Ucp1-CreER) 426) and ROSA26-tdRFP [Luc07]. The Adipoq-CreER (Tg(AdipoqCreER) xxx) strain, was created using bacterial artificial chromosome (BAC) cloning as described previously [Joh10]. The experimental procedures for preparation and purification of the final BAC were identical to those described previously by Johansson and others. We used the Adipoq gene containing BAC RP24-69M4 (BACPAC Resources Center). Cloning primers are listed in SupTab.x. Transgenic mice were generated by pronuclear injection into C57BL/6N oocytes according to standard procedures [Rü10]. The Adipoq-tracer mouse line was generated by crossing the Adipoq-CreER (Tg(AdipoqCreER) xxx) strain with ROSA26-tdRFP strains.

All mice were kept on a C57BL/6N background. Adult male and female Ucp1-tracer-depleter mice aged 13 weeks were used. Unless otherwise indicated, adult male and female mice at the age of 13 weeks were housed at room temperature (20-22°C) on an inverted light-cycle (7 pm - 7 am light on). Standard chow (Kliba-Nafag purified diet #2222; 18% protein, 7% fat, 58% carbohydrate by mass) and water were provided ad libitum. All animal experiments were approved by the Cantonal Veterinary Office of Zurich. All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with EU Directive 2010/63/EU for animal experiments.

**Experimental Animal Procedures**

During cold-exposure, mice were kept at 8°C in groups of three to four animals per cage. Housing and paper tissues were removed to ensure constant cold challenge. According to standard protocol, CreERT2 activity was induced by gavaging mice with 2 mg (80 mg/kg mouse) of Tamoxifen (Sigma-Aldrich) in 100 ul sun flower oil per day for two consecutive days (total dose 160 mg/kg mouse). Diphtheria toxin (Millipore, 2 ug/ml in saline) was injected three times each 100 ng, every six hours to ablate Ucp1 positive cells.

**Sample Harvest**

Animals were euthanized by carbon dioxide asphyxiation and tissues were harvested for histological or genomic DNA recombination analysis. Popliteal lymph nodes were carefully removed from inguinal white adipose tissue depot. The adipose tissue depots were flashfrozen in liquid nitrogen and thereafter stored at -20°C until DNA extraction. The fat depot nomenclature, as described by de Jong et al. was utilized [Jon15].
7.4.2. Quantification of CreERT2 recombination

Genomic DNA (gDNA) was prepared by using the following protocol. The tissue was crushed in a Thermolysis LT (Qiagen) at 50 s-1 for 6 minutes by a metal bead and 1 ml 50 mM NaOH to the 2 ml Eppendorf tube. Subsequently, the tissues were shaken (1000 rpm) at 92 °C for 1 h. Thereafter, 250 ul of 1 M TrisHCl was added to neutralize the pH. The samples were centrifuged (at 12000 rpm for 5 minutes) and 600 ul of the aqueous solution was carefully transferred to a 1.5 ml Eppendorf tube for long-term storage at -20 °C. For analysis, the samples were centrifuged (at 12000 rpm for 5 minutes) and the supernatant was used to create a 1:30 dilution of the DNA solution in Milli-Q water. Quantification of the number of recombined loxPRed and unrecombined ApoB genomic loci copies was performed by qPCR of genomic DNA. Absolute standard curves were prepared from synthesized pUC57recloxPRed-ApoB plasmid (485bp including ApoB-2, recombined-19 and non-recombined-2) CTTGAAGGCGCATGAACTCTTTTGTAGACGCTCTCGGAGGAGGCCAGCATGGATCCAGCGCTAGCTTTGGCTGGACGTAAACTGGAAGCTCTTTGATGAC, loxPRed13RV TCGCGGTTGAGGACAAACTC, ApoB2FW GTCCAGGTTGAATCACGGGT, ApoB2RV AGGATCCTGCAAGGTCAAGC. We schematically depict the primer binding sites and the assay principle in fig. 7.1.

7.4.3. Histological analysis

Adipose tissue depots were incubated in phosphate-buffered saline (PBS) containing 4% paraformaldehyde (PFA) for 24 hours. After overnight washing in PBS, the tissue was dehydrated for five days in 20% sucrose. Subsequently, they were embedded in Shandon Cryomatrix (Thermo Scientific). The fixed cryoblocks were processed to 50 um cryosections and stored at -20 °C. For RFP staining, the sections were washed with PBS containing 0.05% TweenTM (PBS-T) and incubated with 10% donkey serum for 1h, followed by overnight incubation. at 4 °C with primary antibody against RFP (Rockland cat #600-401-379, 1:100 in 10% donkey serum). Samples were then incubated for 1h with an anti-rabbit Alexa Fluor 568 antibody (Cell Signaling Technologies cat #, 1:200 in 10% donkey serum) and then stained with Hoechst 33342 (Invitrogen, 1:10000 in PBS). Slides were then mounted and visualized.
7.4.4. Statistical analysis

Unless otherwise indicated, all results are expressed as mean ± standard error of the mean (SEM). All graphs and statistical analyses were performed using Graphpad Prism (Version 7), we used a two-tailed, unpaired student's t-test (significance cut off: p<0.05) to assess statistical significance.

7.5. Results

7.5.1. Determination of adipocyte numbers with quantitative recombination assay

Quantification assay

The tamoxifen-inducible promoter-specific CreERT2 system was utilized along with the ROSA26-flstop-fltdRFP transgene to permanently trace adiponectin positive or Ucp1 positive cells. Upon administration of tamoxifen, CreERT2 recombinase is induced and a stop cassette is genetically depleted at the Rosa26 locus and as a consequence, the tdRFP transgene is constitutively expressed in all recombed adipocytes. In order to assess the number of labeled adipocytes, primers were designed to identify the number of recombined loxP site loci in genomic DNA, which reflects the amount of Ucp1-positive (for the Ucp1-tracer-depleter) or adiponectin-positive (for Adipoq-tracer) cells. The apolipoprotein B gene, ApoB, was used as an internal control, indicating total cell numbers. A scheme of the design is shown in fig. 7.1A. Absolute quantification of the number of recombined ROSA26-flstop-fltdRFP transgene and ApoB genomic loci was performed by qPCR of genomic DNA using a standard curve generated from a dilution of synthesized plasmid pUC57recloxPRed-ApoB, which contained the recombined loxP and ApoB sequences. As the plasmid has a known molecular weight, we knew the number of molecules of either recombined LoxP or ApoB. Quantification was adjusted for flstop-fltdRFP homozygous and heterozygous genotype of animals. We tested the specificity of CreERT2 expression in the Adipoq-Tracer mice by analyzing the recombination of genomes from other tissues. We could find considerable percentages of recombination only in adipose tissue (fig. 7.1B).

Minimal Dose of Tamoxifen

Adipoq-tracer mice were used to find the minimal dose of tamoxifen needed to label all adipocytes. Tracer mice were subjected to different gavaging frequencies of tamoxifen at 1, 2, 3, or 5 days (daily dose of 80 mg/kg body weight, corresponding to 2 mg/mouse) and the number of recombined cells was assessed 7 days after the first tamoxifen injection. The sufficient dose is hypothesized to be achieved when the recombined cell numbers do not further increase with additional tamoxifen gavaging. In interscapular BAT (iBAT) and inguinal WAT (ingWAT) (fig. 7.2A and B) the number of recombined adiponectin-positive cells remained constant from one to five times gavage. This finding was confirmed histologically and all adipocytes in the tissue section were RFP positive (fig. 7.2E), suggesting efficient
**Figure 7.1.** Quantitative recombination analysis uses two primer pairs and the AdipoqCreERT2 driver line was validated as adipocyte specific using a tissue panel. (A) First primer pair (upper part) is specific for the ApoB locus, an internal control to quantify total number of cells in a depot. (B) Second primer pair (lower part) is specific for the recombined transgene at the Rosa26 locus. The primer pair only produces a PCR product when fstopf-l-cassette is genetically deleted. Mean ± sem.
labeling by our Adipoq-tracer mouse line even at a single dose of tamoxifen. In contrast to iBAT and ingWAT, the number of recombined adipocytes increased by nearly 50% in the epidydimal white adipose tissue (eWAT) of males with increased gavaging.

**Adipocyte Numbers**

Using the recombination assay as a surrogate to quantify the number of labeled adipocytes, the total adipocyte numbers between males and females were compared. The number of adipocytes between male and female mice were equal in both iBAT and ingWAT depots (fig. 7.2A-B). At 13 weeks of age, Bl/6N mice had 28.5 ±3.7×10^6 (each side) adipocytes in the iBAT, 17.1 ±0.6×10^6 adipocytes in the ingWAT and 7.0 ±0.7×10^6 adipocytes in the epidydimal WAT (eWAT) (fig. 7.2A-C). In order to validate the efficiency of the transgenic tracer mouse lines, the labeling efficiency of the Adipoq-tracer was compared to that of the Ucp1-tracer-depleter. Adipoq-tracer and Ucp1-tracer-depleter mice were administered tamoxifen (4 mg/mouse or 160 mg/kg mouse) and their fat pads evaluated for flstopfltdRFP recombination. Notably, at standard housing conditions, Ucp1-tracer-depleter mice had a recombined Ucp1 positive cell population in iBAT which was comparable to the percentage of recombined adipocytes found in Adipoq-tracer mice (fig. 7.2D) suggesting that both lines sufficiently label adipocytes within iBAT.

