Thioredoxin family proteins in the db/db mouse

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Contents

1 Introduction

1.1 The islets of Langerhans

In 1869, German pathologist Paul Langerhans discovered the cell-clusters in the pancreas which we today know as the Islets of Langerhans. Those nests of cells are responsible for the endocrine functions of the organ (Langerhans, 1869). Around 1 million islets are distributed throughout the human pancreas. Their total contribution to the mass of the organ is around 2 % to 3 %. Five types of endocrine cells are known to exist in each of those micro-organs: Alpha-cells produce glucagon, which promotes glycogenolysis in the liver; beta-cells secrete insulin and amylin, which are needed for glucose uptake and inhibition of lipolysis; gamma-cells release pancreatic polypeptide, an important self-regulating factor; delta-cells are responsible for somatostatin, an inhibiting polypeptide; and epsilon-cells account for ghrelin, which has an influence on appetite. The respective amount of every cell type differs between species, but beta-cells usually form the majority, followed by the alpha-fraction (Jones and Persaud, 2010) (table 1.1).

type	**amount**	**product**	**effect**
alpha	20 % to 30 %	glucagon	increases glycogenolysis in the liver
beta	˜60 %	insulin	necessary for glucose uptake, inhibits lipolysis
		amylin	synergistic to insulin, inhibits glucagon
gamma	<5 %	pancreatic polypeptide	regulates pancreatic secretion
delta	˜10 %	somatostatin	inhibits the release of other hormones
epsilon	˜1 %	ghrelin	stimulates appetite and the release of growth hormone

Table 1.1: Endocrine pancreatic cells (Jones and Persaud, 2010).

Insulin is the only hormone which is able to actively lower blood glucose levels and to inhibit lipolysis. Therefore, the insulin-producing beta-cells are the lynchpin of the pathophysiology of diabetes mellitus.

1.2 Diabetes mellitus

1.2.1 Definition and aetiology

Diabetes mellitus is a metabolic disorder defined by chronic hyperglycaemia. However, there are various aetiological reasons for the pathological glucose homeostasis. Most common are

diabetes mellitus type 1 and 2. Type 1 diabetes is characterised by destruction of the beta-cells leading to a lack of insulin. This destruction takes place because of autoimmune processes or happens idiopathically. In contrast, type 2 diabetes occurs due to resistance of tissue against insulin action on the one hand and an inadequate secretion of the hormone on the other hand. Furthermore, diabetes can be caused by various genetic defects, diseases of the exocrine pancreas, endocrinopathies, infections and it can also be induced by drugs. Diabetes occuring during pregnancy is called gestational diabetes (American Diabetes Association, 2003). The different variations of the disease and their aetiology are summarised in table 1.2.

Form	**Aetiology**
I - Type 1	A. Immune mediated B. Idiopathic
II - Type 2	Insulin resistance, relative insulin deficiency, secretory defect
III - Other forms	A. Genetic defects of beta-cell function B. Genetic defects in insulin action C. Diseases of the exocrine pancreas D. Endocrinopathies E. Drug- or chemical-induced F. Infections G. Uncommon forms of immune-mediated diabetes H. Other genetic syndromes sometimes associated with diabetes
IV	Gestational diabetes mellitus

Table 1.2: Aetiology of diabetes mellitus (American Diabetes Association, 2003).

There is an involvement of a genetic component in both type 1 and 2 diabetes. However, the predisposition is even stronger for type 2 than for the autoimmune form of type 1. Genetically characterised forms of type 2 diabetes include maturity onset diabetes of the young (MODY), defects in the insulin or insulin receptor gene as well as mutations in mitochondrial genes and in glucose transporters (Kahn et al., 1996). The genetic predisposition of diabetes can be understood by the following scientific facts: It is estimated that the risk for developing type 2 diabetes is 10-fold higher for a monozygote twin if the other twin has the disease, it is 3.5-fold higher for first-degree relatives and for second-degree relatives 1.5-fold higher in comparison to the general population (Rich, 1990). Furthermore, obesity is an important risk factor for type 2 diabetes and it is suspected that there is an important link between adiposity and diabetes apart from a genetic one (Eckel et al., 2011). Hence, life-style factors and genetics play an important role in predisposing an individual to diabetes mellitus.

1.2.2 Epidemiology

Diabetes mellitus plays a major role in global health. A large meta study by the International Diabetes Federation depicts the global prevalence of the disease and estimates its development for adults aged 20 to 79 years. The so-called Diabetes Atlas was published for the first time in the year 2000. Since then, it has been updated regularly. Data from hundreds of studies worldwide are used. Most of them are taken from peer-reviewed publications, but also national health surveys and official reports from international agencies and government surveys are included (Guariguata et al., 2011).
According to the atlas more than 371 million people worldwide were suffering from diabetes in the year 2012 (International Diabetes Federation, 2012) and incidence is rapidly increasing. In 2003 the federation predicted a total of 334 million diabetic people for 2025 (International Diabetes Federation, 2003). As this number has already been surpassed, new estimates expect a total of 552 million people to be affected by the disease for the year 2030.
Whereas 5 % to 10 % of patients suffer from type 1 diabetes, the vast majority of diseases (around 85 % to 95 %) are categorized as type 2 (International Diabetes Federation, 2012). Thus, the rising incidence in diabetes is mainly caused by new cases of type 2. For example, from 1990 to 2010 the number of patients aged 18 to 79 in the USA almost tripled (Geiss and Gowie, 2010).
Based on data of the AOK, a German health insurance company which represents around one third of Germany's population, the prevalence of diabetes rose from 5.9 % in 1998 to 9.7 % in 2009 (Hauner et al., 2007; Köster et al., 2012). Furthermore, the prevalence turned out to be markedly higher in people aged 60 years and above. While below 5 % at the age of 40 were treated because of diabetes in the year 2004, it was around 20 % aged 60 depending on the sex (Hauner et al., 2007). Specifically regarding the prevalence of diabetes type 2, a meta study merged the data of five regional studies dating from 1997 to 2006. The results showed a different number of type 2 diabetics regionally varying from 5.8 % to 12 % (Schipf et al., 2012). In addition, the rising incidence of diabetes type 2 in children and young adults should be considered. A study with 520 adipose subjects aged 9 to 20 years showed that 1.5 % suffered from type 2 diabetes and 5.8 % had an impaired glucose tolerance (Wabitsch et al., 2004). Both, ageing of the population and the growing number of obese people are main factors of growing diabetes type 2 incidence and prevalence.

1.2.3 Symptoms and long-time consequences

Symptoms of considerable hyperglycaemia are polyuria, polydipsia, loss of weight and blurred vision. Severely high blood sugar levels cause life-threatening ketoacidosis and hyperglycaemic nonketotic coma. In the long run its influence on the vascular system leads to macro- and microangiopathy. As a consequence of the macroangiopathy the incidence of cardial, cerebral and

peripheral vascular disease is increased in patients suffering from diabetes, manifesting in a higher rate of myocardial infarctions and strokes. Symptoms of microangiopathical alterations are retinopathy, nephropathy and neuropathy. In consequence, patients are in danger of losing their eyesight, kidney failure, ulcers and amputations as well as gastrointestinal and genitourinary dysfunction (American Diabetes Association, 2003). An overview of the relative risk for several long-time complications compared to nondiabetics / the general population is shown in table 1.3.

complication	**relative risk**
myocardial infarction	3.7 (males) 5.9 (females)
cardiovascular death	9.1 (patient $<$ 30 years old at diagnosis) 2.3 (patient $>$ 30 years old at diagnosis)
stroke	2 - 4
blindness	5.2
renal failure	12.7 (males)
amputation	22.2 (lower limbs)
foot ulcers	45

Table 1.3: Complications of diabetes mellitus (DDG, 2004).

1.2.4 Treatment of diabetes mellitus

The main aim of diabetes therapy is to reduce acute complications as well as long-term consequences of the disease. This goal is achieved by maintaining blood glucose control. Since the fist extraction of insulin from a canine pancreas (Paulescu, 1921), exogenously produced hormone is used as a subsitute for patients suffering from diabetes (Banting et al., 1922). Type 1 diabetics rely on exogenous insulin in the vast majority of cases. Thus, substitution is the main therapeutic strategy. Other options, which are not in routine clinical practice, are pancreas and islet transplantation (DDG, 2011). Patients suffering from type 2 diabetes are usually treated with oral antidiabetics without or in combination with insulin. Those drugs include sulphonylureas and their analogs, biguanides, alpha-glucosidase inhibitors, glitazones, DPP-4 inhibitors and GLP-1 agonists (DDG, 2009). Several studies show the efficacy of diabetes therapy. In the Diabetes Control and Complications Trial researchers compared the risks of long-term complications between type 1 diabetics who were treated intensively and those who received conventional therapy. The study showed that retinopathy can be reduced by 76 %, nephropathy by 56 % and neuropathy by 69 % (The Diabetes Control and Complications Trial Research Group, 1993). The UK Prospective Diabetes Study revealed a similar benefit for type 2 diabetics. A 25 % risk reduction for microvascular disease, 12 % for any diabetes-related endpoint, 10 % for

any diabetes-related death and 6 % for all-cause mortality were found (UK Prospective Diabetes Study (UKPDS) Group, 1998).
Besides, changes in life-style represent a very important pillar of the treatment. Losing weight, physical activity and healthy nutrition can reduce the symptoms of diabetes or even delay the onset (Narayan et al., 2002).
However, the major risk of therapeutic blood glucose control is hypoglycaemia. This severe draw-back should be accounted for in patient education and individual therapy regimes (DDG, 2009; UK Prospective Diabetes Study (UKPDS) Group, 1998).

1.2.5 Pathophysiology of diabetes mellitus type 2

Diabetes mellitus type 2 is a multi-factorial disease, consisting of a lowered insulin sensitivity in the insulin-dependent organs, raised gluconeogenesis in the liver, an impaired insulin secretion in the pancreas as well as apoptosis of the beta-cells (American Diabetes Association, 2003). Influences are shown in figure 1.1.
There are several mechanisms which lead to insulin resistance. Receptor binding and post-

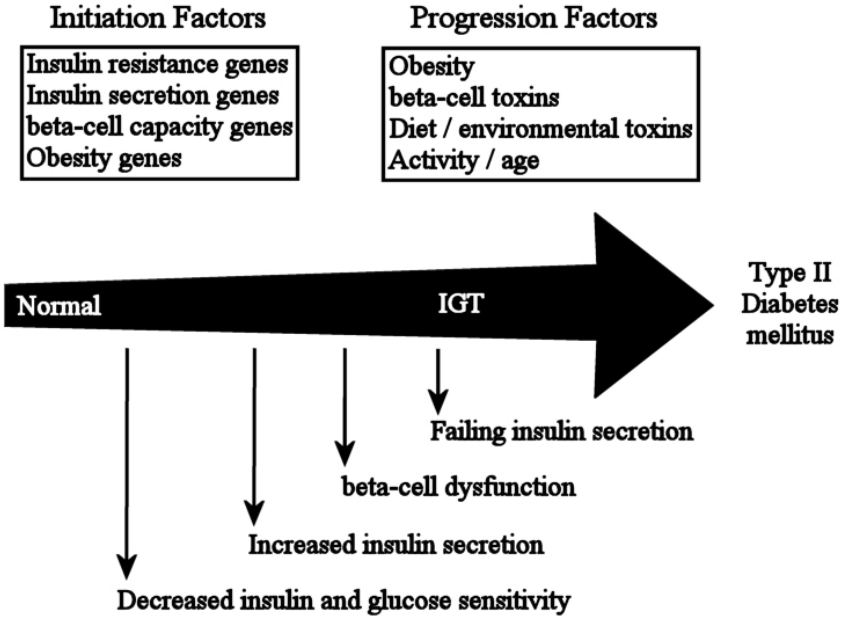


Figure 1.1: Diabetes mellitus type 2 as a multi-factorial disease (Kahn et al., 1996). There are genetic initiation factors which predispose an individual to diabetes. The progress from a physiological glucose homeostasis to a manifest diabetes is furthermore accelerated by several progression factors such as obesity, diet and activity. A relative lack of insulin due to decreased secretion and sensitivity leads to hyperinsulinaemia and a state of impaired glucose tolerance. The beta-cells take damage and become dysfunctional, leading to an absolute lack of insulin and manifesting the disease.

binding defects play an important role, including defective glycogen synthesis and lowered insulin receptor tyrosine kinase activity. Furthermore, the insulin receptor is down-regulated by

chronic hyperinsulinaemia and its signal transduction is impaired.
At the beginning of the disease an elevated insulin secretion compensates for the insulin resistance. In response to raised fasting blood glucose levels the beta-cells are able to increase the insulin concentration up to a blood glucose level of 140 mg dl^{-1}. A peak is reached where the insulin concentration in the patient suffering from diabetes type 2 is up to 2.5-fold higher than in nondiabetic subjects. However, higher blood glucose levels cause the insulin production to lower quickly, resulting in a minimal response from 200 mg dl^{-1} onwards.
Physiologically, the hepatic glucose output is down-regulated by hyperinsulinaemia and hyperglycaemia. For this regulation becomes insufficient, subjects with fasting hyperglycaemia also show an elevated gluconeogenesis in the liver (DeFronzo, 2004).
Another consequence of the chronic influence of nonphysiologically high glucose concentrations is beta-cell dysfunction. This concept is called glucotoxicity. On the one hand, hyperglycaemia has an impact on the gene level. For example the transcription of Pdx1, which plays an important role in terms of insulin promotor regulation, is impaired and islets lose DNA binding activity of Pdx1 when exposed to high blood glucose levels. As a result, insulin gene expression is impaired, too. On the other hand, desensitisation of beta-cells to high glucose concentrations occurs. In contrast to glucose toxicity the cells can recover from desensitisation during which no insulin secretion is possible due to depletion (Robertson et al., 2003). Moreover, it is suspected that elevated levels of fatty acids have a similar impact on the beta cells. Although the exact effects are still not clear, there is evidence that the so-called lipotoxicity also impairs insulin gene expression as well as secretion and induces beta-cell apoptosis (Robertson et al., 2004).
In conclusion, it was shown that in patients with diabetes type 2 beta-cell volume is reduced by up to 63 % and beta-cell apoptosis was up to 10-fold higher compared to nondiabetic subjects (Butler et al., 2003).

1.2.6 Diabetes mellitus and oxidative stress

Other than glucose toxicity and damage by free fatty acids another parameter also plays an important role in the molecular pathophysiology of diabetes: The noxious effect of reactive oxygen (ROS) and nitrogen species (RNS), summarised as oxidative stress. This condition occurs when there is a constant imbalance between the production of free radicals that cause oxidative damage and cellular antioxidant defence mechanisms (Evans et al., 2003).
On the molecular level, oxidative stress arises from molecules with unpaired electrons. Such molecules with unpaired electrons are highly reactive and can cause damage to cellular organelles. Oxidative damage can occur due to heat, ultraviolet and ionising radiation, inflammatory cytokines and redox reactions. An important cellular source of ROS and RNS is the mitochondrial respiratory chain and the redox reactions happening in it. During oxidative phosphorylation molecular oxygen is reduced, resulting in a partly conversion to $\cdot O_2^-$. Superoxide is eliminated by the formation of H_2O_2 by superoxide dismutase 2. Catalase then converts hydro-

gen peroxide to H_2O and O_2. However, the highly reactive $\cdot$OH or other ROS and RNS can also arise from hydrogen peroxide and thus lead to oxidative stress. The formation of $\cdot$OH out of H_2O_2 has already been described in 1894 by Henry Fenton and is thus called "Fenton reaction" (Fenton, 1894; Boveris, 1977). An overview of some reactive species is given in table 1.4.

ROS	**RNS**
$\cdot O_2^-$ (Superoxide)	$\cdot$NO (Nitric oxide)
$\cdot$OH (Hydroxyl)	$\cdot NO_2^-$ (Nitrogen dioxide)
$\cdot RO_2$ (Peroxyl)	
$\cdot HO_2^-$ (Hydroperoxyl)	

Table 1.4: Examples of reactive species (Evans et al., 2002).

While redox reactions are a physiological and vital part of every cell, the imbalance between oxygen free radical formation and its elimination via cellular defence mechanisms can cause oxidative damage to proteins and can change their function. This can trigger stress-sensitive pathways, among which NF-kappaB, c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein. Additionally, lipids and DNA molecules can directly be oxidised and thus get damaged (Evans et al., 2003).
Consequently, oxidative stress plays an important role in many diseases' aetiology, including diabetes. Besides glucose autoxidation and glycation of proteins, hyperglycaemia increments the production of ROS in mitochondria (Wolff and Dean, 1987). Furthermore, free fatty acids increase ROS by promoting beta-oxidation and mitochondrial uncoupling (Carlsson et al., 1999). A good example for the connection between radicals and diabetes is the NF-kappaB-pathway. It is activated by oxidative stress, hyperglycaemia and elevated free fatty acids (Mohamed et al., 1999). NF-kappaB is located in the cytoplasm and activated by phosphorylation of its inhibitory subunit (Karin and Ben-Neriah, 2000). It regulates the expression of several growth and proinflammatory factors, for example VEGF, TNF-alpha and IL-1beta. It was found that NF-kappaB activation correlates with the degree of albuminuria in diabetics (Hofmann et al., 1999).
There is evidence that the formation of ROS is increased and antioxidant defences are reduced in diabetics and that oxidative stress is a part of the early stages of its pathophysiology. The oxidative stress is measurable before the disease becomes clinically apparent and related to its onset (Maxwell et al., 1997; Nourooz-Zadeh et al., 1997). Furthermore, studies found that high levels of reactive species not only correlate with the development of vascular and neurological complications and proteinuria, but also with insulin resistance (Yaqoob et al., 1993; Opara et al., 1999). In conclusion, figure 1.2 summarises the influence of oxidative stress on the pathophysiology of diabetes mellitus type 2.

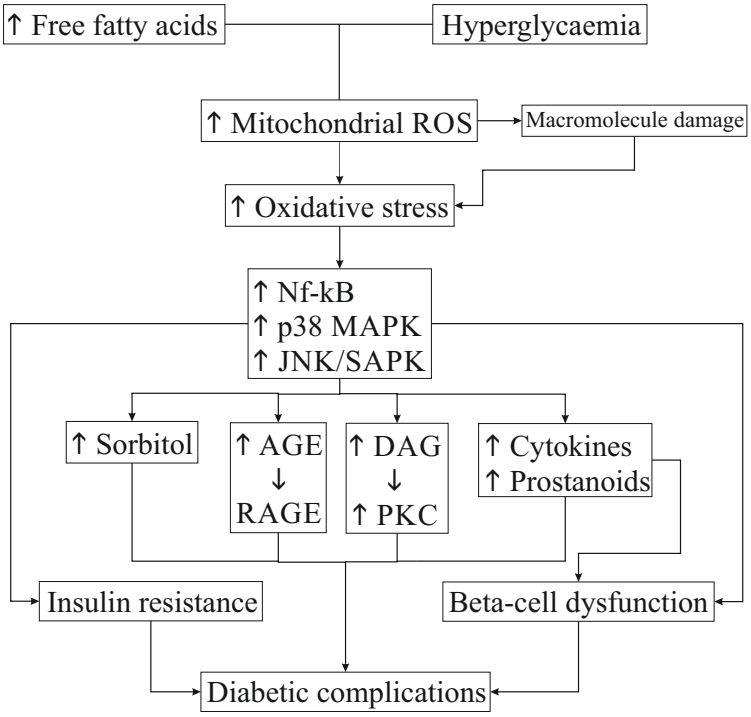


Figure 1.2: The role of oxidative stress in the pathophysiology of diabetes mellitus type 2 (Evans et al., 2003). Gluco- and lipotoxicity induce oxidative stress and thereby trigger stress-sensitive pathways. Ultimately, the beta-cell is rendered dysfunctional.

Regarding the cellular level, it has to be taken account of the fact that the beta-cell has a lower antioxidant defence capacity than other cells. Gene expression of several enzymes, including superoxide dismutase, catalase and glutathione peroxidase was shown to be markedly lower or even not detectable in mouse islets in comparison to other tissues (Lenzen et al., 1996). The physiology of insulin secretion is disrupted by oxidative imbalance in various ways. To begin with, the amount of GLUT2 (Glucose transporter 2) in the membrane of beta-cells is reduced in db/db mice and animals stressed with high carbohydrate diet (Thorens et al., 1992; Kluth et al., 2011). Furthermore, the Krebs cycle is a target for oxidative stress. On the one hand, its enzymes are directly inhibited by oxidants. H_2O_2 for example interferes sharply with the alpha-ketoglutarate dehydrogenase complex (Tretter and Adam-Vizi, 2000). On the other hand, anaplerosis and cataplerosis are sensitive toward oxidants and their disruption has negative impact on insulin secretion. For instance, suppression of pyruvate carboxylase activity results in a lack of substrate for the Krebs cycle and thereby adverses insulin secretion (Hasan et al., 2008). Also, mitochondria themselves are altered in terms of morphology and function, presenting changes in protein expression and density volume (Anello et al., 2005). Moreover, the ATP-sensitive potassium channels in the beta-cell's cell membrane are affected by oxidative stress directly as well due to hyperpolarisation of the cell membrane (Islam et al., 1993; Krippeit-Drews et al., 1994). In conclusion, glucose uptake, processing in the Krebs cycle and coupling between mitochondria and ATP-sensitive potassium channels are influenced by oxidants, accounting for a deteriorated insulin secretion under oxidative stress.