**7.5.2. Tamoxifen washout time assessment with Ucp1-tracer-depleter mouse model**

We employed the Ucp1-tracer-depleter mouse model, in order to determine the time needed for Tamoxifen clearance. This mouse harbors a fusion protein encoding transgene including both green fluorescent protein (GFP) and dipheria toxin receptor (DTR) coding sequences. To test the ablation efficiency, we administered Ucp1-tracer-depleter mice with Tamoxifen to facilitate labeling of Ucp1 positive cells and then injected dipheria toxin (DTA) to ablate these recombined cells, or saline as a control. Indeed, subcutaneous injection of DTA reduced the percentage of Ucp1 positive cells by 97% ±1% percent from 21.2% ±2.5% percent of total cells in control saline-treated mice to 0.6% ±0.3% percent in DTA-treated mice (fig. 7.3C). It can be assumed that Tamoxifen is effectively washed out if newly differentiated cells are no longer recombined. To test this hypothesis, we initially gavaged mice with Tamoxifen (daily 2 mg/mouse) for two days, ablated the recombined cells with DTA and then subjected the animals to varying lengths of time at standard housing conditions (22°C) for washout, before we cold challenged (8°C) them for 1 week. The cold stimulus served to induce new Ucp1 positive cell formation, which would be labeled if Tamoxifen has not been sufficiently cleared from the system. After efficient ablation, the Ucp1 positive cell fraction of iBAT partially recovered to 16% ±2% within 1 week of cold exposure, compared to the initial 23% ±2% at room temperature (fig. 7.3D). Once mice had 3 weeks to recover from the ablation at 22°C, the percentage and amount of Ucp1 positive cells in the iBAT was fully recovered by exposing mice to cold for 1 week (fig. 7.3D). Taken together, an
### Figure 7.2: Effect of tamoxifen dosing on recombination in Adipoq-Tracer was compared to total cell numbers in both sexes and genetic labeling showed similar efficiency in iBAT of Ucp1-Tracer-Depleter and Adipoq-Tracer. (A-C) We analyzed adipocyte numbers in interscapular BAT (A), inguinal WAT (B) and epididymal WAT (C) by quantifying AdipoqCreERT2 recombined cells (left y-axis, black lines and symbols), total cell count (right y-axis, grey lines and symbols); male (filled circles) and female (empty circles) mice. (D) The recombination of adipocytes was confirmed by immunofluorescence microscopy of iBAT and ingWAT. (E) Recombined cell percentage of total cells in Ucp1-Tracer (Ucp1CreERT2) and Adipoq-Tracer (AdipoqCreERT2) was compared in iBAT. Mean ±sem.
interesting step-wise development of the re-growth of iBAT’s Ucp1 positive cell fraction was observed, when only looking at the Ucp1 positive cell percentage of mice that were re-administrated with tamoxifen in the end of the washout period. The first step at the beginning was followed by a 2 week plateau at around half of the Ucp1 positive cell capacity and another plateau at the full capacity established after the second step. The increase in Ucp1 positive cell percentage was a result of the increase of the total Ucp1 positive cell number (fig. 7.3E). Interestingly there were $75\% \pm 7.5\%$ unlabeled Ucp1 positive cells, when Tamoxifen was given 2 weeks to wash out (fig. 7.3D), which is similar to the percentage of unlabeled Ucp1 positive cells after 5 weeks of washout ($85\% \pm 9\%$). When we change the Tamoxifen protocol to an initial three times gavaging, thereby increasing tamoxifen dose to 6 mg (240 mg/kg mouse), we measured only $36\% \pm 6\%$ unlabeled Ucp1 positive cells at the 4 weeks timepoint, suggesting that increased tamoxifen dosage prolongs washout clearance within adipose tissue.

7.5.3. Cold-induced plasticity of iBAT and ingWAT

Cold acclimation strongly remolds adipose tissue through beta-adrenergic stimulation. The adipose tissue depots’ character is defined by the type of adipocytes that it contains. In the following, we counted the number of Ucp1 expressing cells in the course of a chronic cold stimulation. Mice were housed at 22°C room temperature and the cold-stimulation was achieved by decreasing environmental temperature. Compared to $28.9 \pm 7.5 \times 10^6$ Ucp1 positive cells in iBAT, we measured $0.4 \pm 0.1 \times 10^6$ Ucp1 positive cells at 22°C in ingWAT (fig. 7.4A-B). During chronic cold exposure, the number of Ucp1 positive cells changed in both iBAT and ingWAT (fig. 7.4). In iBAT, the total number of Ucp1 positive cells almost doubled during 1 week of cold to $53.2 \pm 5.2 \times 10^6$, but sank back to $26.3 \pm 4.7 \times 10^6$ at 8 weeks in the cold, which was similar to the initial level before the cold. When normalizing the Ucp1 positive cell number to that of all cells, one gets the percentage of Ucp1 positive cells, which developed a plateau after 1 week of cold (fig. 7.4B). In contrast to the iBAT, the percentage-wise increase in Ucp1 positive cells is much stronger in the ingWAT. While two days of cold exposure was not enough to increase Ucp1 positive cell number in ingWAT, it massively increased to $2.3 \pm 0.7 \times 10^6$ at 1 week of cold, and further increased until it saturated after 3 weeks at $4.5 \pm 0.5 \times 10^6$ cells (fig. 7.4C). The saturation curve of the Ucp1 positive cells was also reflected in the shape of the saturation curve when plotting the percentage Ucp1 positive cells per total cells (fig. 7.4D).

7.5.4. Discussion

There is an ongoing debate about the use of tamoxifen-inducible CreERT2 for genetic tracing studies of the adipose tissue, because of reported toxicity of supra-physiological tamoxifen doses. One way to avoid the toxic effect of tamoxifen overdosing is to switch to the doxycycline-inducible Tet-On technology for genetic tracing in the future. Without a question, the doxycycline-inducible Tet-On technology is a great alternative to the CreERT2-loxP technology and has already proven very valuable. The AdipoChaser mouse, as one example, enabled Wang et al. to track adipogenesis and distinguish
Figure 7.3.: Tamoxifen was washed out after two weeks. We determined washout time by quantifying recombination of newly differentiated Ucp1 positive cells that were formed in iBAT during 1 week cold at different times after initial tamoxifen gavage following ablation of old cells with diphteria toxin in Ucp1-tracer-depleter mice. (A) Percentage of recomb. cells at 22°C before and after diphteria toxin injection. (B) Schematic of the 5 week washout time point. (C) Overview of experimental scheme with all time points for the condition with only an initial tamoxifen dose. (D) Percentage of recombined Ucp1 cells in iBAT with only initial tamoxifen (empty circle) or re-administration (positive control, filled squares) and different controls. (E) Same as D but only count of recombined Ucp1 cells in iBAT. Mean ± sem.
Figure 7.4.: Ucp1 cell numbers increased in iBAT and ingWAT at different rates and long term cold exposure (8°C) of up to 8 weeks revealed plasticity differences. We quantified recombined and total cell numbers by qPCR in Ucp1-tracer-depleter mice. Number of Ucp1 positive cells (left y-axis, black triangles) compared to total cell number (right y-axis, grey squares) in iBAT (A) or ingWAT (C). Percentage of Ucp1 cells in iBAT (B) or ingWAT (D). Mean ±sem.
the processes of hyperplasia from hypertrophy in vivo [Wan13].

Another way to prevent toxicity is lowering the tamoxifen dose administered. This alternative seems most suitable for the scientists that already have their genetic mouse models established and do not have the resources or time to switch to the doxycycline-inducible Tet-On technology. We found that a Tamoxifen (Tam) dose as low as 2 mg (80 mg/kg mouse) is sufficient to label all adipocytes in the most important adipose tissue depots for the study of brown and brite adipocytes. This dose is one fifth of the dose usually administered by most studies. Although a single gavage of 2 mg Tam is enough to label all adipocytes, we suggest to gavage two times 2 mg Tam on consecutive days to counter any problem with the gavaging technique. As Ye et al. show, a total of 4 mg is not enough to induce the toxic effect of overdosing tamoxifen [Ye15]. The recombined cell number of epididymal WAT changes when Tamoxifen is given for at least 3 consecutive days, which we hypothesize to be adipogenesis in response to the amount of oil being gavaged.

Another advantage of the CreERT2 technology is that it requires one transgene less, saving costly breeding time. By applying Ucp1-tracer-depleter and Adipoq-tracer mouse lines, we were able to quantitatively analyze the number of recombined adipocytes. By applying the additional depleter function of the Ucp1-tracer-depleter mouse line this study proves, for the first time, that Tamoxifen is washed out from the adipose tissue and does not induce recombination in newly differentiated adipocytes. We found the washout time for 4 mg Tamoxifen per mouse to be 2 weeks and that it is prolonged to more than 4 weeks when gavaging a total 6 mg (240 mg/kg mouse). However, major differences between organs have to be expected, as transgenic pancreas transplantation studies defined the washout time of a 24 mg Tamoxifen dose at 4 weeks [Rei12]. We validated the recombination efficiency of the new Adipoq-tracer mouse line by comparing the fractions of cells recombined in iBAT with that of the previously published Ucp1-tracer-depleter mouse line. Combining these data with the histological analysis, we conclude that all adipocytes are labeled in Adipoq-tracer mice. When comparing our accounting of adipocyte numbers by quantitative genomic DNA recombination analysis with previously published estimations based on microscopic imaging of the adipose organ, we find the adipocyte numbers to be higher by a factor of four both in BAT and WAT [Mur05,Vit12]. The mice were of similar age and Murano et al. used SV129 mice, while Vitali et al. used C57Bl/6 mice [Vit12]. Another study measured cell size in eWAT and modeled the adipose tissue growth [Jo09]. They estimated eWAT’s adipocyte number of C57BL/6 mice at different age, and reported around 5 million cells for 12 week old animals, which is comparable to our result.

One explanation of differences between the histological and recombination analysis might be that histological analysis is biased towards larger cells, because they are better visible and therefore the total cell number is under-estimated. Until now, astonishingly little was known about the exact numbers of adipocytes in relation to the overall cell number, because the total cell number was never fully quantified. In addition, we show that the proportion of the adipocyte fraction is lower than previously assumed.

Furthermore, we investigated the development of brown and subcutaneous white adipose tissue depots’ character upon chronic cold exposure in mature mice. Different scenarios for brown and brite
cell recruitment can be derived from previous hypotheses. The browning of the ingWAT progresses steadily, concomitantly to BAT proliferation, until the animal is fully adapted to cold.