1.3 Thioredoxin family proteins

In defence against oxidative stress, cells use redox signalling in order to re-establish redox balance after exposure to reactive species (Dröge, 2002). Very important actors in redox signalling are thioredoxin (Trx) family proteins. This group of proteins is named after the first discovered thioredoxin. While it has already been found in the year 1964 (Laurent et al., 1964), the common link between Trx family proteins - their basic three-dimensional structure - was only described in 1995. It consists of a four-stranded beta-sheet and three encircled alpha-helices with the motif Cys-x-x-Cys as its active region (Martin, 1995). Among the members of the Trx family are glutaredoxins (Grx), peroxiredoxins (Prx) and thioredoxins.

1.3.1 The thioredoxin system

In 1964, Trx was discovered as an essential factor for DNA synthesis in E. coli. It acts as an electron donor for ribonucleotide reductase and is thus a vital part of every organism (Laurent et al., 1964). Knockout mice in deficiency of TRX1, 2 or the respective reductase are unviable or die as embryos (Matsui et al., 1996; Nonn et al., 2003; Jakupoglu et al., 2005; Conrad et al., 2004). Trx features the active site Cys-Gly-Pro-Cys (Holmgren, 1968) which is present in both major isoforms. Trx1 and TrxR1 are located in the cytoplasm and in the extracellular space (Rubartelli et al., 1992; Söderberg et al., 2000). It was also shown that it can move into the nucleus under certain conditions (Makino et al., 1999). Trx2 and its reductase are found in the mitochondria. Beyond its important role for DNA synthesis, the Trx system acts as a key regulator of redox signalling. Its influence on the redox state is gained by a simple dithiol mechanism. Thioredoxin (Trx-$(SH)_2$) reduces the disulfide in the substrate (P-S_2) and is in turn reduced by thioredoxin reductase (TrxR-$(SH)_2$) which uses NAPDH as electron donor (Figure 1.3) (Holmgren, 1985).

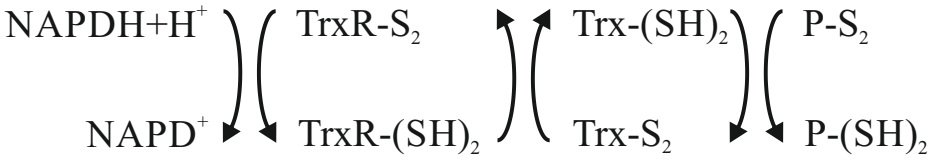


Figure 1.3: The dithiol mechanism of Trx.

Apart from its involvement in maintaining the cellular redox state, Trx is strongly linked to cell proliferation and apoptosis (Powis and Montfort, 2001). Reduced Trx forms a complex with ASK1, a MAP kinase. If oxidative stress occurs and Trx becomes oxidised, ASK1 triggers p38 and JNK pathways, inducing apoptosis (Saitoh et al., 1998). Additionally, several transcription factors are regulated by Trx. An important example is again NF-kappaB, whose DNA binding activity is altered by the dithiol mechanism. Thus, the expression of proinflammatory genes is

indirectly controlled by redox signalling (Hayashi et al., 1993).
Other members of the thioredoxin system are thioredoxin-interacting protein (Txnip) and Nucleoredoxin (Nrx).

1.3.2 Peroxiredoxins

Peroxiredoxins reduce hydrogen peroxide, peroxynitrite and organic hydroperoxides. In contrast to catalase and glutathione peroxidase, Prx use their redox-active cysteines to achieve this goal. There are six different Prx which can be categorized according to the number of cysteinyl residues which take part in their redox reaction. Typical double-cysteine (Prx1 - 4) and atypical double-cysteine Prx (Prx5) have two cysteines in their active site while Prx6 is categorized as single-cysteine. The second part of the two-step reducing reaction depends on the number of available cysteines. Firstly, one active-site cysteine (Cys-SH) is oxidised to cysteine sulfenic acid (Cys-SOH). In 2-Cys Prx this sulfenic acid forms a disulfide with the second cysteine (atypical double-cysteine Prx) or a second Prx molecule (typical double-cysteine Prx) and is then reduced by Trx. Due to the lack of a second cysteine, 1-Cys Prx require GSH for reduction (Rhee et al., 2001) (Figure 1.4).

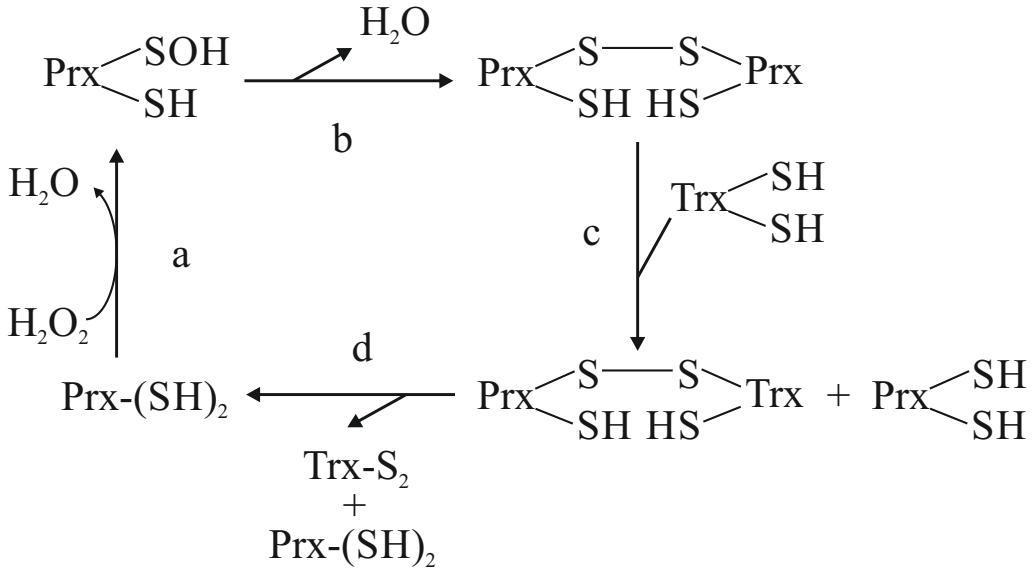


Figure 1.4: The Prx pathway. a) Detoxification of H_2O_2, b - d) Restoration of Prx using the example of a typical double-cysteine Prx.

Due to their function as peroxidase, Prx are important scavengers of cellular H_2O_2. Thereby, they also have influence on cell proliferation and apoptosis (Fujii and Ikeda, 2002). Prx can be found in every cellular compartment and are form around 0.7 % of the soluble protein in mammalian cells (Seo et al., 2000). Prx1 and 2 are mainly found in the cytoplasm and the nucleus, Prx3 only in the mitochondria, Prx4 in the cytosol and the endoplasmatic reticulum, Prx5 in the cytoplasm, mitochondria and peroxisomes and Prx6 in the cytosol. The consequences

of Prx deficiency were demonstrated in several models. Prx1 knockout mice have a decreased lifespan. At about 9 months of age they develop haemolytic anaemia due to raised ROS levels in erythrocytes. Furthermore, the rate of cancer is increased: Mice suffer from lymphomas, sarcomas and carcinomas (Neumann et al., 2003). The effects in Prx2 deficient mice are similar. Anaemia with deformed erythrocytes and elevated ROS levels are present in these animals (Lee et al., 2003). A recent study suggests that Prx3 knockout mice show adipocyte hypertrophy as well as impaired glucose tolerance and insulin resistance (Huh et al., 2012). Moreover, increased intracellular ROS levels were found (Li et al., 2007). Testicular atrophy is observed in Prx4 deficient mice (Iuchi et al., 2009) and overexpression of Prx5 was found to inhibit the p53-dependent generation of ROS and thus to prevent apoptosis (Zhou et al., 2000). Ultimately, mice lacking Prx6 are characterised by low survival rates and raised protein oxidation (Wang et al., 2003).

1.3.3 The glutaredoxin system

The glutaredoxin system had been first described in the 1970s as a hydrogen transport system consisting of NAPDH, glutathione, glutathione reductase and glutaredoxin. It was found to reduce ribonucleotides and thus to ensure DNA synthesis in an E. coli mutant lacking Trx. In mammals, there are four glutaredoxins which can be grouped as dithiol Grx with two cysteine residues in their active site (Grx1 and 2) and monothiol Grx with only one cysteine (Grx3 and 5) (Holmgren, 1976, 1979). By definition, Grx use GSH as substrate. According to the number of cysteine residues in the active site, two different mechanisms in the Grx reaction can be differentiated. In the dithiol mechanism, the N-terminal Cys of the active site and its substrate form a disulfide by nucleophilic substitution. This disulfide is then reduced by the C-terminal Cys of Grx, resulting in oxidised Grx (Grx-S_2) and its reduced substrate (P-$(SH)_2$). Grx then again is reduced by GSH. In the monothiol mechanism the N-terminal Cys interacts with the GSH residue of the substrate (P-S-SG) and creates Grx-S-SG which is again reduced by GSH. Oxidised GSSG then again is reduced by glutathione reductase using NAPDH as electron donor (Figure 1.5) (Holmgren, 1978; Bushweller et al., 1992).

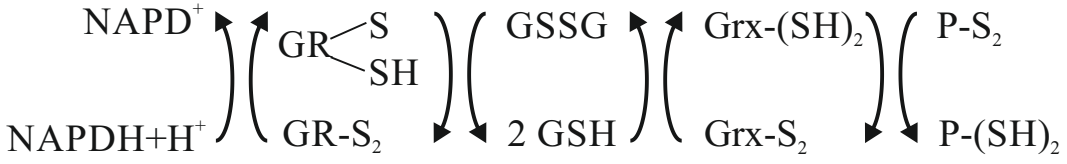


Figure 1.5: The dithiol mechanism of Grx.

Grx1 with the active site Cys-Pro-Tyr-Cys is mainly located in the cytoplasm, but it was also found in the nucleus and in the intermembrane space of mitochondria (Lundberg et al., 2004; Pai et al., 2007). The protein is a major actor in the thiol-disulfide exchange (Holmgren, 1989). Besides, it was shown that it plays a role in cell differentiation (Takashima et al., 1999) and regulates several transcription factors among which NF-kappaB, AP-1, CREB and AKT1 (Hirota et al., 2000; Daily et al., 2001). Thus, it also protects cells from apoptosis (Chrestensen et al., 2000; Murata et al., 2003). Embryonic fibroblasts of Grx1 deficient mice are more susceptible to deleterious effects of some chemical agents (Ho et al., 2007).
There are two isoforms of Grx2 of which one (Grx2a) is located in the mitochondria and the other (Grx2b) was found in the nucleus (Lundberg et al., 2001). Besides, testis- and cancer-specific isoforms exist (Lönn et al., 2008). Grx2 can be reduced by TrxR and forms an iron-sulfur cluster which acts as a redox sensor and activates it (Berndt et al., 2007; Lillig et al., 2005). It regulates the redox state in the mitochondria (Beer et al., 2004) and protects cells from apoptosis (Enoksson et al., 2005). In Grx2 knockout mice it was shown that a lack of this protein leads to increased susceptibility to oxidative stress (Wu et al., 2011).
Grx3 resides in the cytosol and the nucleus. Similar to Grx2, it can also form iron-sulfur clusters (Haunhorst et al., 2010) and is required for the biosynthesis of haem (Haunhorst et al., 2013). Grx3 inhibits NF-kappaB and JNK pathways and thus apoptosis (Witte et al., 2000). Moreover, it was found to be a modulator of T cells (Kato et al., 2008). A recent study found that mice overexpressing Grx3 are protected against cardiac hypertrophy and show enhanced cardiomyocyte contractility (Jeong et al., 2006). In contrast, Grx3 knockout mice are embryonic lethal (Cha et al., 2008).
Grx5 is required for the assembly of iron-sulfur clusters in the mitochondria. A lack of the protein thus leads to an accumulation of iron and inactivation of enzymes which require iron-sulfur clusters (Rodríguez-Manzaneque et al., 2002). It is necessary for physiological haem biosynthesis (Ye et al., 2010): Studies show that anaemia and embryonic death is caused by deficiency of Grx5 in zebrafish (Wingert et al., 2005) and in 2007 a human male lacking Grx5 was reported to suffer from anaemia (Camaschella et al., 2007).

A summary of the previously discussed Trx family proteins is given in table 1.5.

Rx	Location	Function	Knockout effect
Trx1	Cytoplasm, extracellularly, nucleus	DNA-synthesis, regulator of redox signalling, anti-apoptotic	unviable or embryonic lethal
Trx2	Mitochondria		
Prx1	Cytoplasm, nucleus	Detoxification of hydrogen peroxide, peroxynitrite and organic hydroperoxides, anti-apoptotic	anaemia and cancer
Prx2	Cytoplasm, nucleus		anaemia
Prx3	Mitochondria		adipocyte hypertrophy, IGT, insulin resistance
Prx4	Cytoplasm, endoplasmatic reticulum		testicular atrophy
Prx5	Cytoplasm, mitochondria, peroxisomes		overexpression inhibits apoptosis
Prx6	Cytoplasm		low survival rates
Grx1	Cytoplasm, nucleus, mitochondria	DNA-synthesis, anti-apoptotic	increased susceptibility to some agents
Grx2	Mitochondria, nucleus	redox-sensor, anti-apoptotic	increased susceptibility to oxidative stress
Grx3	Cytoplasm, nucleus	immune signalling, anti-apoptotic, haem synthesis	embryonic lethal
Grx5	Mitochondria	assembly of enzymes with iron-sulfur clusters, haem synthesis	embryonic lethal, anaemia

Table 1.5: Summary of the Trx family proteins.

1.4 The db/db mouse

The spontaneous mutant strain of obese black mice was firstly described in the year 1966 (Hummel et al., 1966). However, only after 30 years the underlying genetics was identified. A point mutation in the downstream intron of the transmembrane leptin receptor Ob-Rb gene causes a splicing mutation and renders those animals unresponsive to leptin (Chen et al., 1996). This hormone is released by adipocytes und plays a major role in energy homeostasis and eating behaviour (Friedman and Halaas, 1998). In the hypothalamus it triggers inappetence and thus eventually loss of weight. Therefore, homozygote mice show unrepressed eating behaviour leading to obesity and insulin resistance, hyperinsulinaemia, hyperglycaemia, and ultimately insuffiency of insulin secretion. As a consequence, this strain is used as an animal model for type 2 diabetes (Coleman, 1978; Tuman and Doisy, 1977).

1.5 Aims of the study

The impact of diabetes starting from its very early stages, via the clinical onset through to the late complications has been outlined. There is a marked influence on the physiology of the body, especially on the islets of Langerhans and insulin as their product. Hyperglycaemia, free fatty acids and oxidative stress play a major role as from the early pathophysiology of the disease. The imbalance between reactive oxygen and nitrogen species on the one hand, and cellular antioxidant defences on the other hand have become an important focus of research. It has been shown that reactive molecules are increased in diabetics. Thus, we wanted to study how apoptosis of islets and diabetes are connected with defective redox signalling.
In this study homozygote BKS(D)-Leprdb/JOrlRj (db/db) mice were compared with heterozygote BKS(D)-Leprdb/JOrlRj Témoin (db/+) counterparts in terms of general development and the changes in the islets of Langerhans. Furthermore, the behaviour of Grx, Prx and Trx in the islets over time were observed. In detail, the aims of this study were:

- to observe the changes in body weight and blood glucose level with time in db/db mice and their heterozygote counterparts.
- to qualify and quantify the changes of the islets of Langerhans in terms of morphology, number and area.
- to study the changes in insulin gene and protein expression.
- to describe proliferation and apoptosis of the beta cells.

- to qualitatively analyse the proteins of the Grx, Prx and Trx systems in the islets and the surrounding exocrine tissue.

- to further analyse Grx1 and 5 and their gene and protein expression.

- to detect ROS in isolated islets of db animals.

- to gain insight into the role of leptin by administering of the hormone to MIN6 cells and analysing the protein levels of insulin, Grx1 and 5.

2 Materials and methods

2.1 Materials

2.1.1 Instruments

Centrifuge Biofuge 13	Heraeus (Hanau, Germany)
Centrifuge Universal 320R	Hettich (Tuttlingen, Germany)
Confocal scanning microscope SP2 AOBS	Leica (Bensheim, Germany)
Crysostat CM1850	Leica (Wetzlar, Germany)
Digital microscope camera DFC 420 C	Leica (Wetzlar, Germany)
Fluorescence microscope LB30T	Leica (Wetzlar, Germany)
Glucometer OneTouch Ultra 2	LifeScan (Neckargemünd, Germany)
Incubator T-6030	Heraeus (Hanau, Germany)
Magnetic stirrer Ikamag RCT	Ika (Staufen, Germany)
Microplate Reader Mithra LB940	Berthold (Bad Wildbad, Germany)
Precision scale	Sartorius (Göttingen, Germany)
Real-Time PCR System StepOnePlus	Applied Biosystems (Darmstadt, Germany)
Shaking Water Bath 1083	GFL (Eppelheim, Germany)
Spectrophotometer NanoDrop 1000	Thermo Scientific (Schwerte, Germany)
Sterile Benches	Thermo Scientific (Schwerte, Germany)
Thermal cycler DOPPIO	VWR (Darmstadt, Germany)
Vortexer 34524-200	Cenco (Breda, Netherlands)
Vortexer Reax 2000	Heidolph (Schwabach, Germany)

2.1.2 Chemicals

2',7'-dichlorofluorescein diacetate	Sigma (München, Germany)
5 X First Strand Buffer	Invitrogen (Darmstadt, Germany)
Aqua dest.	B. Braun (Melsungen, Germany)
beta-mercaptoethanol	Life Technologies (Darmstadt, Germany)
BSA, fatty acid free	PAA (Cölbe, Germany)
Ciprofloxacin	Bayer (Leverkusen, Germany)
Collagenase	Roche (Mannheim, Germany)
dimethylsulfoxide (DMSO)	Sigma (München, Germany)
DMEM	Life Technologies (Darmstadt, Germany)
DNase I	Invitrogen (Darmstadt, Germany)
DNase reaction buffer	Invitrogen (Darmstadt, Germany)
DTT 0.1 M	Invitrogen (Darmstadt, Germany)
dNTPs 10 mM	Invitrogen (Darmstadt, Germany)
EDTA 25 mM	Invitrogen (Darmstadt, Germany)

Embedding medium ”Cryoblock”	Biosystems (Nunningen, Switzerland)
FBS	biowest (Nuaillé, France)
Gentamycin	Ratiopharm (Ulm, Germany)
Glucose-40 concentrate	Braun (Melsungen, Germany)
Glycerol	Merck (Darmstadt, Germany)
Hank’s Salt Solution (10x)	Biochrom (Berlin, Germany)
HCl 37 %	Sigma (München, Germany)
Hepes-Buffer (1 M)	Biochrom (Berlin, Germany)
Hoechst 33342	Calbiochem (Darmstadt, Germany)
Medium 199	Gibco (Karlsruhe, Germany)
Na_2HPO_4	Merck (Darmstadt, Germany)
NaH_2PO_4	Merck (Darmstadt, Germany)
NaCl	Roth (Karlsruhe, Germany)
NaCl 0.9 %	B. Braun (Melsungen, Germany)
NaOH	Merck (Darmstadt, Germany)
NP-40	United States Biological (Swampscott, USA)
Oligo (dT) primers	Applied Biosystems (Darmstadt, Germany)
Paraformaldehyde	Merck (Darmstadt, Germany)
PBS	PAA (Cölbe, Germany)
Penicillin-Streptomycin	Lonza (Köln, Germany)
Penicillin-Streptomycin (cell culture)	Life Technologies (Darmstadt, Germany)
Picric acid 1.2 %	Merck (Darmstadt, Germany)
ProLong Gold	Invitrogen (Karlsruhe, Germany)
Protease Inhibitor Cocktail Tablets (Complete, EDTA-free)	Roche (Mannheim, Germany)
Phosphatase Inhibitor Cocktail 3	Sigma (München, Germany)
Recombinant Mouse Leptin, CF	R&D Systems (Wiesbaden, Germany)
RNase-free water	Gibco (Darmstadt, Germany)
TNF-alpha (human rec.)	Bachen (Bubendorf, Schweiz)
Triton X-100	Merck (Darmstadt, Germany)
TRIS base	Merck (Darmstadt, Germany)
TRIS HCl	Sigma (München, Germany)
Trypsin-EDTA	Gibco (Darmstadt, Germany)

2.1.3 Solutions

cell lysis buffer	1.25 ml NP-40 buffer (0.8 g NaCl, 10 ml glycerol, 1 ml NP-40, 2 ml EDTA 100 mM), 50 µl protease inhibitor cocktail (25 x), 12.5 µl phosphatase inhibitor cocktail 3

Hank's solution (islet isolation)	900 ml a.dest., 100 ml Hank's salt solution, 35 ml Hepes-Buffer, 10 ml Ciprofloxacin, 10 ml Penicillin-Streptomycin, 1 ml Gentamycin
master mix (cDNA Synthesis)	4 µl 5 X First strand buffer, 2 µl 0.1 M DTT, 1 µl mM dNTPs, 1 µg µl^{-1} oligo dTs, 1 µl Superscript III Reverse Transcriptase
master mix (qRT-PCR)	10 µl Power SYBR Green PCR Master Mix, 6.4 µl RNase-free water, 0.6 µl Primer (for + rev 1:1, 1:10 in RNase-free water)
narcotic agent	1 ml Ketamine 10 %, 0.8 ml Xylazine 2 %, 8.2 ml NaCl 0.9 %; 0.1 ml / 10 g body weight
P/FCS (islet isolation)	850 ml a. dest., 100 ml Medium 199, 50 ml FBS
TRIS buffer (immunohistology)	60.5 g TRIS base in 700 ml a. dest., 90 g NaCl
Zamboni	20 mg paraformaldehyde in 150 ml picric acid solution, heat to 60 °C for 2h, neutralise with 2.5 % NaOH, filter; fill up to 1000 ml with PBS pH 7.3

2.1.4 Kits

Mouse Insulin ELISA Kit	DRG Instruments GmbH (Marburg, Germany)
Mouse Grx1 ELISA Kit	Wuhan EIAab Science (Wuhan, China)
Mouse Grx5 ELISA Kit	CUSABIO Biotech (Wuhan, China)
Power SYBR Green PCR Master Mix	Applied Biosystem (Darmstadt, Germany)
RNeasy Plus Micro Kit	Qiagen (Düsseldorf, Germany)
SuperScript III Reverse Transcriptase	Invitrogen (Karlsruhe, Germany)

2.1.5 Antibodies

Several antibodies for redoxins were manufactured (Hanschmann, 2011) and kindly provided by the research group of Professor Lillig, Institute of Biochemistry, Ernst-Moritz-Arndt-University, Greifswald.

Name	Source	Dilution	Origin
beta-tubulin	Rabbit	1:10000	Abcam (Cambridge, England)
Insulin	Guinea Pig	1:500	Dako (Hamburg, Germany)
Ki-67	Rat	1:100	Dako (Hamburg, Germany)
Caspase-3	Rabbit	1:1600	Cell Signaling (Frankfurt, Germany)
Grx1	Rabbit	1:200	Santa Cruz Biotechnology (USA)
Grx2	Rabbit	1:100	RG Lillig (Greifswald, Germany)
Grx3	Rabbit	1:1000	RG Lillig (Greifswald, Germany)
Grx5	Rabbit	1:1000	RG Lillig (Greifswald, Germany)
Prx1	Rabbit	1:500	RG Lillig (Greifswald, Germany)
Prx2	Rabbit	1:500	RG Lillig (Greifswald, Germany)
Prx3	Rabbit	1:500	RG Lillig (Greifswald, Germany)
Prx4	Rabbit	1:500	Santa Cruz Biotechnology (USA)
Prx5	Rabbit	1:500	RG Lillig (Greifswald, Germany)
Prx6	Rabbit	1:500	RG Lillig (Greifswald, Germany)
Trx1	Rabbit	1:1000	RG Lillig (Greifswald, Germany)
Trx2	Rabbit	1:1000	RG Lillig (Greifswald, Germany)
TrxR1	Rabbit	1:200	RG Lillig (Greifswald, Germany)
TrxR2	Rabbit	1:1000	Santa Cruz Biotechnology (USA)
FITC (Secondary)	Donkey	1:400	Jackson (Newmarket, England)
Rhodamine Red (Secondary)	Donkey	1:400	Jackson (Newmarket, England)

Table 2.1: List of antibodies and corresponding dilutions.

2.1.6 Primer sequences for qRT-PCR

Target	Sequence (5' to 3')
beta-actin fwd	GTG GGA ATG GGT CAG AAG G
beta-actin rev	GAG GCA TAC AGG GAC AGC A
Ins-1 fwd	TAT AAA GCT GGT GGG CAT CC
Ins-1 rev	GGG ACC ACA AAG ATG CTG TT
Grx1 fwd	GAG CAG TTG GAC GCG CTG G

Grx1 rev	CTC GCC ATT GAG GTA CAC TTG C
Grx2 fwd	CTG CTC TTA CTG TTC CAT GGC CAA GAA G
Grx2 rev	CAC TGA TGA ACC AGA GGC AGC AAT TTC
Grx5 fwd	GAA GAA GGA CAA GGT GGT GGT CTT C
Grx5 rev	GCA TCT GCA GAA GAA TGT CAC AGC

Table 2.2: List of primers.

Sequences for Grx1, 2 and 5 were kindly provided by RG Lillig, Greifswald.

2.1.7 Computer based data handling

2.1.7.1 Processing of immunostained tissues

Images were taken with Leica Application Suite (v 3.8.0) using digital microscope camera DFC 420 C and analysed with ImageJ (Wayne Rasband, National Institutes of Health, USA) and custom macros.

2.1.7.2 Figures

Data were analysed using Graph Pad Prism 5 (GraphPad Software, San Diego, USA).

2.1.8 Research animals

Male BKS(D)-Leprdb/JOrlRj mice (db/db) and BKS(D)-Leprdb/JOrlRj Témoin (db/+) mice aged 5 weeks were bought from Charles River (Sulzfeld, Germany). Animals were housed according to institutional guidelines (room temperature $(22.0 \pm 0.5)\,^{\circ}\mathrm{C}$, 12 hours light / dark cycle, 60 % humidity) with tap water and standard diet pellet food (Altromin, Lage, Germany) ad libitum. Approval of research was obtained under the code GI20/11-Nr.A18/2010 from institutional ethical committee.

2.2 Methods

2.2.1 Pancreatectomy and Immunofluorescence

Animals were sedated using Ketamine and Xylazine (section 2.1.3). The abdominal aorta was cut, the pancreas removed and fixed with Zamboni for four hours. Later the organ was washed and stored in PBS overnight. On the next day, the buffer was replaced with 10 % sucrose solution overnight. Ultimately, the pancreas was embedded in cryoblock embedding medium and frozen at −80 °C.

Sections of 6 µm thickness were cut using Leica Crysostat CM1850. Slides were washed with PBS and blocked with 1 % donkey serum containing 0.3 % Triton X-100 for 20 minutes. For Ki-67 staining, antigen retrieval was performed with NaOH 0.09 M for three minutes followed by another wash cycle.
Sections were incubated with primary antibodies diluted in 1 % donkey serum containing 0.01 % Triton X-100 overnight at 4 °C.
After washing thrice to remove the primary antibodies, sections were incubated with solution containing secondary antibodies in 5 % mouse serum for one hour at room temperature. The procedure was finished by staining nuclei with Hoechst in 0.1 % TRIS buffer pH 7.6 and the sections were mounted with a cover slip using ProLong Gold. The antibody dilutions which were used are listed in table 2.1.

2.2.2 Analysis of images obtained through immunohistology

Immunostained slides were used to analyse mean islet area, to study proliferation and apoptosis of the beta-cells and mean staining intensity of redoxins and insulin. Samples were entirely sectioned and two consecutive sections were placed on a slide and regarded as one (results were divided by two in the analysis). An interval of 140 µm was maintained in order to avoid multiple inclusion of islets and to be able to assess the whole organ.
The islet area (section 3.3.3) was measured as described before (Kilimnik et al., 2009). Images of single or multiple islets were taken from slides stained with insulin in order to identify the islets. ImageJ was calibrated to match their scale and the mean islet area was obtained.
Nuclei stained with Ki-67 (section 3.4.1) were counted manually and caspase-3 (section 3.4.2) was analysed qualitatively.
Intensity measurements in immunostained tissue were done in order to semiquantitatively analyse protein levels. For obtaining the mean staining intensity of insulin (section 3.3.4) Grx1 and 5 (sections 3.6.1.2 and 3.6.2.2) in pancreatic tissue samples were prepared simultaneously in batches using the same solutions. Again, a double stain with insulin was performed in order

to identify islets. Gray scaled pictures were taken under the same conditions and normalised by removing the background using slides without primary antibodies. The mean grey value in islets was now obtained using ImageJ. For reasons of better presentability the second highest measured intensity of every protein which was analysed was respectively set as 100 %.

2.2.3 Islet Isolation

Islet isolation was done according to the protocol established in our group (Lai et al., 2005). Briefly, mice were anaesthesised by intraperitoneal injection of narcotic agent containing Ketamine and Xylazine (section 2.1.3). The abdominal wall was excised to expose the aorta which was cut to drain the blood. The pancreas was removed and perfused with 4 mg collagenase dissolved in 4 ml Hanks solution through the ductus pancreaticus. For better digestion the organ was mechanically chopped with a pair of scissors before 10 minutes of incubation in collagenase solution at 37 °C in a shaking water bath. The sample was vortexed for 10 seconds after every three minutes of digestion. Finally, the digested tissue was shaken by hand for two minutes and the digestion process was eventually stopped by placing the tube containing the tissue on ice and adding cold Hanks solution. The tube was centrifuged for 3 minutes at 1500 RPM and the supernatant was discarded. The pellet was dissolved in P/FCS at room temperature and the islets were hand-picked under stereomicroscope. To overcome the isolation stress, islets were incubated overnight at 37 °C.

2.2.4 Gene expression analysis

2.2.4.1 RNA isolation

RNA was isolated from islets using RNeasy Plus Micro Kit (Qiagen) as per manufacturer's instructions. Concentration and quality of the RNA was measured using NanoDrop 1000 Spectophotometer.

2.2.4.2 cDNA Synthesis

cDNA was synthesised using 1 µg of RNA. Solution containing 1 µg of RNA was added to RNase-free water to a total of 8 µl in a PCR tube. Then 1 µl of DNase I and 1 µl 10 X DNase reaction buffer were added to obtain 10 µl of reaction mixture. The reaction mixture was incubated for 15 minutes at 37 °C to digest the DNA.
The DNase digestion was stopped by adding 1 µl of EDTA. DNA digested RNA was then preincubated at 65 °C for 15 minutes to remove the secondary structure and cooled to 4 °C for 5 minutes.
To this mixture 9 µl of master mix were added. The resulting 20 µl solution was subjected to a

thermal cycler programme which involved incubation for 50 minutes at 50 °C followed by 15 minutes at 72 °C. The resulting cDNA was diluted 1:10 with RNase-free water and used for qRT-PCR (table 2.3).

		x µl solution containing 1 µg of RNA
	+	$(8 - x)$ µl RNase-free water
	=	8 µl solution
DNase digestion	+	1 µl DNase I
	+	1 µl 10 X DNase reaction buffer
	=	10 µl solution
		First incubation:
		15 min, 37 °C
preincubation	+	1 µl EDTA
	=	11 µl solution
		Second incubation:
		15 min, 65 °C
		5 min, 4 °C
cDNA synthesis	+	9 µl master mix
	=	20 µl solution
		Third incubation:
		50 min, 50 °C
		15 min, 72 °C

Table 2.3: The procedure of cDNA synthesis.

2.2.4.3 Quantitative Real-Time PCR

Real-Time PCR System StepOnePlus (Applied Biosystems) was used for gene expression analysis. To 3 µl cDNA (1:10 diluted) 17 µl of master mix containing the respective primers were added to achieve a total volume of 20 µl per well. Respectively, 3 µl of RNase-free water were used as negative control.
The process of amplification was started by activating the enzyme at 95 °C for 10 minutes followed by 40 cycles of denaturation and annealing.
Melting curve analysis was performed for each run (table 2.4).

	step	repeats	temperature	duration
amplification	activation	1	95 °C	10 min
	denaturation	40	95 °C	10 s
	annealing, extension		60 °C	1 min
melting curves	denaturation	1	95 °C	15 s
	melting	40	60 °C	1 min

Table 2.4: The procedure of qRT-PCR.

The normalisation of the target genes' ct values was done with the reference gene using the formula $x = 2^{ct\text{reference} - ct\text{target}}$.

2.2.5 Cell culture

High passage (passage 70 to 80) mouse insulinoma cells, 6th subclone (MIN6 cells) were cultured in Dulbecco's Modified Eagle Medium (DMEM) along with 1 % Penicillin / Streptomycin, 20 % FBS and 0.1 % beta-mercaptoethanol at 37 °C and 5 % CO_2.
Cell splitting was carried out by trypsinisation. Washing was done with PBS before adding 0.5 % Trypsin-EDTA solution. Detached cells were diluted with DMEM, centrifuged for 4 minutes at 1200 RPM and seeded into new flasks.
Prior to analysis leptin was applied for two hours. Leptin was pre-diluted to 0.1 % in 20 mM TRIS-HCl, pH 8.0. Concentrations of 0 ng ml^{-1}, 0.075 ng ml^{-1}, 0.45 ng ml^{-1} and 2 ng ml^{-1} were applied.
For hypoxia treatment, the respective cells were exposed to an atmosphere consisting of 2 % O_2, 5 % CO_2 and 93 % N_2 after application of leptin.
Cells were lysed for Grx1 and 5 ELISA and their supernatant was collected for insulin ELISA.

2.2.6 Protein analysis

Protein expression for insulin was measured in MIN6 cell supernatant, protein expression for Grx1 and 5 in MIN6 cell lysates. Prior to lysis, 1 ml supernatant was extracted before cells were washed in icecold PBS. 200 µl cell lysis buffer (section 2.1.3) were added. Cells were transferred into a tube and incubated on ice for 20 minutes. Supernatant was gathered by centrifugation for 20 minutes at 12000 RPM. Insulin, Grx1 and 5 were analysed using ELISA technique.

2.2.6.1 Insulin ELISA

Insulin content of MIN6 cell supernatant was analysed using a mouse insulin ELISA kit (DRG) as per manufacturer's instructions.

2.2.6.2 Grx1 and 5 ELISA

Grx1 content of MIN6 cell lysate was analysed using a mouse Grx1 ELISA kit (EIAab) and Grx5 content using a mouse Grx5 ELISA kit (CUSABIO) as per manufacturer's instructions.

2.2.7 ROS detection

2,7-dichlorofluorescein diacetate (DCFH-DA) indicator dye was used for detection of intracellular reactive oxygen species production level in isolated islets. Islet cells of 24 weeks old animals were isolated and three groups were formed including an untreated control, high glucose treated (20 mM for 2 hours), and TNF-alpha treated (1 µM for 15 minutes). Samples were incubated in serum-free medium containing 10 µM DCFH-DA indicator dye dissolved in dimethylsulfoxide (DMSO) for 30 minutes at 37 °C in the dark. Afterwards, samples were rinsed with pre-warmed serum-free medium and immediately analysed with a confocal microscope. DCF fluorescence was measured in 3600 μm^2 regions of interest and corrected for background fluorescence. The 488 nm band of the argon ion laser of the confocal laser scanning microscope was used for fluorescence excitation and the emission was recorded using a longpass LP 515 nm filter set. Fluorescence intensities were quantified using Leica Simulator software and images taken of the respective region of interests. Access to his research group's confocal microscope was kindly granted by Prof. Dr. Heinrich Sauer and guidance was provided by Dr. Fatemeh Sharifpanah (Department of Physiology, Justus-Liebig-University, Gießen).

2.2.8 Statistics

Statistics were performed with GraphPad Prism 5 using Mann Whitney test, one-way and two-way ANOVA as appropriate. Results contain mean values ± SEM and a p value <0.05 was considered to be statistically significant. Significance levels were labelled as follows: *: $p<0.05$, **: $p<0.005$, ***: $p<0.0001$.

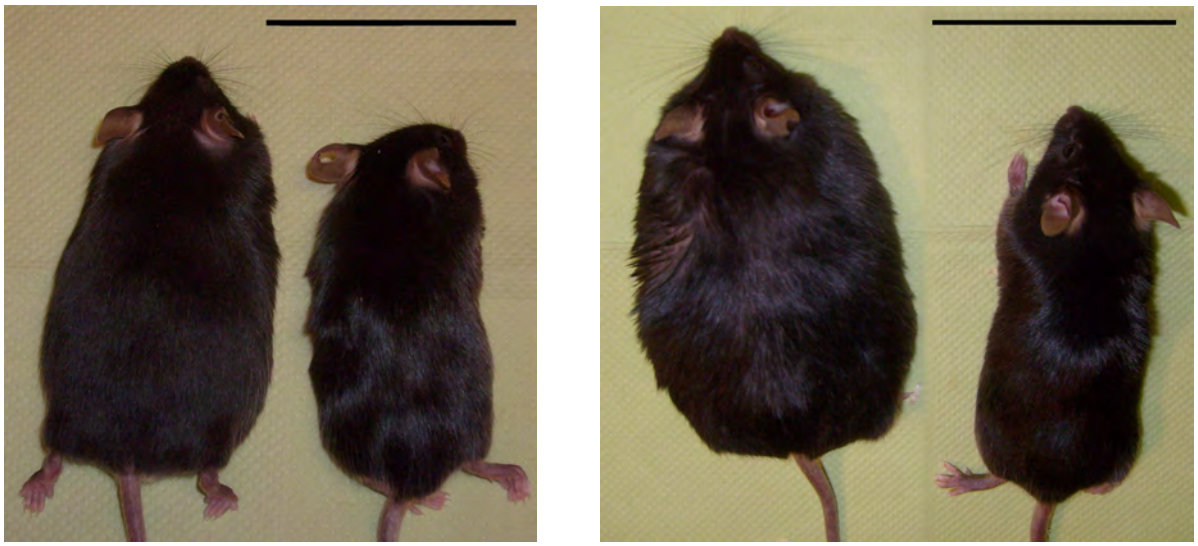
3 Results

3.1 General procedure

40 db/db mice and an equal number of db/+ mice were obtained at the age of 5 weeks and observed from age 6 to 18 weeks. Fasting blood glucose levels and body weight were measured weekly. Blood glucose was measured using a glucometer after overnight fasting. At the age of 6, 12 and 18 weeks mice were sacrificed for islet isolation and organ harvesting. In each group 3 pancreata were harvested for sectioning and immunohistological analysis whereas islets from 4 to 6 animals were used for RNA extraction and qRT-PCR. The number of sacrificed mice varied depending on the yield of collected islets. It was estimated that 800 islets would give sufficient RNA.

3.2 Vital parameters show significant differences

The physical appearance varied between both groups of animals (figure 3.1).



(a) db animals, 6 weeks

(b) db animals, 18 weeks

Figure 3.1: Images of db/db and db/+ mice at the age of 6 (a) and 18 weeks (b). Bars indicate 5 cm. Homozygote mice are on the left side of each image.

Homozygote animals gained weight much faster than heterozygotes. At the age of 6 weeks homozygotes weighed 27.4 g on average, whereas mean weight of their heterozygote counterparts was 18.9 g, corresponding to a 1.5-fold higher body weight. This difference was 2-fold in 12 week old animals (48.1 g and 23.9 g). At the end of the observation period there was a 2.2-fold

difference in the mean body weight between db/db (56.9 g) and db/+ mice (26.2 g). In other words, the mean body weight of db/db mice doubled whereas we only found a 40 % increase in db/+ animals. Body weight was significantly different ($p<0.0001$) between db/db and db/+ mice and between 6, 12 and 18 weeks old db/db animals (figure 3.2). The complete follow-up of the body weight is shown in the appendix (figure 11.1).