Our finding that brown adipose tissue hyperplasia saturates already at 1 week proves for the first time the idea that was suggested by earlier findings [Buk82]. By measuring whole fat depot incorporation of 3H-thymidine, it was found by Rehnmark and colleagues that DNA synthesis induced by b-adrenergic stimulation peaks at around 1 week of cold exposure [Reh89]. Increased 3H-thymidine incorporation could have also resulted from increased non-adipocyte cell number, which we additionally showed to increase in iBAT. When normalizing brown cells to total cell number, it seems as if iBAT does not respond to the cold stimulus, because the percentage of brown cells does not change. This finding further substantiates claims about how to read out BAT adaptation to cold [Ned13].

Furthermore, our long term analysis showed the plateauing brown cell number that is reached in iBAT after 1 week cold exposure reverses when the cold stimulus is extended for long enough. In contrast, brite cell numbers increase in the ingWAT until week 3 and do not decrease with prolongation of the cold stimulus. Based on these results, we hypothesize that BAT adapts faster to the cold-stimulation than WAT, whereas long-term adaptation to cold is mainly mediated via the browning of WAT. We think it worthwhile to follow up this hypothesis in future studies.
8. **Antioxidant cocktail prevents development of Diabetes caused by fat-specific insulin resistance**

### 8.1. Contributions

Leon G. Straub, Luca Truscello, Miro Balaz, Gerald Grandl, Myrtha Arnold and Christian Wolfrum

LGS and CW designed research study  
LGS, LT and MA conducted experiments  
LGS, LT, MB and GG acquired data  
LGS and LT analyzed data  
LGS wrote manuscript  
CW and GG revised manuscript

### 8.2. Abstract

In 2015, the International Diabetes Federation estimated that 415 million humans were diabetic and diabetes health costs accounted for 673 billion dollars worldwide. Adipose tissue insulin resistance is thought to be a leading cause of Type 2 Diabetes Mellitus (T2DM) development. We studied the causal relationship of insulin resistance in adipose tissue with T2DM development.

To explain the early phase of T2DM development, we applied the Tamoxifen inducible fat-specific insulin receptor knockout (iFIRKO) mouse model. The complex sequence of events after the induction of iFIRKO was assessed by metabolic cage measurements of lean and obese iFIRKO mice. Two high fat diet (HFD) feeding paradigms were applied to investigate iFIRKO’s long term effect on adipose tissue mass. To investigate the physiological mechanism behind the establishment of hyperglycemia, we combined radioactive glucose tracing with a hyperinsulinemic euglycemic clamp.

Induction of iFIRKO not only led to partial lipoatrophy, but also changed the predominant metabolic substrate. Radioactive glucose tracing in iFIRKO mice showed that hyperglycemia resulted from reduced muscle glucose uptake and increased hepatic glucose production. The induction of iFIRKO in HFD induced obese mice caused a reversible...
hyperglycemia, beside the heavy lipid load on peripheral organs. We repeatedly induced hyperglycemia in the HFD model. Astonishingly, we found that iFIRKO before HFD prevents obesity development. The pretreatment of mice with an antioxidant cocktail of Apocynin and N-acetylcystein rescued mice from hyperglycemia, hyperphagia and polydipsia in lean iFIRKO mice, as well as hyperglycemia and hyperphagia in genetically obese mice (ob/ob model).

We conclude that adipose tissue insulin resistance protects from obesity, but induces short reversible T2DM. Obese adipose tissue is not insulin resistant, since iFIRKO shows an effect. Apocynin and N-acetylcys-tein can protect from T2DM development, while hyperphagia is regulated independently, or downstream of Leptin. Apocynin not N-acetylcys-tein mediates the blood glucose lowering effect. Herewith we show that the NADH oxidase inhibitor mediates a beneficial health effect during development of T2DM.

8.3. Introduction

Beside courageous efforts to implement dietary guidelines, obesity and diabetes increased world-wide [Org92, Rot04, Ng14]. Body mass index is a risk factor for developing diabetes [Col16, Afs17b], which the International Diabetes Federation (IDF) estimated to be globally prevalent in 415 million people [Atl15]. In 2015, 12 percent of total global health expenditure was spent on diabetes, which translated into 673 billion dollars in cost [Atl15]. In developed countries, Type 2 Diabetes Mellitus (T2DM) accounts for 85-95 percent of diabetes cases [Sac11].

T2DM is defined as a metabolic disorder of systemic insulin resistance that leads to hyperglycemia. Beside elevated fasting blood glucose levels, other common symptoms of T2DM are hyperinsulinemia, hyperphagia and polydipsia. One hypothesis causally links obesity with development of T2DM by excessive fat accumulation-triggered inflammation in the adipose tissue, which in turn reduces insulin sensitivity [Hot95, Kah06]. Insulin insensitivity of adipose tissue reduces lipid clearance and increases plasma free fatty acid (FFA) levels through lipolysis [Bas01]. Developmentally, disruption of insulin signaling cascade leads to lipoatrophy and diabetes development [Sof16, Geo04]. While adipose tissue stores high amounts of fat, other organs as liver or muscle have more limited capacity. Elevated blood FFA concentrations cause insulin resistance through the reduction in glucose oxidation and muscle glycogen synthesis rates [Rod96]. With a prevalence of around 75 percent in obese humans, non-alcoholic fatty liver disease (NAFLD) is the most common consequence of liver lipid overload [Ame02].

The supposed hypothetical paradox that both the absence of adipose tissue (lipodystrophy) [HD10] and the overabundance of adipose tissue (obesity) can lead to T2DM, could be reconciled by the common effect of both disorders [Vir10]. Lipodystrophy as well as obesity induce lipotoxicity by increased storage of lipids in other organs [Par11, Ung10, McQ11]. While obese adipose tissue chronically releases high concentration of FFA into the circulation during fasting periods in between the meals [Hep01], in lipodystrophy the save storage side is lost in the first place [Gav00].

Ectopic lipid storage can cause excessive oxidative stress [Hel86], by exhaustion of mitochondrial
function [New07, Pat10]. Oxidative stress in adipose tissue supposedly leads to development of insulin resistance [Fur04, Lee08]. The blood glucose lowering effect of apocynin, which is an antioxidant structurally related to vanillone [Sto94, Heu08], has been shown first in heavily obese and chronically diabetic KKAy mice [Fur04]. Antioxidant containing food supplements (apple polyphenol extracts) have been suggested to improve insulin resistance in ob/ob mice [Ogu16]. Melatonin, as another antioxidant, was reported to improve NAFLD symptoms in ob/ob mice [Sta16].

After many generations of genetic mouse models, the Tamoxifen-inducible fat specific insulin receptor knockout (iFIRKO) mouse enables researchers to establish the causal link between adipose tissue’s insulin resistance and development of short term T2DM [Sak17]. This mouse model allows fascinating insights into early stages of T2DM development through application of CreERT2, expressed under Adiponectin promoter genetically ablates Insulin receptor gene expression [Brü98, Shi02, Ind99]. After shuttling into the nucleus upon Tamoxifen binding, CreERT2 recombines the floxed Insulin receptor locus. It was shown that insulin resistance in adipose tissue reduces fat mass and Leptin plasma levels. Both the regrowth of newly differentiated adipocytes or the treatment with Leptin reestablished normoglycemia. Leptin deficiency is associated with development of heavy obesity in early life of humans [Mon97], as well as in mice [Col78]. Leptin regulates food intake centrally by stimulating proopiomelanocortin neurons and inhibiting neuropeptide Y and agouti-related peptide neurons in the hypothalamic arcuate nucleus [Mor05].

To test whether obese adipose tissue is insulin resistant, we herewith characterize and apply the iFIRKO genetic mouse model in the context of diet induced obesity. With the clear goal in mind to fight obesity, we tested the hypothesis that temporary strong weight loss could be feasible for therapeutic ventures. Inducing a partial lipatrophy in heavily obese iFIRKO mice causes a long term storage of lipids in liver and muscle. Interestingly, we found that this ectopic lipid storage alone does not cause diabetic symptoms. Since antioxidants were shown to have an influence on chronic T2DM, we tested Apocynin and N-acetylcysteine [Flo85, Per87] for the first time for their preventive effect on T2DM development. Our findings show that both antioxidants together prevent polydipsia and hyperphagia, while hyperglycemia is strongly reduced in the iFIRKO mouse model. We found Apocynin to mediate the blood glucose lowering effect. The treatment of another hyperphagic mouse model with Apocynin and N-acetylcysteine still reduced food intake, which suggests that parts of the antioxidant’s effects are Leptin-independent.

8.4. Methods

8.4.1. Animal work

AdipoqCreERT2 animals were obtained by BAC cloning CreERT2 into RPCI-24-69M4 our laboratory and IRI/lfl animals were purchased from Jackson Laboratory. Both transgenes were held on a C57BL/6N genetic background. Male mice of 10 to 14 weeks of age were used. Unless stated otherwise, animals were housed at 23 °C on an inverted light cycles (7 pm-7 am lights on) and fed a standard chow.
laboratory pellets (18 % proteins, 4.5 % fibers, 4.5 % fat, 6.3 % ashes, Provimi Kliba SA). Unless stated otherwise, water and food were available ad libitum. CreERT2 activity was induced by gavaging of 2 mg Tamoxifen (Sigma-Aldrich) per mouse in 100 µL sunflower oil for three consecutive days (dose 240 mg/kg mouse). Mice were treated with Apocynin (15 mM) and N-acetylcystein (40 mM) by dissolving the powder in their drinking water. Body composition was measured with nuclear magnetic resonance scanning of the mice under a Whole Body Composition Analyzer (EchoMRI) followed by an automated analysis. All experiments have been performed according to national and institutional guidelines. To test insulin signaling in vivo, we fasted mice for 4 hours and 15 minutes before sacrifice injected 10 ul of 100 mg/ml glucose solution intra-peritoneally per gram of mouse body weight.