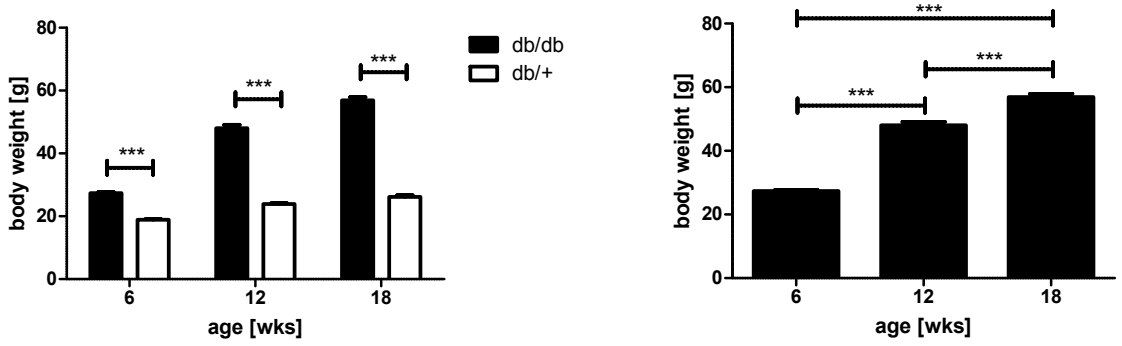
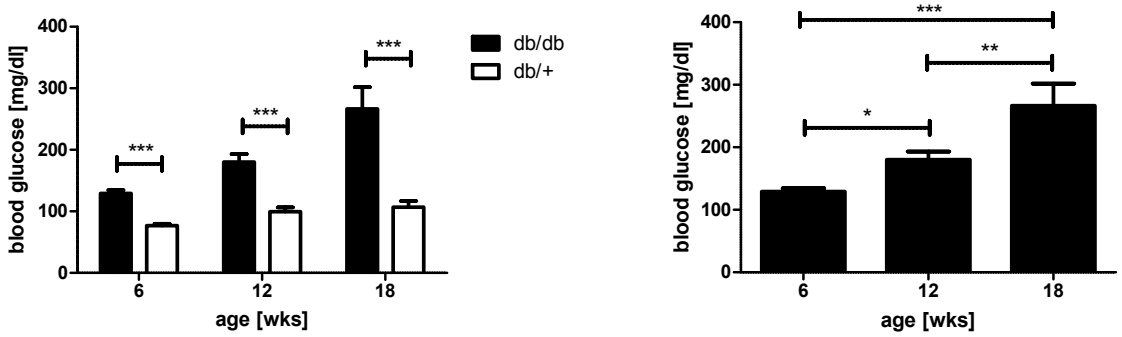


Figure 3.2: Body weight comparison between db/db and db/+ mice. Significant ($p<0.0001$) differences were found between homozygotes and heterozygotes (a) as well as between homozygotes of different age ($p<0.0001$) (b) (n = 11 to 40 per time point).

Furthermore, fasting blood glucose levels were significantly different ($p<0.0001$) between both groups of animals and between 6 and 18 weeks old db/db mice. The mean value for db/db mice was higher than that of the db/+ group in all measurements and it tended to increase during the observation time. In db/db mice it exceeded 200 mg dl^{-1} on average from 13 weeks of age onwards whereas heterozygote mice fluctuated between 60.5 mg dl^{-1} and 106.7 mg dl^{-1} (figure 3.3). The complete follow-up of the blood glucose level is shown in the appendix (figure 11.2).



(a) (b)

Figure 3.3: Blood glucose comparison between db/db and db/+ mice. db/db mice had distinctively ($p<0.0001$) higher blood glucose levels than db/+ animals (a). Also, a significant elevation was found in ageing homozygotes ($p<0.0001$) (b) (n = 11 to 36 per time point).

Homozygote mice were defined as diabetic if their blood glucose level exceeded the fourfold mean deviation of the heterozygotes two weeks in a row. The threshold was $181.22\,\mathrm{mg\,dl^{-1}}$. According to this criterion 49 % of the db/db mice became diabetic. The threshold was exceeded on average from 13 weeks onwards.

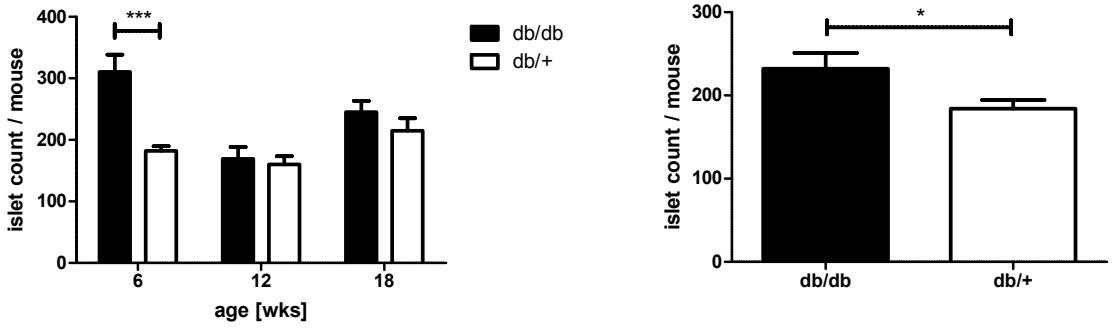
3.3 Islet shape and insulin expression

Islets shape was also observed during the study period. Besides observing their number in the harvested pancreata during isolation, insulin was stained using immunohistology in order to evaluate islet morphology and to measure mean islet area. By the same method, intensity of insulin staining was analysed. The expression of the INS-1 gene was also measured using qRT-PCR.

3.3.1 Changes in number of islets

Islets were counted during the process of isolation and an increased number of islets in homozygote mice at the indicated time points was found. Both groups of animals showed a decrease in the number of islets at 12 weeks of age, before rising again up to the age of 18 weeks. However, this effect was less pronounced in heterozygote animals. The average islet count in db/db mice dropped from 310 at 6 weeks to 169 at 12 weeks and raised again to 245 at 18 weeks, while we found a mean number of 182, 160 and 214 in db/+ animals. At 6 weeks the islet number was significantly ($p<0.0001$) different between both groups of mice. Also the average islet count of

both groups was statistically significant ($p<0.05$) in comparison (figure 3.4).

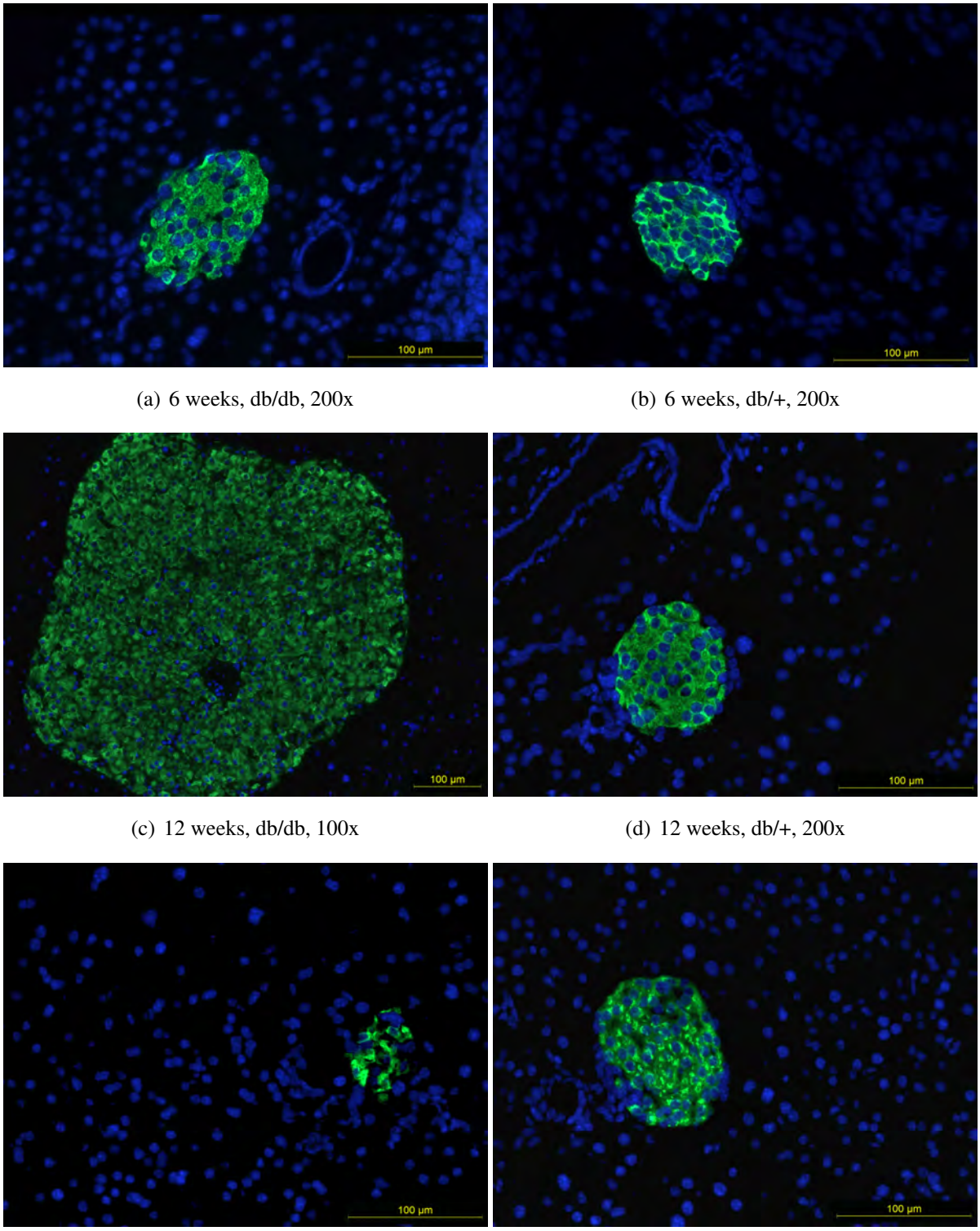


(a) (b)

Figure 3.4: Islet count comparison between db/db and db/+ mice. Homozygotes held more islets at all time points (a). At 6 weeks ($p<0.0001$) (a) and on average ($p<0.05$), the difference was significant (b) (n = 4 to 6 per time point).

3.3.2 Difference in morphology of pancreatic islets

We confirmed changes in morphology of islets in the db/db mouse that had already been described before (Yamazaki et al., 2009). Aging animals had a higher number of small, segmented and irregular shaped islets and also an increase in the number of larger islets (figure 3.5).



(a) 6 weeks, db/db, 200x

(b) 6 weeks, db/+, 200x

(c) 12 weeks, db/db, 100x

(d) 12 weeks, db/+, 200x

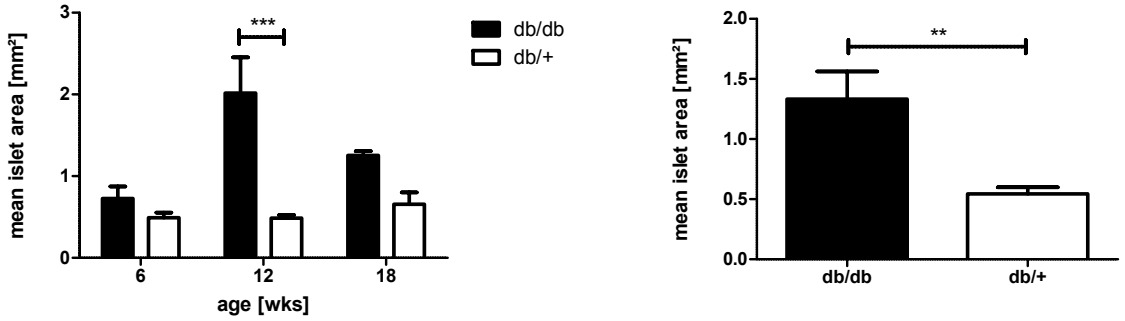
(e) 18 weeks, db/db, 200x

(f) 18 weeks, db/+, 200x

Figure 3.5: Representative images of the morphology of islets in db/db and db/+ mice (green: insulin, blue: nuclei; bars indicate 100 µm). At the age of 6 weeks islets of homozygotes were already enlarged but shaped normally (a). Larger islets in 12 weeks old db/db mice, resulting in huge islet formations (c). As the animals grew older, small abnormally shaped islets increased in number (e). The changes in the islets of heterozygotes were more moderate (b), (d), (f).

3.3.3 Mean area of islet sections was higher in db/db mice

The average area of islets was larger in homozygote mice at all times compared to their heterozygote counterparts. An increased mean islet area could be already found in 6 weeks old animals (0.73 mm^2). At the age of 12 weeks a maximum was reached (2.02 mm^2) before the mean islet area dropped again to 1.25 mm^2 at 18 weeks of age. On the other hand, heterozygote animals showed only a moderate increase in islet area. We observed mean values of 0.49 mm^2 at both 6 and 12 weeks and eventually 0.66 mm^2 at 18 weeks.
The distinction between both groups was significant with $p<0.0001$ at 12 weeks and with $p<0.005$ on average (figure 3.6).



(a) (b)

Figure 3.6: Mean islet area comparison between db/db and db/+ islets. Homozygotes had a higher mean islet area at all time points (a). At 12 weeks ($p<0.0001$) (a) and on average ($p<0.005$), the difference was significant (b) (n = 3 per time point).

3.3.4 Insulin staining intensity

The intensity of insulin staining was evaluated using ImageJ. The staining intensity was significantly higher in db/+ mice with $p<0.0001$ (figure 3.7).

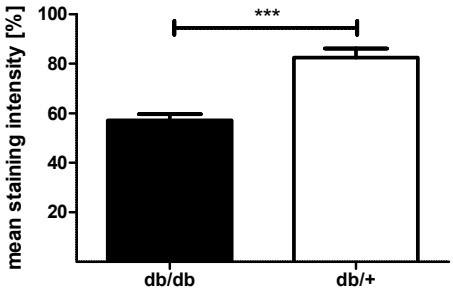
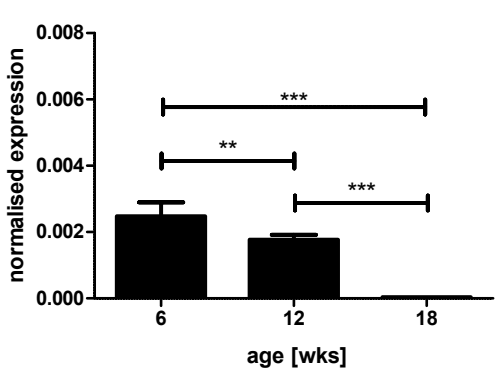


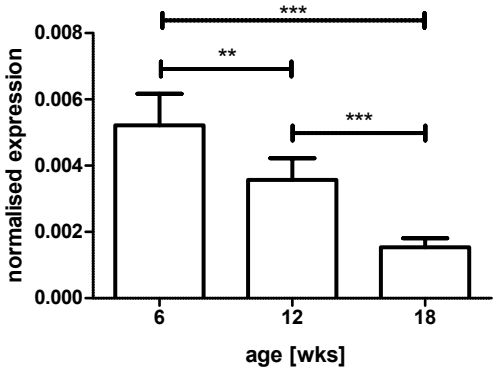
Figure 3.7: Staining intensity of insulin. Analysis detected a significantly ($p<0.0001$) higher staining intensity for heterozygote islets (n = 9).

3.3.5 Expression of the INS-1 gene in homozygotes and heterozygotes

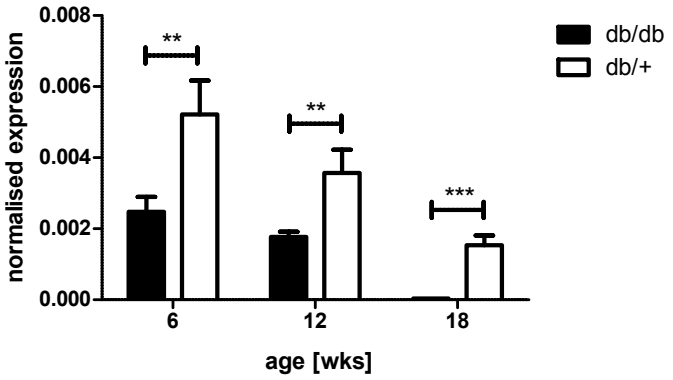
We measured the gene expression of insulin using qRT-PCR to see whether the expression varied between both groups. The insulin gene expression over time showed a similar trend in homozygote and heterozygote mice. We detected a significant ($p<0.0001$) reduction in insulin expression in both animal groups with time. When 12 weeks old db/db mice were compared with 18 week old mice, a 50-fold reduction in INS-1 expression was observed. When 6 weeks old animals were compared with their 18 weeks old counterparts a 70-fold increased insulin expression was detected (figure 3.8a). For heterozygote mice those factors were only 3.4 and 2.4 (figure 3.8b).
A significant ($p<0.005$ at 6 and 12 weeks, $p<0.0001$ at 18 weeks) decrease in the homozygote mice' insulin expression of islets was observed when compared to heterozygote animals. At 6 and 12 weeks, heterozygote mice showed a 2-fold higher expression of insulin than their homozygote counterparts. At 18 weeks, a 44.5-fold higher insulin expression was found in heterozygote mice when compared to homozygotes (figure 3.8c).



(a)



(b)



(c)

Figure 3.8: Gene expression of INS-1. The expression in db/db islets revealed a significant ($p<0.0001$) decrease over time (a), which was also present in in db/+ islets ($p<0.0001$) (b). In direct comparison, heterozygotes featured higher expression at all time points ($p<0.005$ and $p<0.0001$) (c) (relative insulin expression normalised with beta-actin, n = 4 to 6 per time point).

3.4 Changes in the cell cycle

Since islets showed significant variation in size between homozygote and heterozygote mice we studied the proliferation and apoptosis behaviour of beta-cells from both groups.

In order to gain insight into the proliferation rate of the islets of Langerhans we used Ki-67 as a proliferation marker. Respectively, activated caspase-3 was chosen in order to study programmed cell death.

3.4.1 Proliferation plays a major role in islet appearance

The rate of beta-cell proliferation was determined by using an antibody against Ki-67 and insulin was used to clearly identify the beta-cells (figure 3.9).

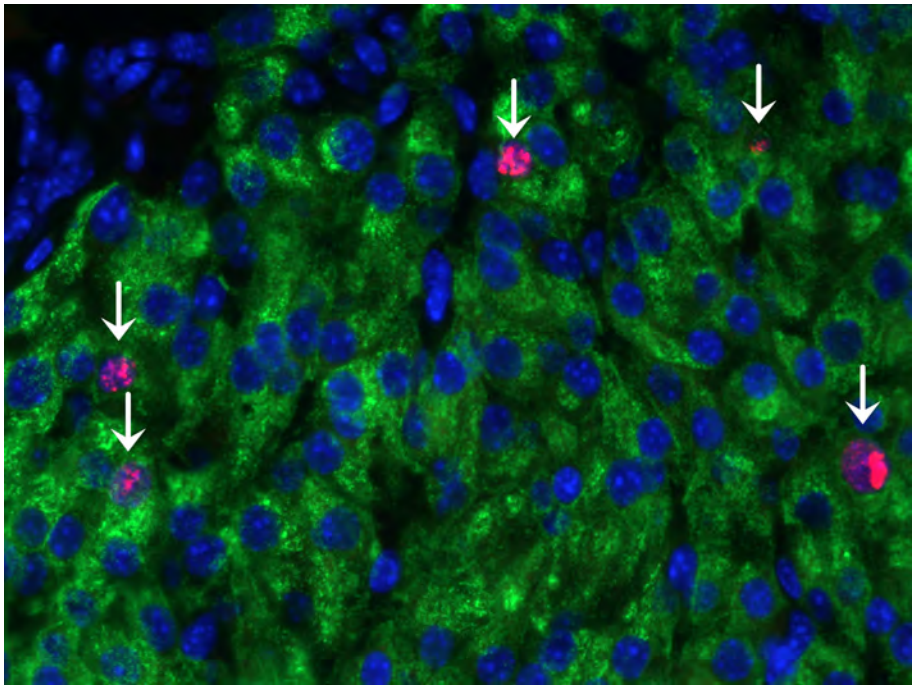
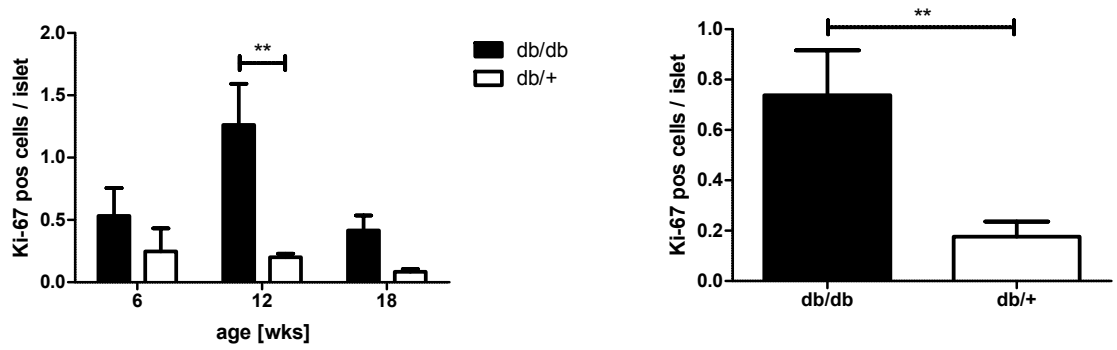


Figure 3.9: Representative image of Ki-67 staining (green: insulin, red: Ki-67, blue: nuclei, 400x, 12 weeks old db/db mouse). Arrows indicate stained nuclei.

The total amount of stained nuclei was manually counted and the number of proliferating cells per islet calculated.
The number of Ki-67 positive cells per islet was significantly ($p<0.005$) higher for homozygote mice at 12 weeks and on average. We found a mean of 0.5 positive cells for 6 week old animals, reaching a maximum of 1.3 at 12 weeks before dropping to 0.4 at the age of 18 weeks. The average values of our heterozygote mice dropped from 0.25 over 0.2 to 0.1 (figure 3.10).

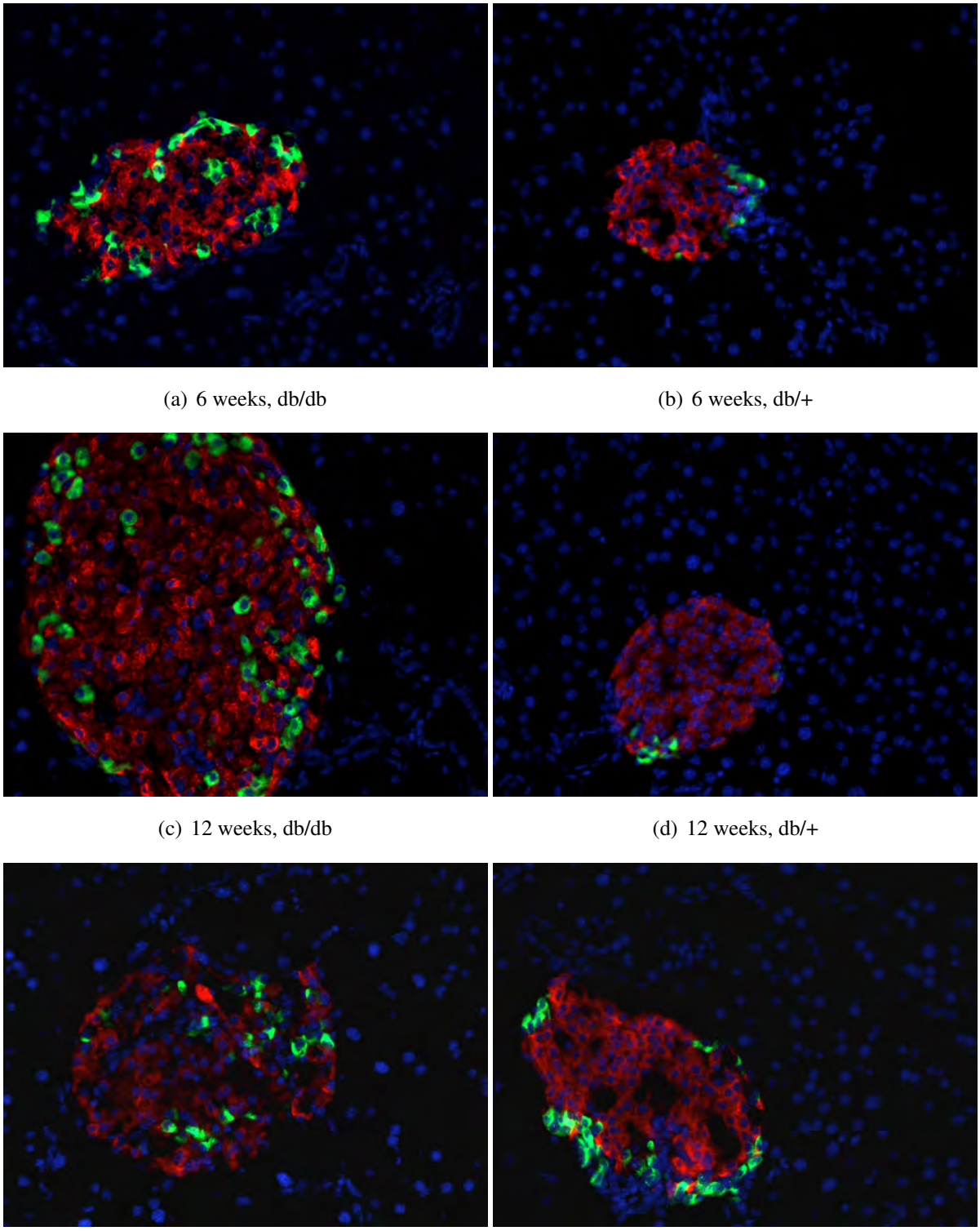


(a) (b)

Figure 3.10: Beta-cell proliferation. Homozygotes featured augmented beta-cell proliferation at all time points (a). At 12 weeks ($p<0.005$) (a) and on average ($p<0.005$), the difference was significant (b) (n = 3 per time point).

3.4.2 Islets of homozygote mice showed a higher rate of apoptosis

Activated caspase-3 is an early marker for apoptosis which was used to analyse the manifestation of regulated cell death. At the age of 6 and 12 weeks islets of homozygote animals generally showed more caspase-3 positive cells than those of their heterozygote counterparts. At the age of 18 weeks this trend was not observed (Figure 3.11).



(a) 6 weeks, db/db

(b) 6 weeks, db/+

(c) 12 weeks, db/db

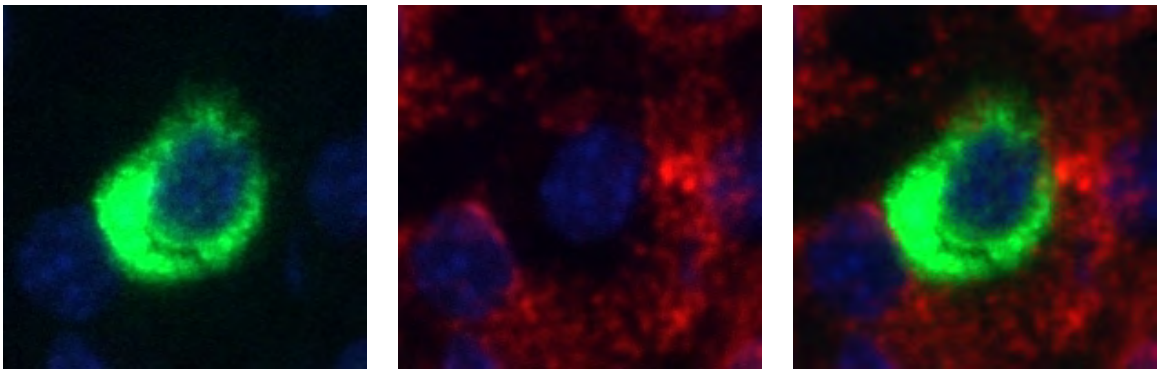
(d) 12 weeks, db/+

(e) 18 weeks, db/db

(f) 18 weeks, db/+

Figure 3.11: Representative images of caspase-3 staining (green: caspase-3, red: insulin, blue: nuclei, 200x).

In general, caspase-3 staining was located mainly in the islets' periphery and did not match insulin staining (Figure 3.12).



(a) (b) (c)

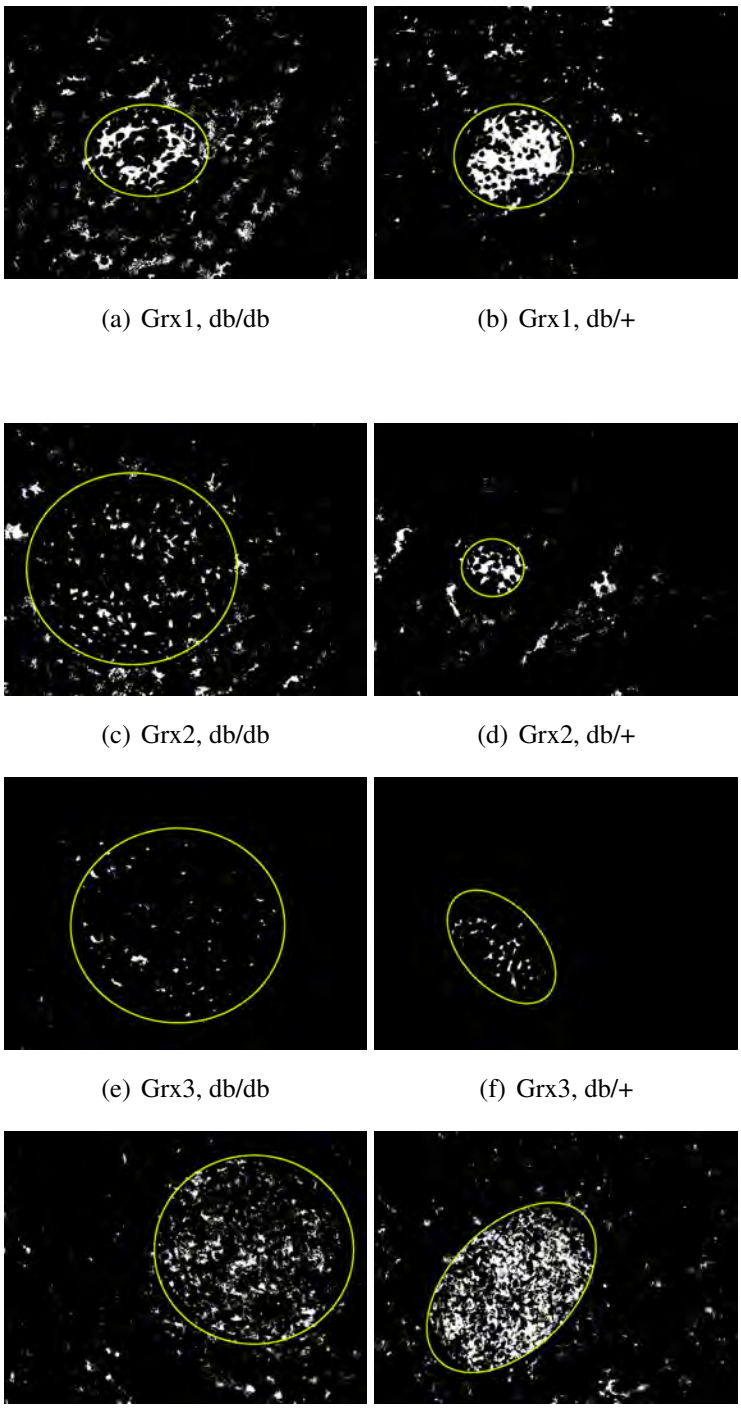
Figure 3.12: Caspase-3 staining, close-up view (green: caspase-3, red: insulin, blue: nuclei). The area stained with caspase-3 shows no insulin staining. a) nuclei and caspase-3 overlay, b) nuclei and insulin overlay, c) nuclei, caspase-3 and insulin overlay.

3.5 Qualitative analysis of Trx family proteins

In the second part we studied Trx family proteins. The expression of the Grx system, Peroxiredoxins and the Trx system was analysed using immunohistochemistry. We compared sections from both groups of mice in order to find out whether there is any expression detected and if so, whether a difference between both groups could be seen. Sections from mice aged 12 weeks were chosen due to the huge intergroup difference in islet area and proliferation rate. Images were transformed to black and white for better contrast.

3.5.1 The Grx system

All of the four Grx proteins were detected in the endocrine and the exocrine tissue of both groups of mice. However, the staining intensity and stained area varied considerably. In general, we found a higher signal in db/+ mice for all glutaredoxins.
Grx1 and 5 showed the most marked staining in islets of all redoxins. Also the distinction between both groups of animals was most pronounced for both proteins. Differences for Grx2 and 3 were less visible, Grx3 being the redoxin with the lowest expression (figure 3.13).



(a) Grx1, db/db (b) Grx1, db/+

(c) Grx2, db/db (d) Grx2, db/+

(e) Grx3, db/db (f) Grx3, db/+

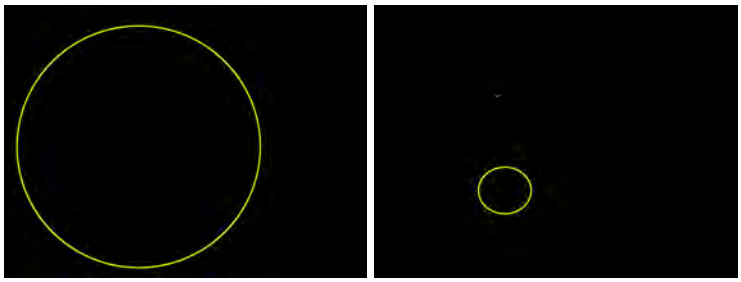
(g) Grx5, db/db (h) Grx5, db/+

Figure 3.13: Qualitative comparison of the Grx system, 200x, yellow circles indicate islets.

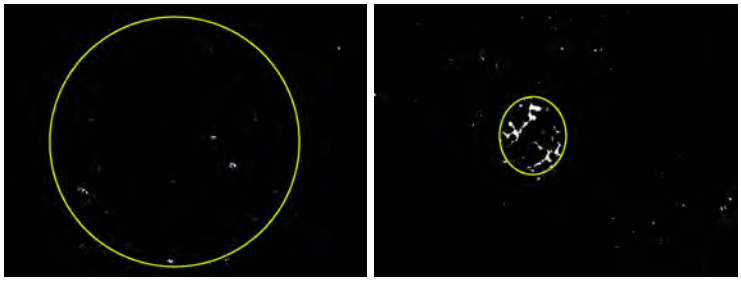
3.5.2 Peroxiredoxins

The expression pattern of the peroxiredoxins was found to be inhomogeneous and scarce except for Prx6. While there was basically no expression of Prx1, the behaviour of Prx2 and 3

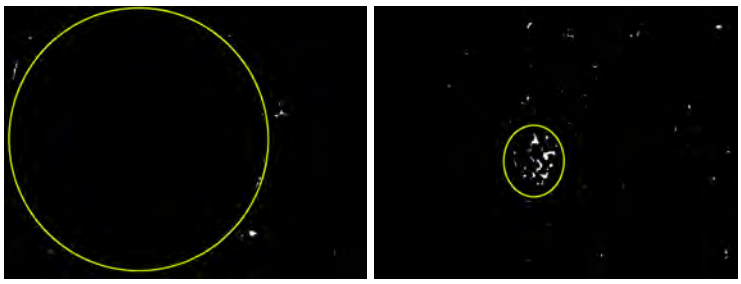
resembled the glutaredoxins. Both proteins were expressed higher in db/+ mice. Ultimately, Prx4 and 5 were expressed similarly in both groups of mice. However, in the exocrine tissue staining seemed to be higher in db/+ animals. A stronger staining without a visible difference between both groups was detected for Prx6 (figure 3.14).



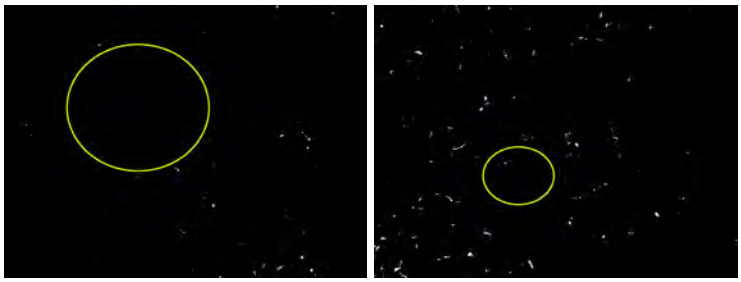
(a) Prx1, db/db (b) Prx1, db/+



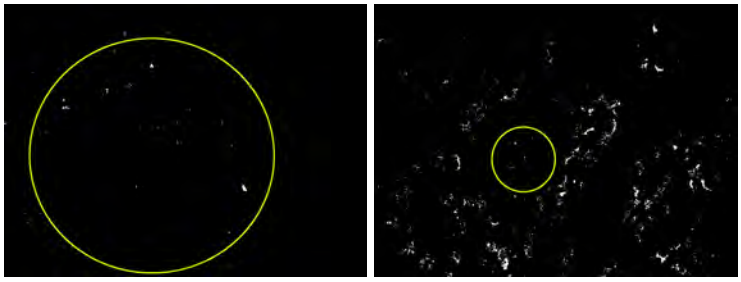
(c) Prx2, db/db (d) Prx2, db/+



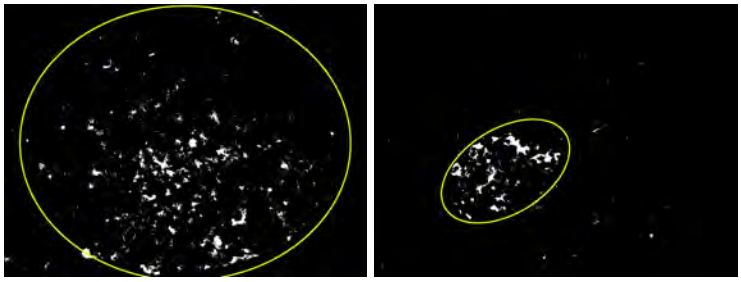
(e) Prx3, db/db (f) Prx3, db/+



(g) Prx4, db/db (h) Prx4, db/+



(i) Prx5, db/db (j) Prx5, db/+

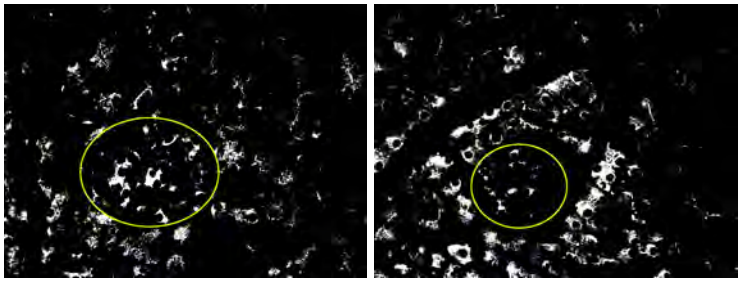


(k) Prx6, db/db (l) Prx6, db/+

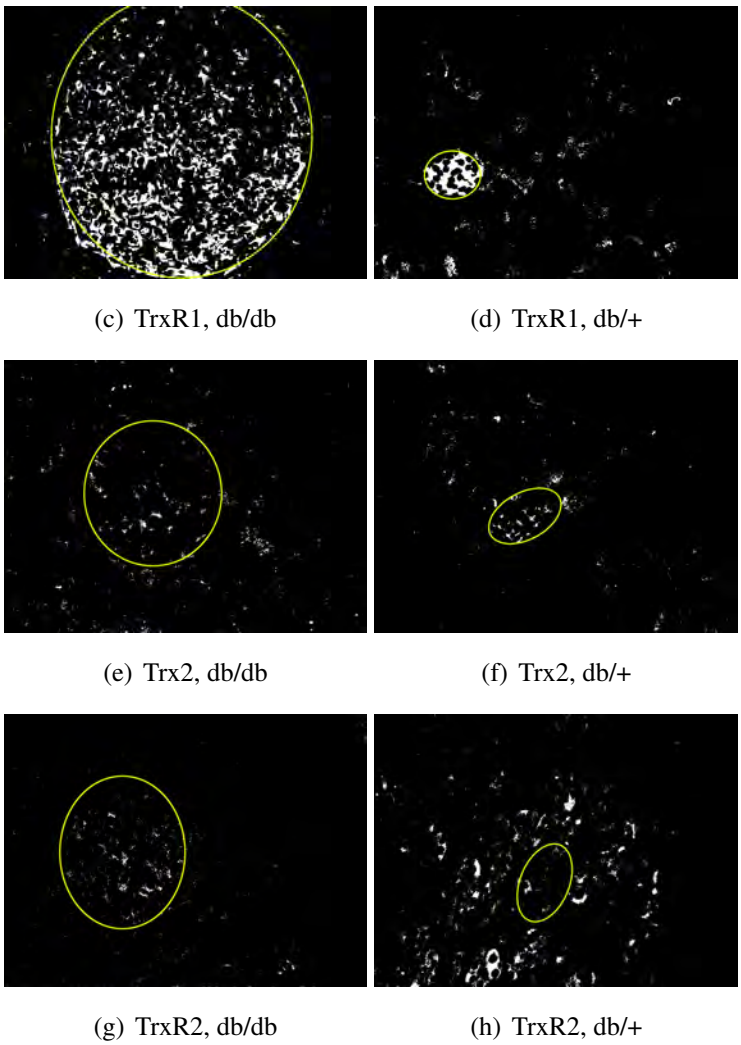
Figure 3.14: Qualitative comparison of the Prx system, 200x, yellow circles indicate islets.