8.4.2. Indirect calorimetry

Indirect calorimetry was measured with a metabolic cage system (PhenoMaster, TSE systems, Bad Homburg, Germany). Activity, water and food consumption were measured continuously. On the basis of sequential measurement points (sample interval 20 min) O2 consumption and CO2 production were calculated by manufacturer software (TSE PhenoMaster V5.6.5) with corresponding coefficients of 3.941 (CVO2) and 1.106 (CVCO2). RER was measured as the ratio of CO2 produced over O2 consumed. Mice were acclimated to the system before measurements. Water and food was available ad libitum during the whole measurement. Mice were acclimated to the system before measurements.

8.4.3. Blood analysis

Fasted and random fed blood glucose were measured from tail vein with an ACCU-Check Aviva Blood Glucose Meter System (Roche). Insulin concentration was measured by ELISA using the Mouse/Rat Insulin kit (Meso Scale Discovery) according to manufacturer’s instructions. Free fatty acid (FFA) levels were measured by colorimetric assay using the NEFA-C kit (WAKO Chemicals GmbH) according to manufacturer’s instructions. Leptin concentration was measured with Leptin Mouse/Rat ELISA (BioVendor, RD291001200R).

8.4.4. Tissue triglyceride content

Liver triglycerides (TG) were extracted using Folch solution adapted from the previously described protocol.(1) Small liver pieces 3-7 mg were lysed using TissueLyser LT (Qiagen) and 2 metal beads for 5 min at 50 Hz in 150 l chloroform:methanol solution (2:1). Tissue homogenate were further dissolved in 1.5 ml of chloroform:methanol solution (2:1) followed by overnight shaking. The following day, the aqueous phases were removed by adding 150 l of citric acid 0.5 M and centrifuged at 4000 rpm for 15 min. The organic phase was completely dried using SpeedVac sc100 (Savant) and lipids were solubilized in 2 ml chloroform. TG content was measured with Trig/GB quantification kit (ROCHE/ITACHI) according to the manufacturer’s instructions.
8.4.5. Glucose Tolerance Test

Glucose tolerance test (GTT) was performed on conscious unrestrained mice after a 4h fast. Mice were weighed and fasting blood glucose was measured with ACCU-Check Aviva Blood Glucose Meter System (Roche) from the tail vein. Glucose solution (1 g Glucose/kg body weight, Sigma-Aldrich in 0.9 % NaCl, Braun) was injected intraperitoneally and blood glucose was measured following at 15, 30, 60 and 90 minutes.

8.4.6. Insulin Tolerance Test

Insulin tolerance test (ITT) was performed on conscious unrestrained mice after a 5h fast. Mice were weighed and fasting blood glucose was measured with ACCU-Check Aviva Blood Glucose Meter System (Roche) from the tail vein. Insulin solution (1 IU or 2 IU insulin Actrapid HM/kg body weight, Novo Nordisk in 0.9 % NaCl, Braun) was injected Intra-peritoneally and blood glucose was measured following.

8.4.7. Jugular Vein (JV) catheter

Chronic jugular vein catheters (RAUP 12.3B) for mice consisted of a poly-urethane tubing Renathane, 0.3 mm ID x 0.6 mm OD, length 42mm) and a bent stainless-steel tubing (12 mm, 26G cannula, slightly bent to a 120 angle, protruding end). The catheter tubing was attached to the steel tubing and reinforced with silicon tubings. The reinforced end with the steel tubing was led through a piece of soft surgical mesh (0.7 x 1 cm, Premilene or Optilene) to improve adhesion to skin and fascia and fixed to it with non-absorbable monofil suture (6-0 Prolene). The mesh was placed subcutaneously between the scapulae, with the external end of the catheter protruding. The catheter was then led subcutaneously from the neck to the right clavicle. The catheter was filled with sterile NaCl and, 12 mm distant from the tip, a suture was placed around the catheter. The jugular vein was exposed and two ligatures (the caudal ligature is a loose loop) with 5/0 silk were placed around the vein. An incision was made on the ventral surface of the vein, and the catheter was inserted and advanced until the tip reached the right atrium (about 12mm). The catheter was fixed in place with 5/0 silk around the catheter and connected with the caudal suture thread. Both incisions were sutured with 6/0 Vicryl. The catheter was filled with heparinized 50 % glycerol solution (100 - 200 IU heparin/mL). After surgery catheters were flushed every every 3-4 days.

8.4.8. Gavage of mice with jugular vein catheters

Two days after implantation of jugular vein catheters we gavaged mice for two consecutive to induce the knockout. Since the catheter tubing exits at the back of the neck, special gavaging handling procedure was necessary. Mice were anaesthetized with isoflurane (1.5 - 2.5 %), carefully handled and gavaged just when they recovered their reflexes indicated by the movement of their nose hairs.
8.4.9. Anaesthesia and analgesia

One hour prior to surgery 50 ug/kg Fentanyl was given subcutaneously. Mice were anaesthetized for surgery with isoflurane (1.5 - 2.5 %) and surgery was performed under constant flow. Post-surgery animals were injected with glucosaline (300 ul) and 5 mg/kg Norocarp subcutaneously. The first 2 days after surgery animals received daily subcutaneous injections of 5 mg/kg Norocarp for analgesia. Animal welfare and body weight was assessed daily following a score-sheet.

8.4.10. Hyperinsulinemic euglycemic clamp

One week after Tamoxifen was given, mice were deprived of food for 5h and jugular vein catheters were connected to an infusion pump immediately after water deprivation. Blood glucose levels were measured from tail vein blood with Accu-Check (Roche). Starting with a bolus of 2.5 μCi in a volume of 50 ul, within the last 80 min of fasting time, 3H-glucose (0.05 μCi/min; PerkinElmer) was infused to measure baseline glucose turnover. This was followed by an initial bolus of 900 mU/kg inulin in a volume of 50 ul. In parallel to insulin infusion, variable amounts of 20 % glucose were infused and infusion rate was adjusted every 5 min to reach a constant blood glucose concentration (euglycemia). The effect of insulin (18 mU/kg/min) on glucose turnover was measured by infusion of 3H-glucose (0.1 μCi/min; PerkinElmer). This is necessary to calculate endogenous rates of glucose production. At steady state (euglycemia without changing GIR for more than 15 min) a bolus of 14C-glucose was administered through the catheter to measure glucose uptake into peripheral tissues for 35 minutes. Mice were euthanized by cervical dislocation. We calculate the glucose turnover rate by dividing 3H-glucose infusion rate by the plasma 3H-glucose specific activity. To calculate the hepatic glucose production, we subtracted glucose infusion rate from glucose turnover rate.

8.4.11. Protein analysis

Extracted protein concentration was first measured with the DC Protein quantification assay (Bio-Rad) according to manufacturer’s instructions. The following primary antibodies were used for protein recognition: IR β-subunit (Santa Cruz Biotechnology #SC-711), Phospho-IRS (Ser1101) (Cell Signaling Technology #2382S), Phospho-AKT (Ser473) XP (Cell Signaling Technology #4060), Phospho-AKT (Thr308) XP (Cell Signaling Technology #13038), panActin (Cell Signaling Technology #8456), HSP90 (Cell Signaling Technology #4877), GAPDH (Cell signaling Technology #5174), ACC (Cell Signaling Technology #3676), GYK (Abcam #126599).

8.4.12. RNA analysis

RNA was isolated with TRIZOL (Invitrogen) reagent according to manufacturer’s instructions. Total RNA was then reverse transcribed using randomized primers and the High Capacity cDNA Reverse
Transcription kit (Applied Biosystem) according to manufacturer’s instructions. Quantitative measurement was performed with Syber Green qPCR (Life Science Technology). mRNA levels were calculated by normalization to TBP (housekeeping gene) and expression of IRfl/fl tissue was set to 1. Primers sequences are stated in supplementary tab. 1.

8.4.13. Statistics

All results are expressed in mean±SEM, all graphics and statistical analysis were performed using Graphpad Prism. Statistical significance was calculated using multiple two tailed unpaired student’s t-test.

8.4.14. ROS (H2O2) measurement

We used a protocol based on (Furukawa et al., 2004). Animals were sacrificed by cervical dislocation, pieces of not bigger than 5x5 mm of hypothalamus, interscapular BAT, inguinal WAT, epidydimal WAT Liver and musculus soleus were dissected, placed in Krebs-Ringer HEPES buffer (pH 7.4) with 100 M Amplex Red (Sigma) and 0.2 U/ml Horse Radish Peroxidase (Sigma), cut into small pieces and left for 30 min at 37°C in the dark. The solution was analysed on a SynergyMX (BioTek) plate reader at 544 nm excitation and 590 nm emission with a standard of H2O2 in the same buffer.
8.5. Results

8.5.1. Induction of adipocyte-specific knockout of IR receptor (iFIRKO) led to lipoatrophy

One week after first Tamoxifen gavage, protein levels of IR-beta chain were markedly reduced in the different fat depots of iFIRKO mice, but not in liver (fig. 8.1A).

Induction of adipocyte-specific knockout of IR receptor (iFIRKO) reduced total fat mass later than 1 week after induction with Tamoxifen. A strong difference in fat mass was observed three weeks after iFIRKO (fig. 8.1B), but neither body mass or lean mass of chow fed animals changes in iFIRKO mice (fig. 8.2A-B). Subcutaneous adipose tissue depots as iBAT and ingWAT were reduced in wet mass already after 5 days (fig. 8.1C). At four weeks the epiWAT was also markedly reduced (fig. 8.1C).

Assuming a strong induction of lipolysis after iFIRKO, we measured free fatty acid (FFA) concentrations of blood plasma from mice that were randomly fed. One day after a first Tamoxifen gavage, FFA concentrations increased by 50 % comparing iFIRKO mice with IRfl/fl mice. At all other time points FFA levels were similar (fig. 8.1D). However, when weighing liver at different time points after Tamoxifen gavaging, we found a slight increase in liver wet weight on day 5 that persists until day 28 (fig. 8.2C). Unexpectedly this increase in liver mass did not coincide with total triacylglyceride (TAG) concentration of liver lipid extracts normalized to liver weight (fig. 8.2D). Even the total TAG load was not different between the two groups (data not shown). While plasma Leptin concentration was unchanged 3 days after iFIRKO, we measured a transient strong reduction by around 70 % 5 days after KO, but mice recovered to starting Leptin concentrations on week 4 of the KO (fig. 8.1E). The reduction in adipose tissue depot mass of the ingWAT was accompanied by reduced Leptin, Adiponectin and Resistin gene expression (Sfig. 1C), which recovered 4 weeks after iFIRKO, although the depot mass was still reduced (Fig. 1C).