3.5.3 The Trx system

A visible difference between homozygote and heterozygote animals could not be found for both thioredoxins. In general, staining of Trx1 could be found extensively in both the endocrine and exocrine pancreas. The same applied for thioredoxin 1 as compared to thioredoxin 2 reductase (figure 3.15).



(a) Trx1, db/db (b) Trx1, db/+



(c) TrxR1, db/db (d) TrxR1, db/+

(e) Trx2, db/db (f) Trx2, db/+

(g) TrxR2, db/db (h) TrxR2, db/+

Figure 3.15: Qualitative comparison of the Trx system, 200x, yellow circles indicate islets.

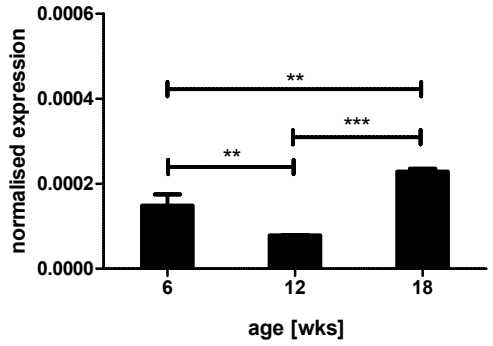
3.6 Analysis of glutaredoxin 1 and 5 expression

To conclude the study of redoxins, we analysed the properties of the glutaredoxin isoforms 1 and 5. Both proteins featured the most evident expression in pancreatic islets in immunohistological staining and also a strong difference between db/db and db/+ islets (section 3.5). Gene expression was quantified by running qRT-PCR and protein expression by immunohistochemistry.

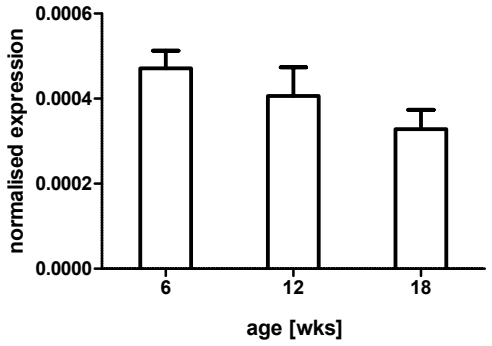
3.6.1 Grx1

3.6.1.1 qRT-PCR

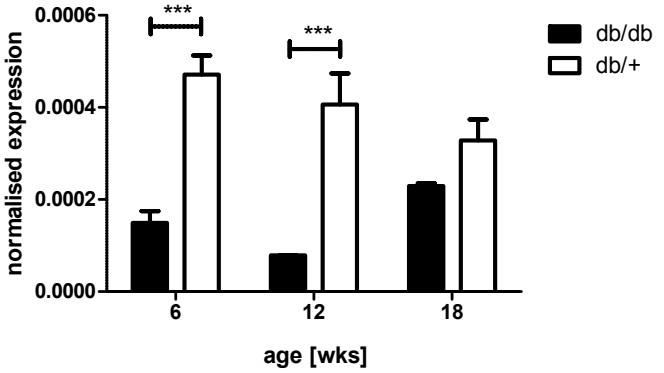
The expression of Grx1 was significantly higher ($p<0.0001$) in heterozygote compared to homozygote mice at 6 and 12 weeks. The difference between 6, 12 and 18 week old db/db animals was also significant ($p<0.005$ for 6 and 12 as well as 6 and 18 weeks, $p<0.0001$ for 12 and 18 weeks). Heterozygotes showed a higher expression at all time points, while homozygotes presented huge variations over time. As displayed in figure 3.16a we found a lower expression at the age of 12 weeks for the latter group. At 6 weeks the expression was on average around 1.9-fold and at 18 weeks around 2.9-fold higher than at the second time point (12 weeks). By contrast, there was a non-significant decrease in the expression of Grx1 in db/+ animals (figure 3.16b). We compared the expression separately for each time point and found a 3.3, 5.2 and 1.5-fold higher value for db/+ animals (figure 3.16c).



(a)



(b)

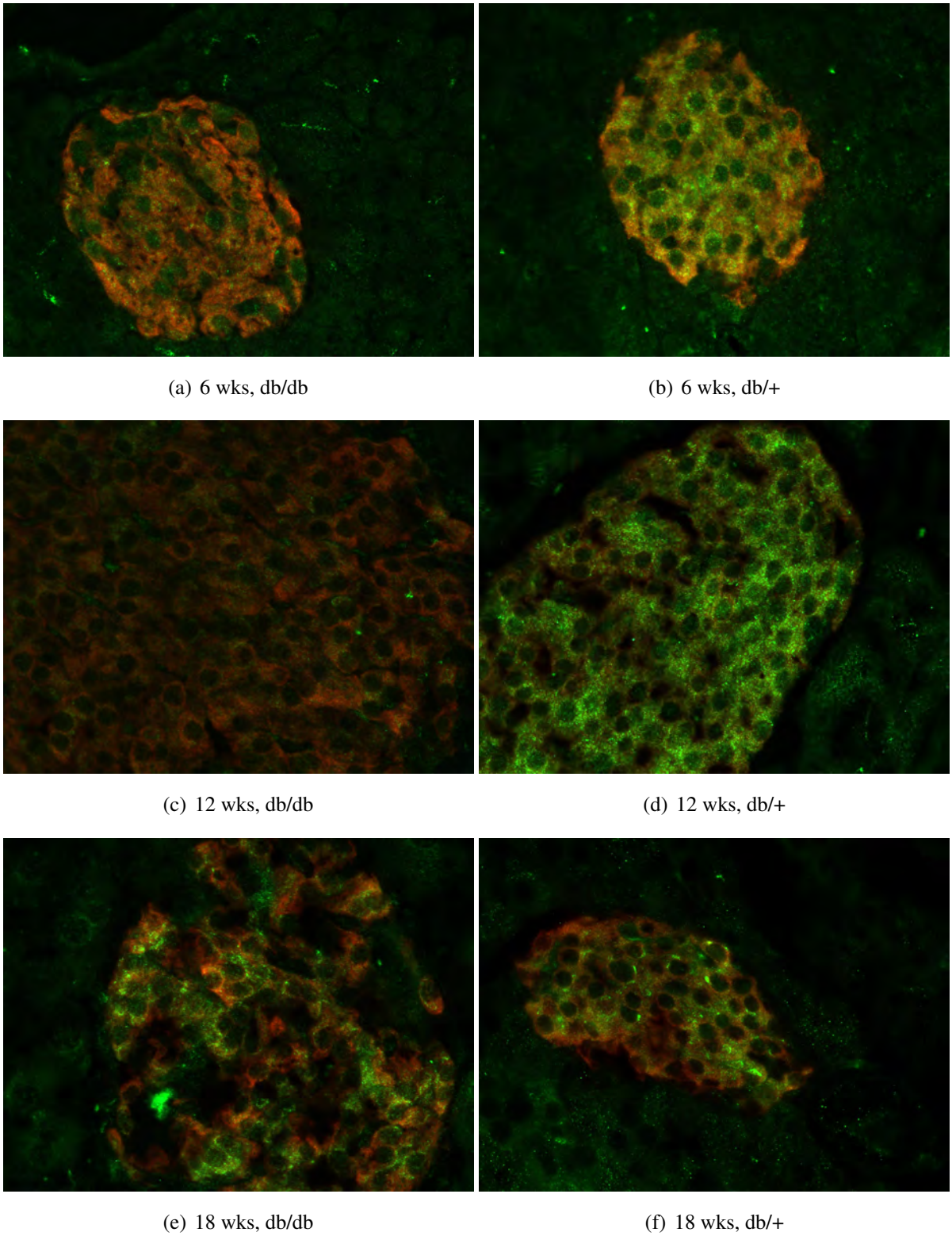


(c)

Figure 3.16: Gene expression of Grx1. The expression in db/db animals showed a significant ($p<0.005$ and $p<0.0001$) gap at 12 weeks (a), a constant decrease in their db/+ counterparts (b) and a significantly higher expression ($p<0.0001$) at 6 and 12 weeks in direct comparison per time point (c) (relative Grx1 expression normalised with beta-actin, n = 4 to 6 per time point).

3.6.1.2 Staining

The analysis of immunostained tissue confirmed the previously described alterations on the gene level. In figure 3.17 examples of both groups of animal at all three time points are shown. Staining was more dense and intense in db/+ mice. Also, a very faint and scarce staining in 12 week old db/db mice is apparent.



(a) 6 wks, db/db

(b) 6 wks, db/+

(c) 12 wks, db/db

(d) 12 wks, db/+

(e) 18 wks, db/db

(f) 18 wks, db/+

Figure 3.17: Representative images of Grx1 staining (green: Grx1, red: insulin, 400x).

Furthermore, mean intensity of all stained slides was analysed and found to be significantly ($p<0.0001$) higher in heterozygote mice compared to homozygotes (figure 3.18).

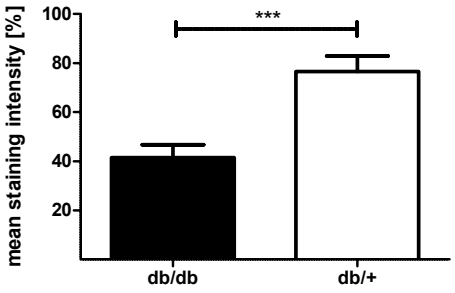
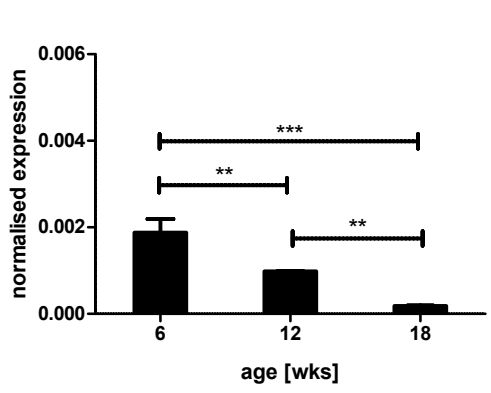


Figure 3.18: Staining intensity of Grx1. Analysis detected a significantly ($p<0.0001$) higher staining intensity for heterozygote islets (n = 9).

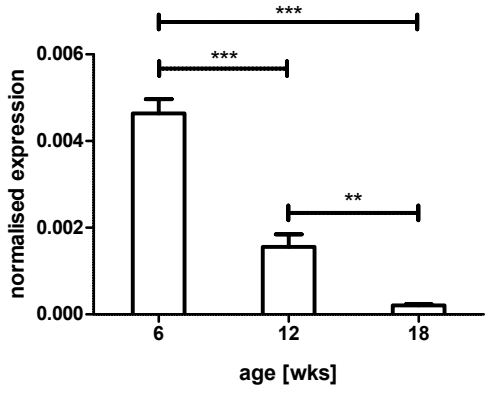
3.6.2 Grx5

3.6.2.1 qRT-PCR

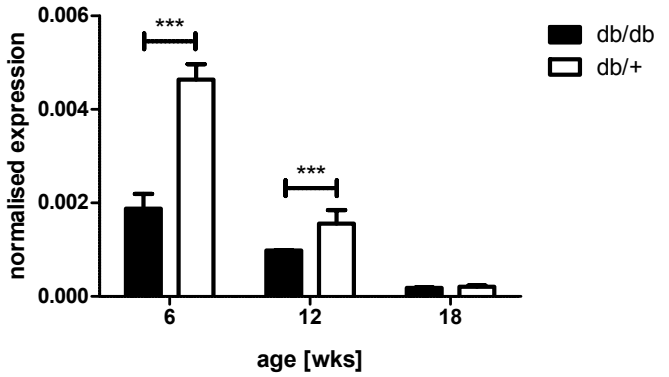
The results of the PCR showed a decrease in the expression of Grx5 in homozygote and heterozygote animals with time. The expression was 10.4 (6 weeks) and 5.4-fold (12 weeks) higher in db/db mice compared to 18 weeks old animals (figure 3.19a). A larger decrease in expression was found for db/+ mice when compared to db/db animals. In heterozygote mice the expression was 22.6 (6 weeks) and 7.6-fold (12 weeks) higher compared with 18 week old animals (figure 3.19b). In both groups the decrease in expression was significant with $p<0.0001$. Also the difference in Grx5 expression between homozygotes and heterozygotes at 6 and 12 weeks was significant ($p<0.0001$) with a mean factor of 2.6 and 1.6. At the age of 18 weeks the difference in expression became negligible (3.19c).



(a)



(b)

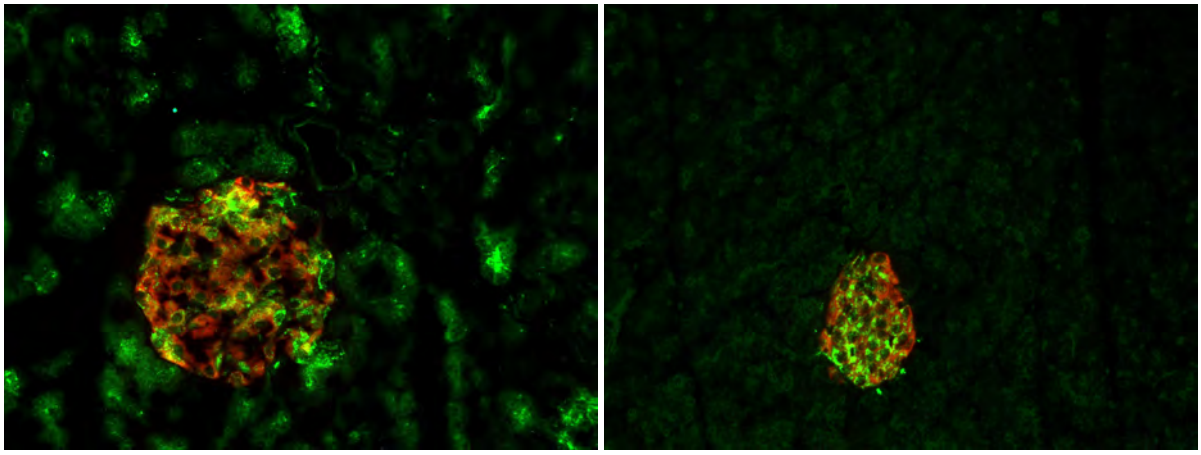


(c)

Figure 3.19: Gene expression of Grx5. Expression in homozygotes (a) and heterozygotes (b) followed a significant downward trend ($p<0.0001$). Direct comparison revealed a significantly ($p<0.0001$) higher expression in 6 and 12 weeks old db/+ islets (c) (relative Grx5 expression normalised with beta-actin, n = 4 to 6 per time point).

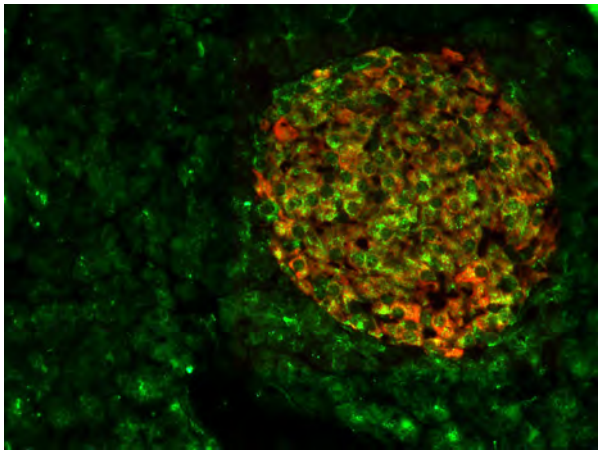
3.6.2.2 Staining

We immunostained pancreatic tissue for Grx5. A stronger staining was found in db/+ mice at the age of 6 and 12 weeks when compared to the homozygotes. At 18 weeks of age the expression of Grx5 in both groups was similar (figure 3.17).

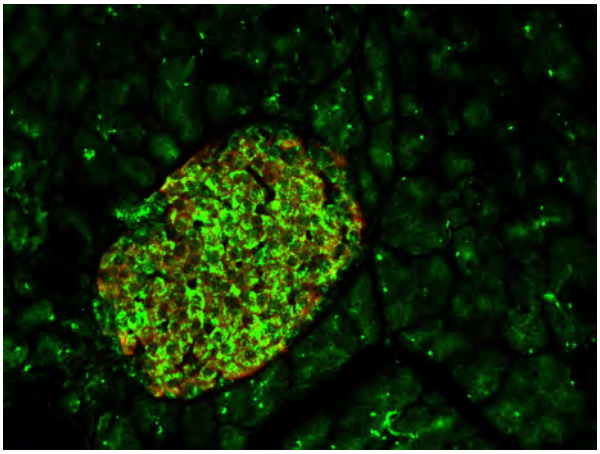


(a) 6 wks, db/db

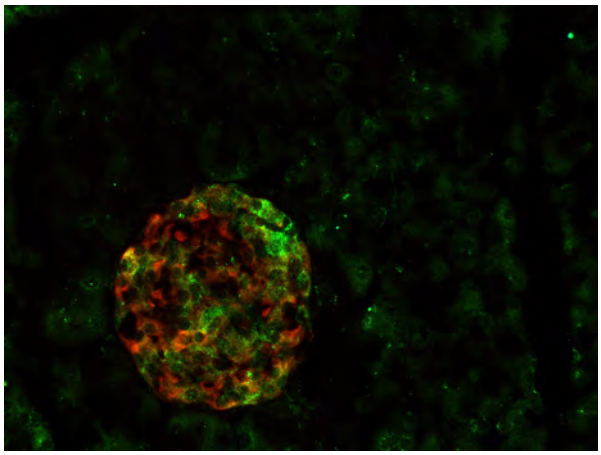
(b) 6 wks, db/+



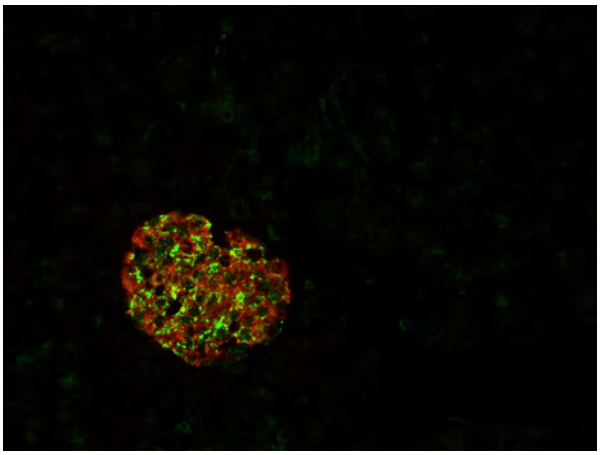
(c) 12 wks, db/db



(d) 12 wks, db/+



(e) 18 wks, db/db



(f) 18 wks, db/+

Figure 3.20: Representative images of Grx5 staining (green: Grx5, red: insulin, 200x).

Also, the staining intensity of Grx5 was compared using images taken from immunostained slides. The expression of Grx5 was significantly ($p<0.0001$) higher in db/+ mice compared to

db/db animals (figure 3.21).

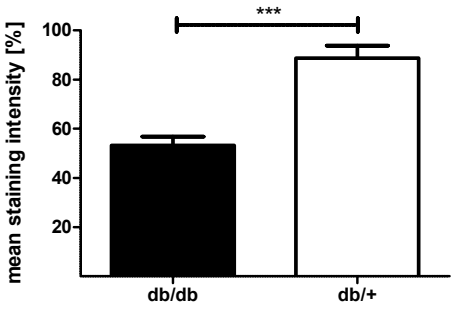
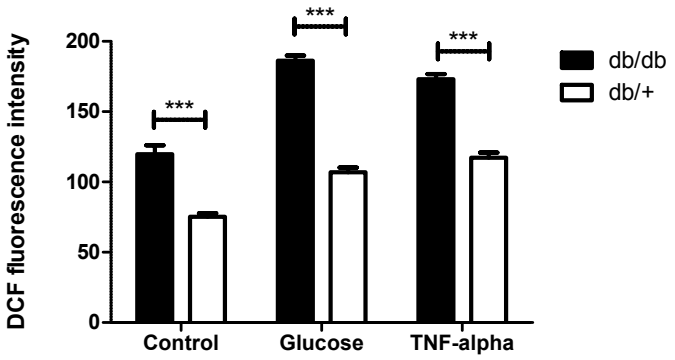


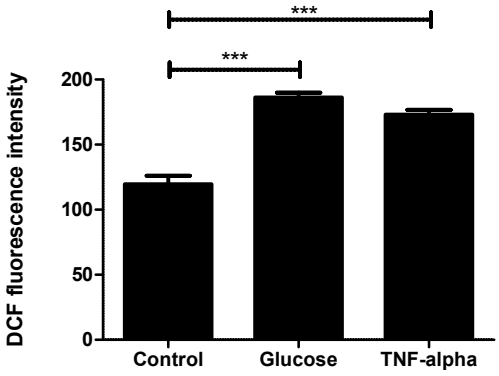
Figure 3.21: Staining intensity of Grx5. Analysis detected a significantly ($p<0.0001$) higher staining intensity for heterozygote islets (n = 9 per time point).