8.5.2. iFIRKO changed metabolism and induced hyperglycemia, polydipsia and hyperphagia

We performed metabolic cage studies, in which oxygen consumption (VO2) and carbon dioxide production (VCO2) were assessed, to measure potential metabolic disturbances caused by the insulin resistance in the adipose tissue. The metabolic cage system is based upon indirect calorimetry. As a ratio of CO2 production over O2 consumption, respiratory exchange ratio (RER) informs about the main energy substrate on which metabolism operates.

First, two days after iFIRKO, we measured a strong decrease in RER (fig. 8.3A), as the oxygen consumption stayed the same (fig. 8.3B) and the mice were equally active (fig. 8.3A). Of note, the RER changes took place one day after the plasma FFA levels were briefly increased (day 1) and 3 days before the plasma Leptin concentration (day 5) was reduced. This decrease in RER hints at a substrate change to lipid metabolism.
Figure 8.1: Inducible fat-specific insulin receptor KO (iFIRKO) lead to reduced fat mass and reduced circulating Leptin conc. compared to IRfl/fl control. (A) Western Blot of IR-beta and Pan-Actin protein in adipose tissue and liver lysate. (B) Total fat mass measured by EchoMRI  7 and 21 days after Tamoxifen. (C) Interscapular BAT, inguinal WAT, epididymal WAT wet weight 5 or 28 days after Tamoxifen. (D) Time course of plasma free fatty acid (FFA) conc. (E) Plasma Leptin conc. 3, 5 and 28 days after Tamoxifen. Mean ± sem. p-value of unpaired, 2-tailed Student’s t-test ≤ 0.05 : *, ≤ 0.01 : **, ≤ 0.005 : ***.
Figure 8.2.: While lean and body mass remained unchanged in inducible fat-specific insulin receptor KO (iFIRKO) mice, liver weight slightly increased and Adipokine mRNA expression was reduced. (A) Lean body mass measured by EchoMRI. (B) Body mass. (C) Liver mass in mg 3, 5 and 28 days after Tamoxifen. (D) Liver triacylglycerides (TAG) conc. in mg per g Liver 5 and 28 days after Tamoxifen. (E) Expression in ingWAT of Leptin, Pparg2, Adipoq and Resistin mRNA normalized to TBP measured by qPCR 1 and 4 weeks after Tamoxifen. Mean ± sem. p-value of unpaired, 2-tailed Student’s t-test ≤ 0.05 : *, ≤ 0.01 : **.
Second, shortly after RER was reduced, mice developed hyperglycemia four days after iFIRKO (fig. 8.3C). The tripling of random fed blood glucose concentration from 7.7 ± 0.2 mmol/L to 23.3 ± 0.6 mmol/L was established within 2 days on day 6 after KO. The strong hyperglycemia (>19 mmol/L) lasted for 7 days until day 12, then it decreased for 3 days until it was no longer different on day 15 of iFIRKO. The hyperglycemia correlated with hyperinsulinemia, which was found by measuring plasma insulin concentration both 7 days and 4 weeks after the iFIRKO. The 3.9 fold increase of plasma insulin concentration in iFIRKO mice at day 7 of KO was fully reversed at week 4 of KO, when we also no longer observed a difference in blood glucose concentration (fig. 8.4B).

Third, with a 2 day delay compared to development of hyperglycemia, iFIRKO mice showed a massive increase of water consumption. 8 days after KO, iFIRKO mice drank with 15.5 ± 2.0 ml 3 fold as much water per day as IRfl/fl mice that drank 5.2 ± 0.4 ml per day (fig. 8.3D).

We monitored food intake daily, because Leptin is a well known satiety regulator and we observed it to be downregulated 5 days after the KO. Unexpectedly, food intake was initially reduced in iFIRKO mice throughout day 2 to 5 of KO (fig. 8.3E). It took two days that the reduced plasma Leptin concentration translated into increased food intake that was persisting for three days. From day 6 to day 9 after KO, iFIRKO mice (4.9 ±0.4 g/day) ate 24 percent more than IR fl/fl mice (3.9 ±0.2 g/day). From day 10 onwards both groups ate similarly again.
Figure 8.3.: Inducible fat-specific insulin receptor KO (iFIRKO) changed respiratory exchange ratio (RER) and caused heavy insulin resistance and reversible symptoms sequentially, first hyperglycemia developed, then polydipsia and finally hyperphagia. continued on next page.
To test the glucose tolerance on day 7 of iFIRKO mice, we fasted mice for 4 hours before intraperitoneally injecting 1 mg glucose per g of mouse. In iFIRKO mice, fasting blood glucose concentration was strongly elevated with 16 mmol/L (fig. 8.4C). Although iFIRKO mice had elevated blood glucose levels, they consistently showed much higher total blood glucose concentration over all time points. However, the percentage increase between the two groups is similar. Interestingly, 200 minutes after glucose injection, blood glucose concentration of iFIRKO dropped below the fasting concentration that they showed at the baseline. To test insulin sensitivity of iFIRKO mice at day 7 of the KO, we fasted mice for 4 hours and peritoneally injected 1.5 U insulin per kg mouse mass. Insulin injection did lower the blood glucose concentration only in IR/Il/Il mice (fig. 8.4D). Throughout the 80 min time period of the insulin tolerance test, iFIRKO mice’s blood glucose were strongly elevated compared to IR Il/Il mice.

Since fasting glucose concentrations differed strongly between IR/Il/Il and iFIRKO mice, we found it necessary to perform a hyperinsulinemic euglycemic clamp, to further delineate if hyperglycemia is caused by changes in muscle glucose uptake or hepatic glucose production. We clamped mice at a blood glucose concentration of 5 mmol/L by continuously infusing insulin and glucose through the jugular vein (fig.8.4 E). The glucose infusion rate was gradually increased until it reached a stable value, this state in which the blood glucose concentration remains between 4 and 5 mmol/L without further adjustment of the glucose infusion rate is the steady state (fig.8.4 F). Seven days after the first Tamoxifen gavage, iFIRKO mice at steady state showed extremely reduced glucose infusion rates (fig.8.3 F). In the following, we tested the glucose uptake of muscle (M. soleus) or ingWAT tissue (fig.8.3 G), by injecting 14C labelled glucose into the jugular vein at steady state. The total glucose uptake into the ingWAT was strongly reduced in iFIRKO, but since the ingWAT mass is also strongly reduced and we normalize the counts per minute (CPM) by the tissue mass, it seems to not be changed although the IR was knocked out in that tissue. The muscle’s (M. soleus) glucose uptake at steady state was reduced in iFIRKO mice by 35 percent (fig.8.3 G). Hepatic glucose production at basal state, which means 4 hours fasted state, was not different. Insulin is supposed to inhibit hepatic glucose production. When a liver is insulin resistant, the insulin cannot reduce the hepatic glucose production rate. The insulin stimulated hepatic glucose production rate was strongly increased in iFIRKO mice compared to the IR/Il/Il mouse control (fig.8.3 H).
Figure 8.4.: Inducible fat-specific insulin receptor KO (iFIRKO) caused reversible hyperinsulinemia and both glucose tolerance and insulin tolerance tests indicate insulin resistance. (A) Total movement (XT+YT) as readout of spontaneous locomotor activity measured with infrared light beams. (B) Plasma Insulin conc. measured 7 and 28 days measured with ELISA. (C) Day 7 of iFIRKO, kinetics of blood glucose conc. after intraperitoneal injection of 1 mg/g of mouse. (D) Day 7 of iFIRKO, kinetics of blood glucose conc. After intraperitoneal injection of 1.5 U/kg Insulin. Day 7 of iFIRKO, blood glucose conc. (E) and glucose infusion rate (GIR) (F) during hyperinsulinemic euglycemic clamp. Mean ±SEM. p-value of unpaired, 2-tailed Student’s t-test ≤ 0.05 : *, ≤ 0.01 : **.
8.5.3. iFIRKO in high fat diet-induced obese mice repeatedly caused hyperglycemia

While, at an age of 12 weeks our chow fed mice had a moderate fat mass with around 3 g, mice fed a high fat diet (HFD) for 16 weeks developed fat masses around 20 g. To study the effect of iFIRKO in a state of heavy obesity, we induced the fat-specific knockout after 16 weeks of HFD (fig. 8.5). In heavily obese mice, the genetic ablation of IR strongly reduced the body weight from 50.2 ±0.8 g to 39.8 ±0.8 within 20 days (fig. 8.5A). By measuring the body composition with the EchoMRI, we found that 10.4 g loss of body weight was due to 13 g loss in fat mass from 19.9 ±0.6 g before to 7.2 ±0.3 g after iFIRKO (fig. 8.5B). In addition, we also measured mass reduction of several adipose tissue depots (fig. 8.6A). Again, as in chow fed iFIRKO mice, epidydimal WAT was less reduced then the subcutaneous depots of iBAT and ingWAT. Along with the heavy fat mass loss, the lean mass slightly increased in iFIRKO mice, which explains the differing 2.6 g of body weight (fig. 8.6B). Similar to what we found in chow iFIRKO mice, the Leptin concentration in the plasma strongly dropped in heavily obese mice on HFD after iFIRKO 70 percent (data not shown). Addressing the question if the fat became re-distributed and where the 2.6 g increase in body weight comes from, we weighed the liver and confirmed that its weight was increased by around 0.8 g, or 50 percent 4 weeks after iFIRKO (fig. 8.5C).

One week after induction of fat-specific IR-KO in heavily obese mice, we tested them for insulin resistance by injection of 2 international units of Insulin per kilogram body weight (fig. 8.6C). Whereas the obese IRfl/fl mice were insulin sensitive, even that high dose could not reduce the blood glucose concentration in obese iFIRKO mice. The fasting of obese iFIRKO mice for 6 hours lead to an unexpected reduction of the blood glucose concentration to 8 mmol/L, which was similar to the obese IRfl/fl control. Additionally, ad libitum high fat diet fed obese iFIRKO mice developed hyperglycemia 1 week after induction of IR-KO and returned to normoglycemia at 4 weeks of iFIRKO, which was similar to the development in their chow fed counterparts (fig. 8.5D).
Figure 8.5.: Repeated induction of fat-specific insulin receptor KO (iFIRKO) caused hyperglycemia also in high fat diet (HFD) induced obese mice, while it dramatically reduced fat mass only at the first induction, strong increases of liver mass did not correlate with hyperglycemia. (A) Body weight over time of HFD feeding. (A-D) Grey columns in graphs indicate time of Tamoxifen gavage. (B) Fat mass before and after first and second Tamoxifen gavage. continued on next page
When observing that fat mass of iFIRKO did not recover 4 weeks after the IR-KO, while the blood glucose concentration normalized, we induced the knockout again to see if we could reduce the fat mass even further. The fat mass was further reduced from $8.3 \pm 0.5$ g to $6.1 \pm 0.7$, although with only 25 percent reduction resulting from the second IR-KO induction, its percentage-wise impact was only half as strong as during the first iFIRKO (fig. 8.5B). The second iFIRKO again induced the development of another hyperglycemic phase, which was again reversible, since we measured normoglycemia 4 weeks after the second iFIRKO (fig. 8.5D). When we compared the liver mass from before the second iFIRKO, with that after the second iFIRKO, we found that there was no correlation between increased liver mass and development of hyperglycemia (fig. 8.5C-D).

In the following, we analyzed the liver and muscle protein lysates by western blotting to see the insulin sensitivity of these tissues. The mice were first fasted for 4 hours and then 15 minutes before the tissue harvest injected intraperitoneally with glucose to trigger an internal insulin response. We were specifically interested in the comparison of two time points of this experiment we sacrificed mice 4 weeks after the first iFIRKO and 1 week after the second iFIRKO. At these time points the HFD induced obese mice reside in a normoglycemic, or hyperglycemic state, respectively. We analyzed phosphorylation states of Akt at threonine 308 (pAkt(T308)), Foxo1 at threonine 24 (pFoxo1(T24)) and AS160 at threonine 642 (AS160(T642)), which are all downstream targets of insulin receptor signaling cascade.

We unexpectedly found reduced pAkt(T308)/Akt ratio in the livers of iFIRKO mice at the normoglycemic state 4 weeks after first iFIRKO (fig. 8.5E). No differences in phosphorylation of any of the analyzed proteins from the M. soleus muscle was found at the normoglycemic state (fig. 8.5F). In the hyperglycemic state of mice 1 week after a second iFIRKO, the liver pAkt(T308)/Akt as well as pFoxo1(T24)/Foxo1 protein ratios showed no differences (fig. 8.5G). However in the muscle of hyperglycemic iFIRKO mice we could detect an increased pAkt(T308)/Akt, but reduced pFoxo1(T24)/Foxo ratio (fig. 8.5H), while pAS160(T642)/AS160 stayed unchanged. The western blot bands are shown in a separate figure (fig. 8.6D-G).
Figure 8.6.: Fat mass reduction in HFD induced obese mice through induction of fat-specific insulin receptor KO (iFIRKO) was reflected by mass reduction of interscapular BAT, inguinal WAT and epidydimal WAT depots, but increased lean body mass and caused insulin resistance. (A) Weight of iBAT, ingWAT and epiWAT 4 weeks after 1st and 1 week after 2nd Tamoxifen gavage. (B) Kinetics of lean mass. (C) Kinetics of blood glucose after injection of 2 U/kg Insulin. continued on next page
8.5.4. iFIRKO in obese mice reduced RER and increased oxygen consumption

The same heavily obese mice that were described previously (fig. 8.5), underwent indirect calorimetric analysis during parts of the experimental time course. When mice were fed a HFD their RER was generally reduced, because the main energy source in the food consisted of lipids. Although starting already at an unusually low RER around 0.74 (which might be caused by the oil gavage and single housing stress) the RER of obese iFIRKO mice was further reduced to around 0.71 within 2 days after the induction of KO (fig. 8.7A). The RER difference between obese IRfl/fl control and obese iFIRKO mice further increased, because the obese control group strongly increased its RER between day 3 and day 11 after the Tamoxifen oil gavage. Three weeks after the KO-induction, when the iFIRKO mice were normoglycemic, the RER of iFIRKO mice was no different from IRfl/fl controls. Notably, the oxygen consumption measurement, which is a measure for determining energy expenditure of an organism. It took three days after iFIRKO in obese HFD fed mice to observe increased oxygen consumption rates in relation to lean body mass (fig. 8.7B). The increased oxygen consumption was still measured 13 days after the induction of insulin resistance of adipose tissue. Carbon dioxide (CO2) production was not different between obese IRfl/fl and obese iFIRKO mice until d21, when the iFIRKO mice’s CO2 production (VCO2) showed similar reduction as the oxygen consumption (fig. 8.7C). Three weeks after iFIRKO however, obese iFIRKO mice showed decreased oxygen consumption that was below that of the obese IRfl/fl mice, which coincides with the establishment of a normoglycemia. Both groups of mice showed no difference in their movement (fig. 8.7D).
Figure 8.7: Inducible fat-specific insulin receptor KO (iFIRKO) first reduced respiratory exchange ratio (RER) then increased oxygen consumption (VO2) in HFD induced obese mice. (A-D) Indirect calorimetry over time after first Tamoxifen. Three time segments connected: 1st segment day -1 to d4, 2nd segment d10 to d13 and 3rd segment d21 to d25. RER (A), VO2 (B), VCO2 (C) and movement (D). Black arrows indicate time points of Tamoxifen gavage. Mean ± sem. p-value of unpaired, 2-tailed Student’s t-test ≤ 0.05 : *, ≤ 0.01 : **, ≤ 0.005 : ***.
8.5.5. *iFIRKO before start of high fat diet feeding prevented the obesogenic effect of HFD feeding*

Next we induced the IR-KO in the adipose tissue of chow fat mice and changed their diet to an obesogenic HFD afterwards, to test the re-growth potential of the adipose tissue after it was rendered insulin resistant. As a result of the inducible character, newly differentiated cells should contain a functional IR once the Tamoxifen is washed out. Starting at equal body and fat mass before the induction of KO, body mass of *iFIRKO* mice grew less strong on HFD than IR *fl/fl* mice and therefore diverged 18 days after the KO (fig. 8.8). The body mass difference increased over time and reached its maximum on day 82 (week 11) of HFD (fig. 8.8A). After around 11 weeks the *iFIRKO* mice were still partially protected from the obesogenic effect of HFD and their body mass of $37.1 \pm 0.8$ g was $11.3$ g lower than that of the IR *fl/fl* mice with $48.4 \pm 1$ g. At that time point *iFIRKO* mice had a fat mass of $6.6 \pm 0.7$ g and a lean mass of $29.3 \pm 0.2$ g, while the IR *fl/fl* mice had a fat mass of $21.3 \pm 0.9$ g and a lean mass of $26.2 \pm 0.4$ g (fig. 8.8B).
8.5.6. Antioxidant cocktail rescued hyperglycemia, hyperphagia and polydipsia in iFIRKO

ROS concentrations were measured in different tissues 7 days after the induction of IR-KO in adipose tissue. We could not detect differences in H2O2 levels in hypothalamus, iBAT, iWAT, epiWAT, or liver when mice were fasted for 4 hours (fig. 8.9A). Only the muscle of iFIRKO mice showed ROS levels that were 3.4 fold increased compared to IR/fl/fl mice. Assuming that it is oxidative stress that caused hyperglycemia, hyperphagia and polydipsia, we dissolved two well known anti-oxidants Apocynin (Acetovanillon) and N-acetyl cysteine (Nac) in the mice’s drinking water at a concentration of 15 mM Apocynin and 40 mM N-acetyl cysteine. Assuming that mice drink 5 ml per day they were treated with a daily dose of 12 mg Apocynin in addition to the 32.5 mg N-acetyl cysteine.

Astonishingly, not only did the antioxidant cocktail delay the development of hyperglycemia in iFIRKO mice by 1 day, it also strongly reduced the hyperglycemia to a maximum of 17.0 ± 1.7 mmol/L compared to 26.5 ± 1.6 mmol/L in the untreated iFIRKO mice (fig. 8.9B). When we compared the effect of each antioxidant separately, Apocynin was found to be the substance that reduced blood glucose in iFIRKO mice, while N-acetyl cysteine had no effect on blood glucose concentration (fig. 8.10A). Another effect of the antioxidant cocktail on iFIRKO mice was that it reduced the elevated food intake by 40 percent, from average 4.9 ± 0.4 g per day in iFIRKO mice to 2.9 ± 0.4 g per day in iFIRKO mice treated with Apocynin and N-acetyl cystein between day 6 and day 9 after Tamoxifen gavage. Both the reduction of hyperglycemia and food intake was only present in iFIRKO mice (fig. 8.9D), because both parameters stayed unchanged in the IR/fl/fl control mice when treated with antioxidants (fig. 8.10B). Also the increase in water intake, which fully developed in iFIRKO mice 7 days after Tamoxifen gavage, was completely blunted by antioxidant treatment (fig. 8.9C). Although antioxidants did not reduce fat mass in IRfl/fl mice, antioxidants counteracted an increase of fat mass that was measured in IRfl/fl mice during the 21 days after Tamoxifen/oil gavage (fig. 8.9E). Reduction of fat mass caused by iFIRKO was not affected by antioxidant treatment. However, 7 days after antioxidant cocktail treatment, body mass was reduced in both iFIRKO and IRfl/fl mice by 10 and 15 percent, respectively (fig. 8.10C). This reduction in body mass on day 7 of treatment is reflected in the lean mass reduction of both iFIRKO and IRfl/fl mice by 10 and 13 percent, respectively (fig. 8.10D).