3.7 Marked differences in cellular ROS production

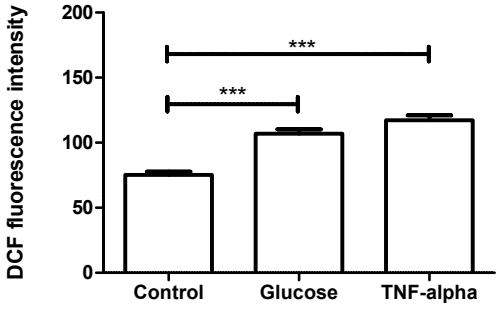
In an effort to further apprehend the pathophysiology of the islets of Langerhans in db mice, and to conclude our previous results, we aimed to detect ROS in isolated islets. Harvested islets were grouped according to the treatment which they received. Control islets underwent no special treatment. In order to observe the impact of glucose- and TNF-alpha-induced stress, two groups were exposed to a high concentration of glucose (20 mM for 2 hours) and TNF-alpha (1 µM for 15 minutes), respectively (section 2.2.7).
Already unstimulated homozygote islets turned out to exhibit significantly higher ROS production than their heterozygote counterparts ($p<0.0001$). Islets treated with glucose or TNF-alpha responded in the same way (figure 3.22a). Furthermore, both kinds of islets underwent a distinctive increase ($p<0.0001$) in ROS when stimulated by glucose and TNF-alpha. This increase in reactive species was most notable in db/db islets, while it was less pronounced in islets of db/+ animals (figure 3.22b, c).
Examples of images used for ROS measurements are displayed in figure 3.23.



(a)

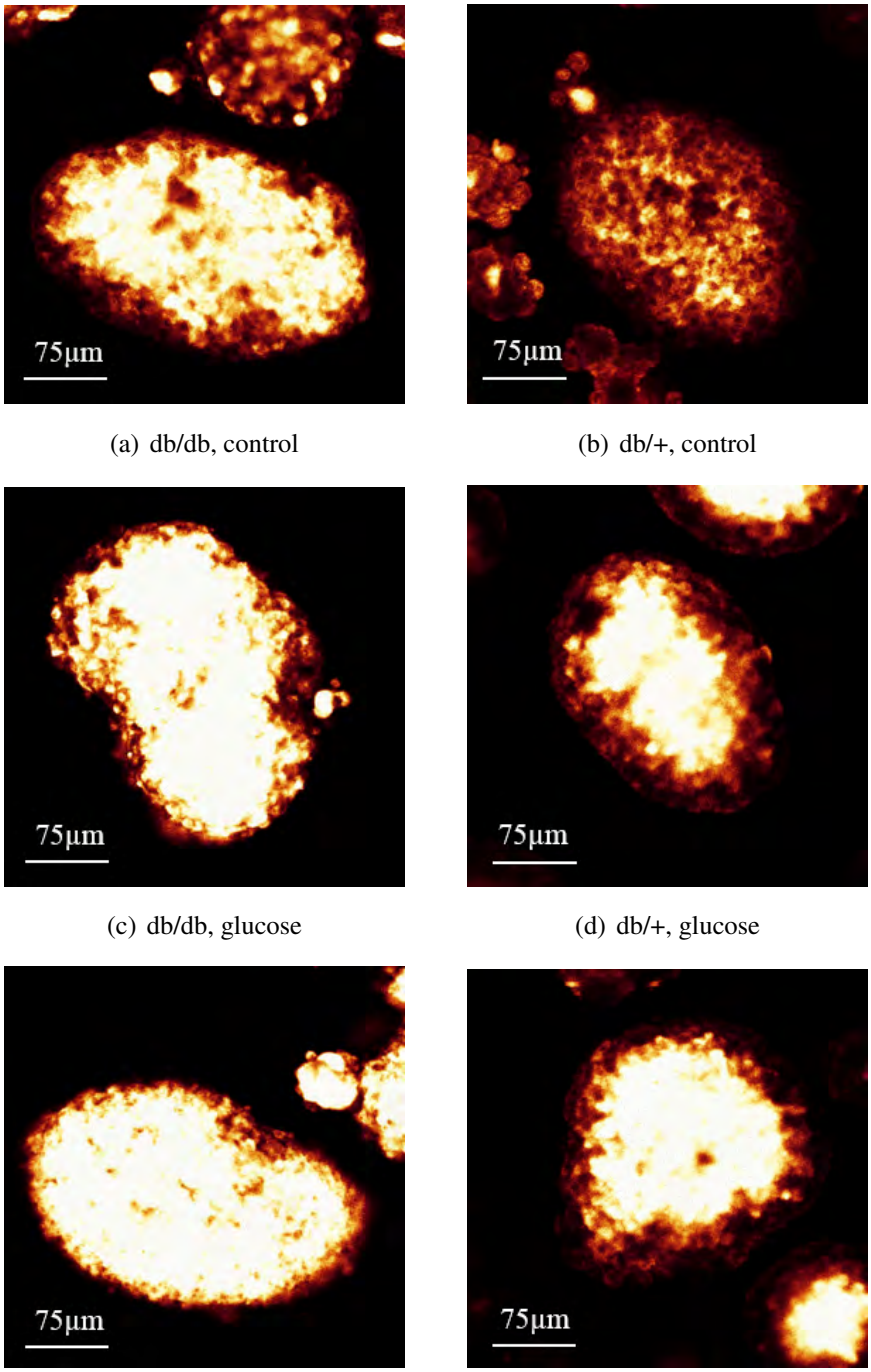


(b)



(c)

Figure 3.22: Basal ROS production and the influence of glucose- and TNF-alpha-induced stress on ROS production in db/db and db/+ islets. ROS production was significantly higher in homozygotes under all conditions ($p<0.0001$) (a). Both groups featured an increase in ROS production when stimulated conditions were compared to controls ($p<0.0001$), although homozygote islets underwent a more pronounced rise than heterozygote ones (b, c) (n = 54 to 139).



(a) db/db, control

(b) db/+, control

(c) db/db, glucose

(d) db/+, glucose

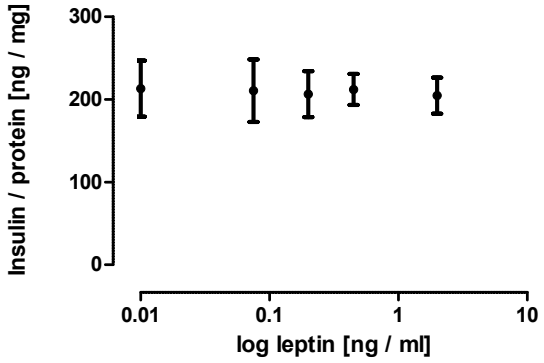
(e) db/db, tnf-alpha

(f) db/+, tnf-alpha

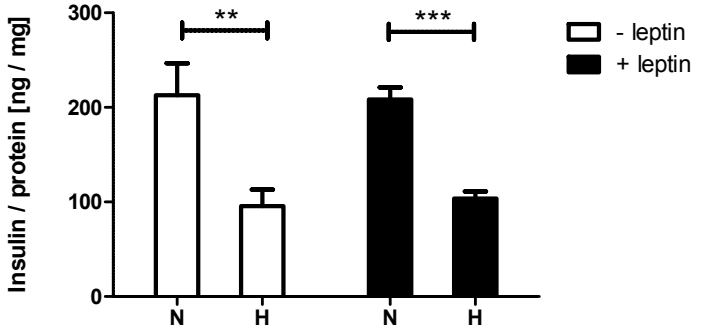
Figure 3.23: Representative images of ROS measurements. Left column: db/db islets, right column: db/+ islets. First row: control islets, second row: glucose treated islets, third row: TNF-alpha treated islets.

3.8 The influence of leptin on MIN6 protein expression

MIN6 cells were exposed to four different concentrations of leptin (0 ng ml^{-1}, 0.075 ng ml^{-1}, 0.45 ng ml^{-1} and 2 ng ml^{-1}) for two hours during cell culture prior to cell lysis as described earlier (section 2.2.5). MIN6 cells were either subjected to normal or hypoxic conditions, because 2 % hypoxia induces the emergence of ROS in pancreatic beta cells.
Insulin content was analysed by ELISA in supernatant. No significant difference in the amount of secreted insulin in dependence with different leptin concentrations could be detected (figure 3.24a). However, significant differences ($p<0.0001$ and $p<0.005$) were found when supernatant of MIN6 cells which had been cultivated under standard conditions were compared with ones which had been exposed to hypoxia (figure 3.24b).



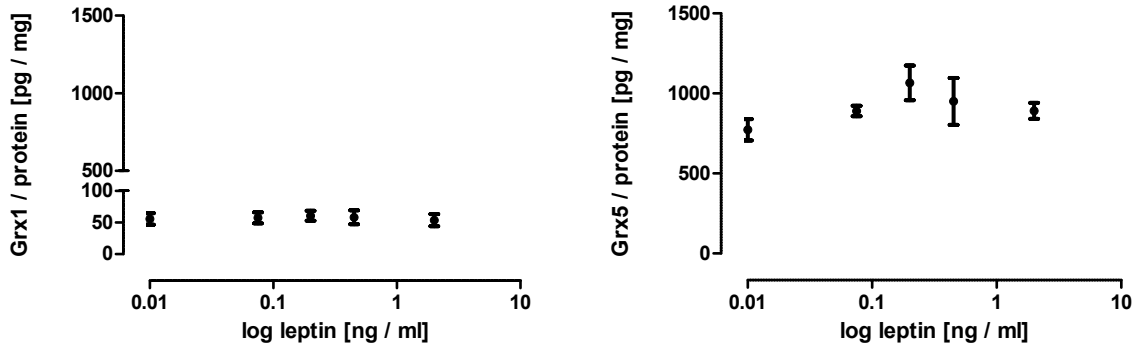
(a)



(b)

Figure 3.24: Leptin effect and insulin synthesis of MIN6 cells. Insulin content did not differ significantly between different concentrations of leptin (a) (n = 6 runs per concentration). Significantly higher insulin secretion could be detected for MIN6 cells which had been cultivated under normoxia in comparison to ones which had been exposed to hypoxia ($p<0.0001$ and $p<0.005$). Leptin concentrations were pooled. (b) (N = normoxia, H = hypoxia; n = 3 to 24 runs per oxygen level).

Grx1 and 5 contents in MIN6 cell lysates were analysed by ELISA. As for insulin, no significant difference was found in Redoxin content for different concentrations of leptin. However, markedly higher protein levels were detected for Grx5 than for Grx1 (figure 3.25).



(a) (b)

Figure 3.25: Grx 1 and 5 protein concentration at different leptin concentrations. No significant difference was detected between Grx1 (a) and 5 (b) levels for different concentrations of leptin (n = 5 runs per concentration).

3.9 Summary of results

	Comparison between db/db and db/+ mice
Body weight [g]	***
Blood glucose [mg dl^{-1}]	***
Islet number	*
Islet area [mm^2]	**
qRT-PCR INS-1	** / *** (6, 12 / 18 wks)
Intensity Insulin	***
Beta-cell proliferation	**
qRT-PCR Grx1	*** (6, 12 wks)
Intensity Grx1	***
qRT-PCR Grx5	*** (6, 12 weeks)
Intensity Grx5	***
ROS basal rate	***
ROS glucose-stimulated	***
ROS TNF-alpha-stimulated	***

	Comparison between leptin concentrations (MIN6)
Insulin ELISA	no significance
Grx1 ELISA	no significance
Grx5 ELISA	no significance

	Comparison between normoxia and hypoxia (MIN6)
Insulin ELISA	** / *** (- leptin / + leptin)

Table 3.1: Summary of the collected results and their significance.

4 Discussion

The various functions of Trx family proteins and their significance in the pathophysiology of diseases has been a subject of research for the past years. It is suspected that they play an important role in the early preclinical stages of diabetes mellitus type 2. Therefore, we explored and compared the expression of glutaredoxins, peroxiredoxins and thioredoxins in obese, diabetic db/db and lean, non-diabetic db/+ mice. Despite marked islet hyperplasia and a new generation of islets, homozygotes lost blood glucose control as the insulin expression in their islets decreased distinctively over time. Visible differences in the expression patterns of Trx family proteins were demonstrated. A clear distinction between both groups of mice is marked by the expression of glutaredoxins 1 and 5. Thus, we believe that there is a strong correlation between them and the development and progress of the pathological changes in diabetes mellitus type 2 in our research animals.

4.1 The influence of the obese phenotype on the islets of Langerhans

There are several animal models for diabetes. We were interested in the consequences of the obese phenotype on pancreatic islets in contrast to the lean one. Two commonly used types of mice on the C57BL/6 background are db/db and ob/ob animals, which both have a defective leptin signalling resulting in obesity. Leptin-deficient ob/ob mice hold a mutation in the hormone itself. C57BL/6 wild type ob/ob animals develop diabetes but regain blood glucose control after stages of hyperglycaemia and hyperinsulinaemia (Bell and Hye, 1983). For this reason, we decided to use db/db mice for our study. The BKS(D)-Leprdb/JOrlRj strain which we utilised originates from a mixture of the C57BL/KsJ (diabetes susceptible) substrain and C57BL/6 wild type mice (Hummel et al., 1972; Coleman, 1978). The development of both separate strains was described recently, showing significant differences between homozygotes and heterozygotes, respectively, in terms of body weight, blood glucose, plasma insulin, beta-cell mass, beta-cell proliferation and apoptosis. (Puff et al., 2011).
Accordingly, we observed that obese homozygote db/db mice lost blood glucose control (figure 3.3) while gaining weight rapidly. Both, fasting blood glucose level as well as body weight (figure 3.2) marked a significant difference between them and heterozygotes. In contrast, those heterozygotes resembled wild type C57BL/6 mice in body weight and blood glucose levels which makes them a reasonable control group (data shown in appendix, figure 11.3 and 11.4). In this way we had the opportunity to study viable homozygote genotypes for 18 weeks and compare them to their heterozygote counterparts.

As a correlate of raising blood glucose levels we found a strong decrease in the INS-1 gene expression in homozygotes (figure 3.8) together with less insulin staining in islets (figure 3.7).

In contrast, heterozygotes only presented a slight decrease in insulin gene expression, matching their stable fasting blood glucose. We neither determined their plasma insulin level nor their glycated haemoglobin. However, it was shown that insulin levels were dropping and the HbA_{1c} was rising significantly over time in db/db animals (Yamazaki et al., 2009). Furthermore, pancreas weight and pancreatic insulin levels were found to be considerably different when db/db and heterozygote control animals were compared (Garris and Garris, 2004).
In compensatory response to declining insulin secretion and raising blood glucose levels, there were considerable cellular changes in the pancreas of our db/db animals. Mean islet area (figure 3.6), islet proliferation (figure 3.10) and islet count (figure 3.4) revealed significant differences between both groups of animals, which was consistent with the previously described studies (Puff et al., 2011; Kawasaki et al., 2005). Elevated blood glucose levels may explain these findings, since it was reported that glucose promotes proliferation in several cell lines (Hügl et al., 1998), in beta-cells of rodents (Swenne, 1982) and humans (Tyrberg et al., 1996), and also in a rat model of diet-induced diabetes (Del Zotto et al., 2002).
Concordantly, our data (figure 3.11) suggest that cell death was especially higher in young homozygotes than in heterozygotes. Caspase staining was mainly located in the periphery of islets. Additionally, caspase and insulin staining did rarely match. This staining pattern can be found throughout literature in mouse and rat models (Reddy et al., 2003).
Thus, it has to be suspected that caspase-3 staining covered primarily non-beta-cells or dedifferentiated beta-cells. Beta-cell dedifferentiation is known to play an important role in beta-cell dysfunction. In the pathophysiology of diabetes, an early switch in beta-cell gene expression leads to downregulation of physiologically active genes and upregulation of suppressed genes. Beta-cells lose their differentiation and cease to produce insulin. (Weir and Bonner-Weir, 2004). Studies indicate that beta-cell dedifferentiation is closely connected to a loss of Forkhead box protein O1 (FOXO1) expression. Transcription factor FOXO1 has influence on beta-cell mass and stress response, as well as cell differentiation in several cell types. While dedifferentiated beta-cells show reduced FOXO1 expression, they express transcription factors usually found in progenitor cells. These findings were confirmed for islets of db/db mice (Talchai et al., 2012). At states of severe hyperglycaemia, dedifferentiation is accelerated. Thus, the concept of beta-cell dedifferentiation gives an explanation for the loss in insulin producing cell mass while beta-cell apoptosis is an rarely observed event in db/db specimen.
An explanation for the loss in islets due to beta-cell apoptosis and dedifferentiation can be found in the concepts of glucotoxicity and lipotoxicity (section 1.2.5). The formation of ROS, the triggering of stress-sensitive pathways such as signalling via NF-kappaB, glucose autoxidation and the alterations on the gene level caused by chronic hyperglycaemia have a detrimental impact on beta-cells and glucose metabolism. It was reported that beta cells in db/db mice have a reduced expression of GLUT2 and thereby an impaired reaction to serum blood glucose (Thorens et al., 1992). In Zucker rats defective reaction to glucose in combination with hyperglycaemia was observed when the ratio of GLUT2-negative beta-cells exceeded 40 % (Johnson

et al., 1990). Those findings might account for loss in proliferation and insulin secretion in ageing homozygotes. Furthermore, a study with New Zealand Obese (NZO) mice revealed results similar to those in homozygote db/db animals. NZO mice were rendered obese by diet and then separated into two groups, receiving either a carbohydrate-free diet or a diet containing carbohydrates. Compared to the control group, obese NZO mice fed with carbohydrates had high blood glucose levels combined with a reduced pancreatic insulin content, down-regulated GLUT2 receptors on beta-cells, increased beta-cell apoptosis and decreased Pdx1 and FOXO1 expression (Kluth et al., 2011).

4.2 Trx family protein expression in dependency of the diabetic environment

We explored the expression of Trx family proteins in our research animals over time. The stainings of thioredoxins (figure 3.13), peroxiredoxins (figure 3.14) and glutaredoxins (figure 3.15) revealed varying expression patterns and differences between homozygote and heterozygote animals.

We focused on Grx1 and 5, because of all analysed redoxins their expression was most pronounced in pancreatic islets. All other redoxins merely showed a faint expression in both homozygote and heterozygote islets. Moreover, there was a marked difference in Grx1 and 5 expression between islets of both groups of mice. No such evident distinction was apparent for any other redoxin. Apart from that, both isoforms appeared to be promising targets to study because of their common features and differences. While they belong to the same subsystem of glutathione dependent Trx family proteins, the glutaredoxins, they differ regarding their intracellular location and purpose and their catalytic activity. Grx1, on the one hand, is a mainly cytoplasmatically located glutaredoxin with impact on cell differentiation and several transcription factors, expressing high catalytic activity in the thiol-disulfide exchange. The main task of Grx5, on the other hand, is maintaining iron homeostasis in the cell. It is located in the mitochondria and required for the assembly of iron-sulfur clusters and thereby also for the activation of several respective enzymes.

Our qRT-PCR data for Grx1 revealed that the level of Grx1 expression varied with the amount of islets and the mean islet area. Between 6^{th} and 18^{th} week there was a gap in the number of islets as well as in Grx1 expression in homozygotes. Mean islet area was largest at this time point. Heterozygotes, on the contrary, showed a significantly higher, stable expression of the protein (figure 3.16) as well as higher protein levels (figures 3.17 and 3.18). Since Grx1 is anti-apoptotic and functions as a promotor for cell proliferation (section 1.3.3), the expression of Grx1 may facilitate the generation of new islets in db/db animals in response to hyperglycaemia. Similar effects were witnessed in hearts of diabetic mice where increased Grx1 levels achieved by gene therapy prevented cardiac damage caused by ischaemia in diabetic mice (Lekli

et al., 2010).
The expression of Grx5 in db/db mice followed the same downward trend as the expression of the INS-1 gene (figure 3.19). We found a constant decrease in both groups of animals with the heterozygotes having a higher expression in general (figures 3.20 and 3.21). The function of the redoxin was studied in yeast mutants lacking Grx5. They were found to be much more susceptible to oxidative and osmotic stress. They suffered from increased ROS, an accumulation of iron in the cell and an inactivation of enzymes containing Fe-S clusters whose generation requires Grx5 (Rodríguez-Manzaneque et al., 1999, 2002). The role of Grx5 in obese db/db mice might be similar, as ageing animals suffer from islet apoptosis and dysfunction while the Grx5 expression recedes.
In conclusion, heterozygote db/+ mice had a higher gene expression for Grx1, Grx2 (data shown in appendix, figure 11.5) and Grx5. Also the protein level was found to be higher for Grx1 and 5 (section 3.6.1.2 and 3.6.2.2). Especially for Grx5 our results revealed a strong correlation with age. Both diabetic db/db and non-diabetic db/+ mice exhibited a drastic decrease in Grx5 gene expression, which leads to the assumption that Grx5 is even more dependent on age than on diabetes and obesity, although non-diabetic lean heterozygotes expressed more Grx5 than their diabetic obese homozygote counterparts. Grx1, by contrast, seems to correlate with the metabolic situation, exhibiting varying expression together with marked alterations of islet area and diabetes in homozygote db mice.
These findings suggest that there is a correlation between the Trx family of proteins on the one hand and the obese phenotype and diabetic environment on the other hand.