8.5.7. Antioxidant cocktail rescued hyperglycemia and hyperphagia in genetically obese mice (mouse model of Leptin mutation)

Mice with a mutation in the Leptin gene (ob/ob mice) develop hyperphagia, which leads to obesity. We applied this obesity mouse model to further elucidate the effect of the antioxidant cocktail on hyperphagia and hyperglycemia. At 20 weeks of age, ob/ob mice developed extreme obesity and elevated blood glucose (fig. 8.12A), which was accompanied by hyperphagia when fed ad libitum (fig. 8.11B-C). In the following ob/ob mice are compared to a littermate group containing both genotypes ob/wt and wt/wt as control.
Figure 8.9.: Antioxidant cocktail rescued hyperglycemia, hyperphagia and polydipsia in adult chow fed inducible fat-specific insulin receptor KO (iFIRKO) mice when added to drinking water. (A) H2O2 production in Hypothalamus, iBAT, ingWAT, Liver, Musculus (M.) soleus and M. gastrocnemius normalized to protein content. (B) Blood glucose conc., (C) water intake and (D) food intake of ad libitum fed mice upon iFIRKO and or treatment with antioxidants 14 mM Apocynin (Apo) and 40 mM N-acetylcystein (Nac) containing drinking water. Mice were pre-treated with antioxidants 3 d before Tamoxifen gavage. (E) Total fat mass before, 7 and 21 days after Tamoxifen gavage. Mean ± sem. p-value of unpaired, 2-tailed Student’s t-test ≤ 0.05 : *, ≤ 0.01 : **, ≤ 0.005 : ***. IRfl/fl vs iFIRKO:*; iFIRKO vs iFIRKO(Nac+Apo):€; IRfl/fl vs IRfl/fl(Nac+Apo): $.
Figure 8.10.: Apocynin mediated blood glucose reducing effect of the antioxidant cocktail. When added to drinking water 14 mM Apocynin (Apo) and 40 mM N-acetylcystein (Nac) reduced lean body mass in IRfl/fl control and iFIRKO mice. (A) Blood glucose conc. 9 days after Tamoxifen gavage when either Apocynin or N-acetylcystein were given separately for 2 days starting 7 days after Tamoxifen. Body mass (B) and lean mass (C) before, 7 and 21 days after Tamoxifen gavage. Mean ± sem. IRfl/fl vs iFIRKO: *, IRfl/fl vs IRfl/fl(Nac+Apo): §.

IRfl/fl (Nac+Apo)
Figure 8.11.: Antioxidant cocktail of 14 mM Apocynin (Apo) and 40 mM N-Acetyl cysteine (N-acetyl) given ad libitum via drinking water reduced Teflin deficiency induced hyperphagia and established normoglycemia within 2 days in 20 week old ob/ob mice. (A) Body composition (FM= fat mass, LBM= lean body mass, BW= body weight) analysis 15 days before and 8 days after treatment with antioxidant cocktail. (B) Daily food intake before, 1 and 2 days after treatment with antioxidant cocktail. (C) Ad libitum fed blood glucose concentration (mmol/L) Days after treatment with antioxidant cocktail. Black arrows indicate supply with freshly prepared antioxidant cocktail. Mean ± sem. wt vs ob/ob: * p<0.05, ** p<0.01, *** p<0.001.
Figure 8.12: Leptin deficient ob/ob mice had established hyperglycemia at 20 weeks of age and showed reduced insulin sensitivity. (A) Ad libitum fed blood glucose of measurement started 14 days before antioxidant treatment. Time line continues in fig. 8.11C. (B) Insulin tolerance test with 1.5 U/kg mouse after 4 hours fasting. Mean ± sem. wt vs ob/ob: *

Unexpectedly, when fasting ob/ob mice for 5 hours, their blood glucose levels were no longer different (fig. 8.12B). The injection of 1.5 U/kg of Insulin heavily reduced the blood glucose levels of the control group, while it was not enough Insulin to reduce the blood glucose levels of 20 week old ob/ob mice (fig. 8.12B). One day antioxidant treatment of ob/ob mice was enough to rescue ob/ob mice from hyperglycemia (fig. 8.11B). Refreshing antioxidants in drinking water 5 days afterwards ensured this rescue could be prolonged for 15 days. Thereafter, we did not refresh the antioxidants in the drinking water, resulting in a re-establishment of hyperglycemia in ob/ob mice within 10 days. Heavily obese ob/ob mice ate 5.5 ± 0.4 g, which is 60 percent more than the 3.4 ± 0.2 g of the control group. Two days of antioxidant treatment were sufficient to reduce food intake by 26 percent to 4.1 ± 0.3 g which was no longer different from the control group (fig. 8.11B).
8.6. Discussion

Societal relevance of lipoatrophy syndromes seem small, because of low incidence numbers. However, these serious disease states of human kind need to be addressed by biomedical research [Rei00], especially because physiological mechanisms involved in development of lipoatrophy can increase our understanding of obesity related T2DM development [O’R02]. The induction of fat specific insulin receptor KO (iFIRKO) [Sak17], which was mediated by our Adiponectin-CreERT2 driver line, leads to short term reduction of subcutaneous adipose tissue depots in chow fed iFIRKO mice. Correlating with this fat loss, blood Leptin concentration was heavily reduced, but the epidydimal WAT depot and total fat mass was only changed after the metabolic phenotype of iFIRKO mice had already developed. This might be explained by depot specific differences in compensation for IR signalling loss by IGF-1R, which shows overlapping signaling pathway capacity, but needs to be tested further [Zie98, Bou10].

This induction of an incomplete reduction of adipose tissue depots, which rather compares to partial lipodystrophy [Sha00] distinguishes the here presented iFIRKO mouse model from other lipodystrophic mouse models [Moi98, Dua07], which were driven under aP2 promoter elements or could not be induced. Our results differ strongly from these published mouse models of inducible lipodystrophy, in which apoptosis of adipocytes was mediated by the induction of transgenic caspase 8 that was expressed under the aP2 promoter elements [Paj05]. Panjvani et al. showed that heavy lipodystrophy coincided with reduced serum insulin concentration, while in our model insulin concentration positively correlates with blood glucose concentration.

An observation from population-based longitudinal studies suggest a link between T2DM development and insulin resistance of adipose tissue T2DM [Loo01]. After a long phase of obesity development the diagnosis of T2DM coincided with a drop in body weight. A hypothesis describes short term insulin resistance in adipose tissue as a natural obesity defense mechanism that leads to T2DM when chronically extended [Sal12, Nol15]. Our findings that iFIRKO induces a reversible T2DM support this hypothesis. Although we could only observe differences in blood FFA concentration one day after Tamoxifen gavage, the overall energy metabolism switched from glucose to fat as the main energy source, what suggests that lipids reach the muscle where a fuel change from glucose to lipid takes place. The change of respiratory exchange ratio precedes the reduction of serum Leptin levels.

The sequence of unfoldng diabetic symptoms starts with hyperglycemia, is followed by hyperphagia and finally ends with the development of polydipsia. While the polydipsia is not unexpected, it has not been previously reported [Sak17]. The development of hyperphagia in ad libitum chow fed iFIRKO mice is more complicated than previously reported. In iFIRKO mice, we found, a hypophagic phase that coincides with the start of metabolic fuel change. The following hyperphagic phase starts some days after blood Leptin concentration is blunted. Extremely reduced glucose infusion rates at the steady state in iFIRKO mice during hyperinsulinemic euglycemic clamps prove that hyperglycemia is a consequence of peripheral insulin insensitivity. Furthermore, we showed that both muscle and liver are affected by iFIRKO. When hyperglycemia peaks, we found that muscle’s glucose uptake is reduced and hepatic glucose production is strongly increased. A lipid-induced mitochondrial oxidative stress was linked to
skeletal muscle insulin resistance and could explain reduced glucose uptake in muscle [Kov08]. The increase of hepatic glucose production rate in iFIRKO supports the hypothesis that liver might play a role in establishing the hyperglycemia of iFIRKO mice [Sak17].

Sakagushi et al. explained the reversibility of diabetes symptoms in iFIRKO mouse model by adipocyte redifferentiation, which correlates with reconstitution of normoglycemia of iFIRKO mice [Sak17]. Furthermore they attributed the diabetes development in iFIRKO mouse model to Leptin reduction, which they supported by Leptin replacement therapy mediated rescue from hyperglycemia. Leptin therapy also reverses hyperglycemia in lipoatrophic humans [Pet02]. As previously discussed, hyperglycemia was linked to ectopic lipid storage in liver and insulin resistance [Sof16]. While this might explain hyperglycemia in midterm or longterm mouse models, our findings in the obese iFIRKO mouse model, which allows the study of short term and reversible T2DM development, question the direct causative link of hyperglycemia and ectopic lipid storage in liver. We found that an extreme fatty liver develops by induction of fat specific insulin receptor KO in already obese iFIRKO mice. While the rescue from HFD induced obesity can be achieved by iFIRKO, in turn diabetes develops. Astonishingly, these once-obese iFIRKO mice with a massive fatty liver can recover from hyperglycemia similar to chow-fed iFIRKO mice within 4 weeks, although their adipose tissue does not fully regrow. This is in accordance with our findings in the chow fed mice that can also not fully recover their adipose tissue mass although normoglycemia is already established. When inducing the insulin receptor KO in adipose tissue before long term HFD feeding, mice are protected from obesity, which is further evidence for the inability of the adipose organ to fully recover. This finding especially highlights the differences between iFIRKO and the combined knockout of both IR and IGF-1R [Sak17].

Our experiments with diet induced obese iFIRKO mice address a major aspect of the question how obesity and insulin resistance in adipose tissue are connected. Adipose tissue, as other peripheral tissues, is supposed to become insulin resistant with the development of obesity. Our finding that heavily obese iFIRKO mice still react to the genetic ablation of IR in fat tissue with heavy reduction of fat mass suggests that adipose tissue is not fully insulin resistant in diet induced obesity. This is in line with the idea that adipose tissue differs between the obese and T2DM state regarding their insulin sensitivity and that T2DM correlates with insulin resistance of adipose tissue [Fra85, Che87, Zav94, Rea95].

Many studies have focused on antioxidants and their beneficial health effect regarding diabetes [Cab93, Ful02, Hir00, For01, Gol02]. In 2004, Furukawa et al. performed one of the most important experiments to address the question of how oxidative stress affect diabetes. One has to bear in mind that these experiments were limited by the complexity of KKAy mouse model. The KKAy mouse model is a combination of the KK strain, that develops diabetes of polygenic origin, with the Ay strain that becomes obese and mildly diabetic already before maturity [Iwa70, Kle95]. Based on their finding that antioxidants can reduce hyperglycemia of chronically obese and diabetic mice, and that this correlates with ROS production in adipose tissue, they proposed that oxidative stress in adipose tissue could trigger T2DM [Fur04]. Unfortunately, larger interventional trials with a wide variety of antioxidant food supplements could not confirm the proposed health benefit of antioxidants in general [Che06, Cze06, GP95, Liu06], what guided the American Diabetes Association to question the useful-
ness of antioxidants for treatment of T2DM in 2007 [Ass07]. The postulate that reactive oxygen species (ROS) can have beneficial health effects further questions the potential of antioxidants to protect from diabetes [Bje14]. A prominent example is that the diabetes-preventive effect of exercise was shown to be impeded when antioxidants were prospectively given to healthy men [Ris09]. These key findings lead to the hypothesis that Mitohormesis could explain the beneficial health effects of ROS [Ris14]. Only recently, further supportive evidence was given by two key studies that focused on the adipose tissue. When supplementing food of obese Zucker rats with polyunsaturated alpha linolenic acid (ALA) accompanying chronic exercise periods, epidydimal white adipose tissue was identified as the organ, in which ALA increases ROS production and decreases mitochondrial bioenergetics and attenuated insulin signalling [Mon17]. In contrast, ALA did not influence skeletal muscle’s adaptation to exercise effects on insulin sensitivity and mitochondrial bioenergetics. To genetically target adipocyte ROS production, Han et al. created the genetic mouse model with adipose tissue-specific knockout of manganese superoxide dismutase (MnSOD). The genetic ablation of the antioxidative enzyme MnSOD prevented diet-induced obesity [Han16], speaking in favor of a beneficial health effect of ROS in adipose tissue.

However, antioxidant-containing food supplements (e.g. apple poly-phenol extracts) have been suggested to improve insulin resistance in ob/ob mice [Ogu16] and Melatonin (as another antioxidant) was reported to improve non-alcoholic fatty liver disease (NAFLD) symptoms in ob/ob mice [Sta16]. Neither of these studies have mentioned food intake regulatory effects. Herewith, we report the food intake effect of the antioxidants Apocynin and Nac for the first time. Of note, initially observed during the hyperphagic phase of iFIRKO mice, we confirmed this hyperphagia-rescuing effect of Apocynin and Nac in the obese hyperphagic model of Leptin-deficient mice. The ob/ob mice lack Leptin, which lets us conclude that the antioxidants’ anti-hyperphagic effect is either Leptin-independent, or that Apocynin and Nac act downstream of Leptin. Other studies have already established a type of Leptin-independent hyperphagia. The mutation of serotonin 5-HT2C receptor gene can lead to Leptin-independent hyperphagia and T2DM in mice [Non98]. The hypophagic phase that preceeds the development of hyperglycemia and coincides with the RER change, lets us assume that the central nervous system and especially hypothalamic hunger regulatory regions are involved early on in the mediation of iFIRKO’s phenotype.

Our findings are in line with the hypothesis that some antioxidants can mediate beneficial health effects. While Furukawa et al. showed that Apocynin improves already established hyperglycemia, our experimental approach goes further and allows us to propose a health effect during development of T2DM. When testing the two antioxidants of our cocktail, Apocynin and N-acetylcystein seperately, we found that only Apocynin had the capacity to reduce hyperglycemia in iFIRKO mice. This suggests that the observed effect of Apocynin might be independent of general antioxidative capacity. Although it has been questioned by some experiments [Heu08, Vej05], Apocynin is widely used as an inhibitor of the NADPH oxidase (Nox) [Ste08,Pet13]. The assembly of Nox2 was suggested to be inhibited by Apocynin’s effect on Ncf1 (p47phox) translocation from cytosol to membrane [Ste08]. In microglia, Nox2 is a key superoxide producing enzyme [Bed07]. We hypothesize that Apocynin’s anti-inflammatory
effects could be responsible for the rescue of T2DM development. Several studies have already ad-
dressed the effect of Apocynin and derivatives on different organs. Apocynin dimerizes to diapocynin,
which effects many tissues e.g. muscle, liver and brain function. For the muscle, diapocynin was shown
to reduce ROS production and force loss in eccentrically contracting dystrophic muscle [Ism14]. Ini-
tially seen as a neutrophil oxidative burst antagonist, a joint swelling reducing effect of apocynin was
found in a mouse model of collagen-induced arthritis [Har90]. In rats, apocynin’s anti-inflammatory
effect was confirmed by the reduction of ulcerative skin lesions in a skin inflammation model [AH92].
Furthermore, Apocynin was shown to reverse the inflammation-induced inhibition of cartilage proteo-
glycan synthesis [Hou06a]. In a transgenic mouse model of Parkinson Disease, diapocynin was found
to prevent deficits in motor coordination [Dra13]. An anti-inflammatory effect of Apocynin in brain
was reported in a stroke mouse model, which is based on a transient middle cerebral artery occlu-
sion [Tan08]. Especially since focal cerebral ischemia causes the activation of microglia and thereby
inflammation of the brain, apocynin could counteract an inflammation of hypothalamus. Inflammation
of hypothalamus might be the consequence of lipid accumulation [Pos09]. The involvement of
hypothalamic inflammation would make sense, because it is proposed to be a starting point of hyper-
phagia development that leads to obesity [Tha12] and there is strong evidence for a brain-centered
gluoregulatory system (BCGS) [Sch13b]. Unlike Furukawa et al., we hypothesize that the target tissue
of Apocynin is not adipose tissue, but rather the hypothalamus, muscle and liver. Our findings suggest
that it could be worthwhile to clinically test beneficial health effects of Apocynin in both pre-diabetic
and diabetic patients.
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A. List of publications

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## B. Abbreviations

<table>
<thead>
<tr>
<th>abbreviation</th>
<th>word</th>
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<tbody>
<tr>
<td>ADA</td>
<td>American Diabetes Association</td>
</tr>
<tr>
<td>Adipoq</td>
<td>Adiponectin</td>
</tr>
<tr>
<td>BAC</td>
<td>bacterial artificial chromosome</td>
</tr>
<tr>
<td>BAT</td>
<td>brown adipose tissue</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>conc.</td>
<td>concentration</td>
</tr>
<tr>
<td>d</td>
<td>day</td>
</tr>
<tr>
<td>DNA</td>
<td>desoxyribonucleic acid</td>
</tr>
<tr>
<td>epiWAT</td>
<td>epidydimal white adipose tissue</td>
</tr>
<tr>
<td>et al.</td>
<td>et alii (and others)</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acid</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
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<tr>
<td>HbA1c</td>
<td>haemoglobin A1c</td>
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<tr>
<td>HFD</td>
<td>high fat diet</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>H2O</td>
<td>water</td>
</tr>
<tr>
<td>H2O2</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>iBAT</td>
<td>interscapular brown adipose tissue</td>
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<tr>
<td>icv</td>
<td>intra cerebro ventricular</td>
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<tr>
<td>iFIRKO</td>
<td>inducible fat-specific insulin receptor knockout</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>ingWAT</td>
<td>inguinal white adipose tissue</td>
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<tr>
<td>IR</td>
<td>Insulin Receptor</td>
</tr>
<tr>
<td>IR-KO</td>
<td>Insulin Receptor knockout</td>
</tr>
<tr>
<td>IU</td>
<td>international units</td>
</tr>
<tr>
<td>kg</td>
<td>kilo gram</td>
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<tr>
<td>KO</td>
<td>knockout</td>
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### Continuation of Table B.1

<table>
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<tr>
<td>M</td>
<td>mole per liter</td>
</tr>
<tr>
<td>m</td>
<td>milli</td>
</tr>
<tr>
<td>μ</td>
<td>micro</td>
</tr>
<tr>
<td>mg</td>
<td>milli gram</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>Nac</td>
<td>N-acetylcystein</td>
</tr>
<tr>
<td>O2</td>
<td>Oxygen</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisomal proliferator activated receptor</td>
</tr>
<tr>
<td>Tam</td>
<td>Tamoxifen</td>
</tr>
<tr>
<td>TAG</td>
<td>triacylglyceride</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 Diabetes Mellitus</td>
</tr>
<tr>
<td>TNFa</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>Ucp1</td>
<td>Uncoupling protein 1</td>
</tr>
<tr>
<td>WAT</td>
<td>white adipose tissue</td>
</tr>
<tr>
<td>WB</td>
<td>western blot</td>
</tr>
</tbody>
</table>
C. Closing remarks

"Faust: Habe nun, ach! Philosophie, Juristerei und Medizin, Und leider auch Theologie Durchaus studiert, mit heissem Bemühn. Da steh ich nun, ich armer Tor! Und bin so klug als wie zuvor;"

Monologue from Faust I (Johann Wolfgang Goethe)

Zusätzlich möchte ich noch meinem Onkel Wanja danken, der mir im Aufzug in Tschechow die Inspiration für das dritte Kapiel meiner Doktorarbeit lieferte.