Researchers have begun to gain insight into that link. It was revealed that NADPH promotes insulin secretion in MIN6 cells and isolated islets of rats and that Grx1 and Trx1 have influence on the process. While Grx1 has a positive effect on NADPH-mediated exocytosis of insulin, Trx1 was found to oppose its release (Ivarsson et al., 2005). Furthermore, another study showed that Grx1 knockout in INS-1 cells and rat islets results in an impaired insulin secretion (Reinbothe et al., 2009). A protective effect in db/db mice overexpressing Trx1 could also be detected. The gain in body weight and the reduction of insulin secretion were counteracted in those animals and thus the progression of hyperglycaemia was slowed down (Yamamoto et al., 2008). Moreover, studies suggest a positive effect of Peroxiredoxins. Prx3 is an antagonist against several noxious agents and a lack of the protein affects insulin secretion in rat insulinoma cells (Wolf et al., 2010). Similar protection was witnessed for Prx4 (Ding et al., 2010).
These studies suggest that the observed differences between homozygote and heterozygote db mice including phenotype, blood glucose levels, islet count, area, proliferation and apoptosis, as well as insulin expression are not merely a result of their phenotype, but also of a diverging redoxin expression. Furthermore, the significance of Trx family proteins in humans has been investigated in several studies. A lowered Grx1 activity was observed in platelets of patients suffering from diabetes (Di Simplicio et al., 1995), and Japanese researchers detected a corre-

lation between a polymorphism of the human thioredoxin gene (TXN) and diabetes (Ikegami et al., 2008). Besides, diabetic patients showed increased expression of Txnip, which is up-regulated by glucose (Parikh et al., 2007) and prevents proper Trx functioning (Schulze et al., 2004).

We did not only observe lower Grx expression in homozygote db mice, but also increased basal ROS levels (section 3.7). Stimulation by glucose and TNF-alpha lead to even higher formation of reactive species, especially in homozygotes. On the one hand, ROS occur in aerobic metabolism, represent physiological reactions of a healthy cell, and an elevation implies an increased metabolic activity (Fleury et al., 2002). On the other hand, an imbalance between reactive species and defense mechanisms causes oxidative stress. Due to this imbalance, cells take damage and ROS connote with harmful effects (Evans et al., 2002). It is to be suspected that db/db islets underwent pronounced metabolism in these diabetic obese animals, represented in our experimental setup by exposure to glucose and TNF-alpha. Even at basal conditions, they featured elevated ROS. At a certain stage, the formation of ROS could not be countered anymore by the inherently weak cellular defense mechanisms of the beta cell, resulting in cell damage and apoptosis. In contrast, db/+ islets featured lower basal and stimulated ROS formation, indicating physiological cell metabolism and an operational redox regulation.

4.3 Adipokines in the pathophysiology of diabetes mellitus type 2

Another main influence factor which has to be considered is the effect of messengers released by white adipose tissue (WAT). Autocrine, paracrine and endocrine regulation is exerted by so-called adipokines and adipose-derived hormones. Such products of WAT had already been described in the 1980s (Flier et al., 1987). In 1994, researchers identified leptin as an adipose-derived hormone (Zhang et al., 1994) and subsequently, its receptors, too (Tartaglia et al., 1995). Being mainly produced by WAT, the amount of leptin correlates with fat mass and is thus elevated in obese subjects (Fain et al., 2004). Accordingly, obese animal models such as the db/db mouse express high levels of ob mRNA and leptin (Maffei et al., 1995). As a key regulator of energy homeostasis (Friedman and Halaas, 1998), leptin has an impact on glucose metabolism and on the beta-cell. On the one hand, leptin inhibits the secretion of insulin (Kulkarni et al., 1997) as well as insulin gene expression (Seufert et al., 1999), but on the other hand, insulin stimulates leptin secretion (Laferrère et al., 2002) and gene expression (Moreno-Aliaga et al., 2001). Those findings lead to the postulation of a so-called adipo-insular axis which consists of a feedback loop between WAT and the pancreas (figure 4.1).

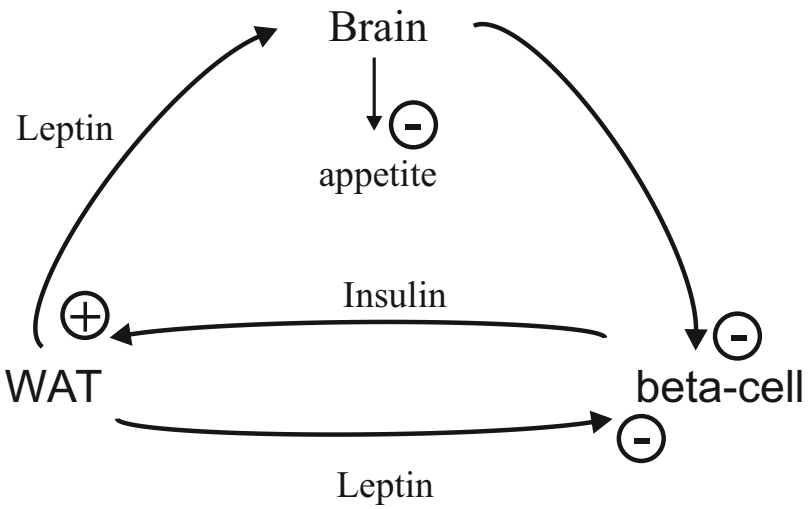


Figure 4.1: The adipo-insular feedback loop. Apart from the regulation between WAT and the pancreas, leptin restricts appetite and the autonomous nervous system inhibits insulin secretion, too (Kieffer and Habener, 2000).

As a confirmation of this hypothesis, a normalisation of body weight, body fat content, food intake, blood glucose level and hyperinsulinaemia is achieved within a few weeks by administering leptin to ob/ob mice. Furthermore, an intraperitoneal or intracerebroventricular administration of the hormone on homozygote db/db animals has no effect due to the defect of the Ob-Rb receptor (Halaas et al., 1995), while pregnant db/+ animals show benefit, namely an ease of the symptoms of gestational diabetes mellitus (Yamashita et al., 2001).
Accordingly, a supraphysiological release of insulin resulting in hyperinsulinaemia, typical for the pathophysiology of diabetes mellitus type 2 (section 1.2.5), is found in ob/ob and db/db mice alike. This stage of the disease is reached already at a young age in those animals and precedes obesity and insulin resistance (Coleman and Hummel, 1974). When we started observing our research animals, they were already past this stage and presented beta-cell dysfunction and failing insulin secretion.
An important observation that has to be considered in this context is leptin resistance. Both central as well as peripheral resistance (Halaas et al., 1997), including the beta-cell (Seufert, 2004), have been observed. Impaired leptin signalling, as functionally present in db/db animals, poses an explanation for the fact that there is no effect of leptin in obese subjects from a certain point in the development of obesity onwards. Although impressive effects of exogenously administered leptin, including weight loss, were described in two children suffering from leptin deficiency (Farooqi et al., 2002), no significant reduction of weight was found in a randomized, placebo-controlled trial with 284 subjects (BMI 27 to 37 $kg\,m^{-2}$, age 46.8 ± 10.4 years) in 2005 (Zelissen et al., 2005).

However, our experiments with MIN6-cells exposed to leptin showed no regulation of neither insulin nor Grx (section 3.8). Regarding these findings, it has to be taken account of the fact that we used high passage MIN6-cells (passage 70 to 80), which are known to be non-gluco-responsive (Cheng et al., 2012). Thus, our results suggest that glucose is a required cofactor in leptin-triggered regulation of insulin and redoxins. On the one hand, we observed a marked decline in insulin secretion when MIN6 cells were exposed to hypoxia. On the other hand, our experiments were missing the physiological and biochemical context of the islet of Langerhans as a micro organ, like in experiments carried out with isolated islets.

Apart from the regulation of energy homeostasis, leptin is also a modulator of immune responses. The phagocytic function of macrophages of ob and db mice was already evaluated in 1998. Results revealed that significantly less macrophages from homocygote animals were active in comparison to heterozygotes. Furthermore, application of leptin improved phagocytosis in ob/ob, ob/+ and db/+ animals, whereas db/db macrophages turned out to kill significantly less targets, suggesting a strong effect of administered leptin. Those observations could also be confirmed in vivo when labeled E. coli bacteria were injected into ob animals. Clearance was significantly more efficient in lean control animals, suggesting an important function of leptin in cellular immune defence (Loffreda et al., 1998). Moreover, a study revealed a strong effect of leptin on the expression of inflammation-related genes. Fibrinogen-beta, Lipocalin-2, manganese-dependent superoxide dismutase, pancreatitis-associated protein, preprotachykinin-1 and tissue-type plasminogen activator were shown to be upregulated by leptin in insulinoma cells. Its authors emphasize that those transcripts encode for both protective as well as toxic products (Hekerman et al., 2007). It is still a matter of research to what extent and under which conditions leptin carries out a protective function and when its effects are rather harmful. Some researchers were able to present protective effects, for example a certain resistance against apoptosis induced by fatty acids in Zucker diabetic rat (Shimabukuro et al., 1998) and a prolongation of viability of isolated rat beta-cells by administration of leptin (Okuya et al., 2001). However, there are opposing results. An in-vitro study targeting human islets revealed a leptin-induced increase in beta-cell production of interleukin 1 (IL-1) while a decrease in IL-1 receptor antagonist (IL-1Ra) was observed. The authors demonstrated a protective effect of exogenously administered IL1Ra while antagonising it resulted in corrupted insulin release and increased apoptosis measured by caspase-3 activation and fragmentation of DNA (Maedler et al., 2004). Altogether it can therefore be suspected that leptin plays a major role in the pathophysiology of diabetes and is certainly involved in pathologies of db and ob animals, giving an explanation for the fact that db mice present very early changes in comparison with lean phenotypes.

4.4 Limitations

Some limitations have to be considered. On the one hand, there is a limited possibility of transferring results gained from mouse experiments to human subjects. The animals' metabolism runs much faster, resulting in an accelerated cell cycle and a quick progress of the pathologies associated with obesity and hyperglycaemia. Also, the complete resistance to leptin represents an extreme genetic variant which is generally not present in human diabetic patients. On the other hand, the choice of our model allowed us to observe a distinct phenotype which take years to develop in human subjects.
When we started our observations, homozygote animals already displayed marked phenotypical alterations. Body weight, blood glucose level, islet area and INS-1 expression had already been pathological. Starting our study at an earlier age would thus have enabled us to observe early development of especially homozygote db/db animals. By contrast, we finished our study at 18 weeks of age. Regarding our data for heterozygotes, it appears promising to follow their development beyond 18 weeks of age. They weighed more than wild type animals and their blood glucose level and islet area followed an upward trend while a decrease in insulin and Grx expression was noticed, leaving open the question whether they would eventually progress into diabetes like their homozygote counterparts. Nonetheless, the selected time period allowed us to sufficiently study the selected mouse model and outline the differences between db/db and db/+ animals. In the consulted literature, db animals were usually studied from 5 to 16 weeks of age on average. Thus, we concluded that a respective observation period can be considered sufficient for our intended analysis of Trx family proteins and the islets of Langerhans. Rarely, animals were kept for longer than 20 weeks. The progression of the body weight, blood glucose level including HbA_{1c} and the morphology of islets appeared to show now more marked shift from 18 weeks of age onwards in homozygotes. It must also be taken into account that there was a logistical limitation regarding the start of our study. Charles River provided the animals at the age of 5 weeks. After being deployed to our animal housing, the mice were given time to get accustomed to their new environment. Thus, 6 weeks of age appeared to be a reasonable start. Starting earlier would subsequently have required us to breed the animals ourselves, which was not possible in our laboratory.

Regarding our methods, it has to be taken into account that we used qRT-PCR, not revealing the absolute gene expression levels. Despite not knowing to what exact extent gene expression was constrained in the studied research animals, we were able to point out the severe impact of a homozygote db/db mutation on INS-1 and Grx expression in islets by comparison with heterozygotes.
We also did not determine absolute protein levels for insulin and redoxins in pancreatic tissue, but established a semiquantitative approach using normalised pictures taken of immunostained tissue (section 2.2.2). The data were consistent with results gained from PCR and plasma in-

sulin levels as well as pancreatic insulin content (section 4.1).
For the immunohistological analysis of islets, an interval of 140 µm was maintained between sections in order to avoid multiple inclusion of identical islets. During our studies a mean islet area of 200 µm was detected for 12 weeks old homozygotes. Thus, measuring the same islet twice was avoided by careful observation of consecutive slides.
We have not succeeded in quantitatively analysing apoptosis in islets. A TUNEL kit was used for staining, but there were too few TUNEL positive cells for a reasonable evaluation. Corresponding results to our qualitative approach were mentioned in section 4.1.

4.5 Conclusion

In summary, we defined the pancreatic pathologies of obese, diabetic and leptin-resistant homozygote db/db mice and delineated them to their lean, non-diabetic heterozygote counterparts. In addition, we demonstrated a significantly different expression of Trx family proteins between both groups of mice, especially a lower one for Grx1 and 5 in homozygotes, which has not yet been described before to our knowledge. Moreover, we detected an increased formation of ROS in pancreatic islets of db/db animals. A direct influence of leptin on Grx and insulin protein levels could not be observed.
On the one hand, we suspect that obesity with all its implications, such as elevated reactive species, is an important factor behind early blood glucose dysregulation in db/db mice. The adipo-insular feedback loop is interrupted and insulin secretion cannot be inhibited by leptin and satiety after ingestion. On the other hand, insulin secretion and beta-cell survival is impaired in several ways. A lack of Grx 1 is connected to reduction of numerous transcription factors, for example Nf-kappaB, AP-1, CREB, Nuclear Factor I and ASK1. Additionally, the redoxin is missing in the Grx-pathway, interfering with cellular antioxidant capacity, and its role in promoting exocytosis of insulin is affected as well. Reduced levels of Grx5 cause iron overload as well as inactivation of proteins requiring iron-sulphur clusters, including enzymes of the Krebs cycle. The amount of GLUT2 is reduced under oxidative stress. Enzymes of the Krebs cycle as well as anaplerosis and cataplerosis are corrupted directly. Also, deleterious effects on ATP-sensitive potassium channels have been observed. Taken together, these influence factors deteriorate the beta-cells' physiological response to glucose. A summary is given in figure 4.2.

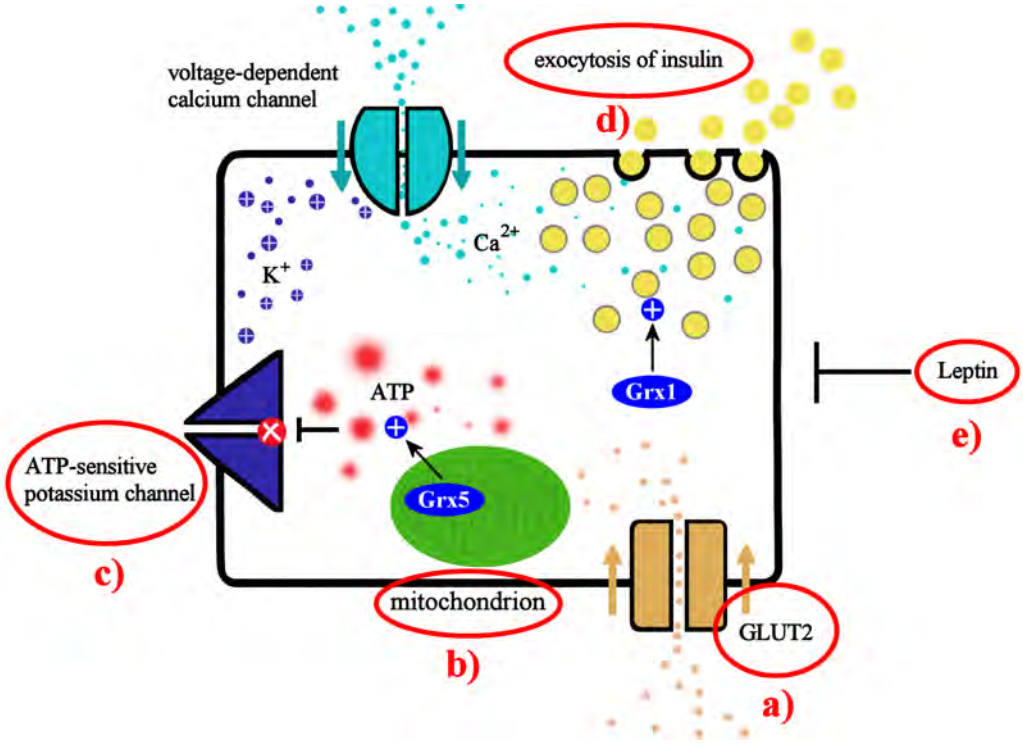


Figure 4.2: Implications of our results on the physiology of insulin secretion of the beta-cell in correspondence with literature. a) Under oxidative stress, the amount of GLUT2 in the beta-cell's membrane was found to be decreased. b) Mitochondria develop pathologies regarding morphology and function. Anaplerosis and cataplerosis of the Krebs cycle are corrupted and its enzymes are directly inhibited by oxidants. A lack of iron-sulphur clusters caused by a deficiency of Grx5 further adverses production of ATP. Thus, a functional isolation of glucose uptake, processing and ATP production from ATP-sensitive potassium channels is observed. c) Those channels are furthermore directly and indirectly harmed by free radicals. d) Also, a shortage of Grx1 is supposed to impair exocytosis of insulin. While these facts contribute to defective insulin secretion, leptin exhibits an inhibitory effect. e) However, in leptin-resistant db/db mice as well as in adipose subjects the hormone cannot exert its function, resulting in enhanced insulin secretion.

In conclusion, we propose that the redox state of the beta-cell and Grx1 and 5 are closely connected to impaired insulin secretion and destruction of the islets of Langerhans.

However, the mechanisms underlying induction and depletion of redoxins as well as their role in protection and regulation of cellular processes require further investigation. Although there are several studies with animals over- or underexpressing Trx family proteins, leading to partly severe, and partly mild phenotypical consequences (section 1.3), the exact role of redoxins in diabetes remains vague. Pancreas or beta-cell specific knock-down or -up models could supply powerful tools to illuminate the functions of those factors. On the one hand, the effects of a

lack of Grx1 are suspected to depend on the respective target proteins. Mice deficient in Grx1 show neither increased myocardial damage after ischemia, nor significantly higher lung injury after hyperoxia. Embryonic fibroblasts of Grx1 deficient mice are protected against apoptosis induced by TNF-alpha, and are more susceptible to deleterious effects of diquat and paraquat but not to those of H_2O_2 and diamide (Ho et al., 2007). On the other hand, growth defects, iron overload and increased susceptibility to oxidants are well documented in yeast mutants lacking Grx5 (Rodríguez-Manzaneque et al., 2002). Thus, observation of redoxin expression and protein levels as well as blood glucose level and development of islets, including morphology, islet insulin content, proliferation and apoptosis in such a pancreas or beta-cell specific animal model should provide important information regarding the role of redoxins. A conceivable approach could be to chose beta-cell specific Grx1 and 5 knockout models, respectively, and stress homozygotes and heterozygotes by high-carbohydrate diet to induce hyperglycaemia and oxidative imbalance. Based on our data, we predict that decreased levels of Grx1 or 5 will lead to elevated islet apoptosis and dedifferentiation, defective insulin secretion and thereby corrupted glucose homeostasis, while elevated levels of Grx1 or 5 should have rather protective effects.
Furthermore, parameters which give information about the redox state of the pancreas and isolated beta-cells should be investigated. GSH / GSSG levels, glutathionylation of proteins, redox modifications of the proteome and identification of targets for redoxins should provide more detailed insights into connections between diabetic pathologies and impaired redox signalling. Moreover, it has to be discussed whether Trx family proteins could be used as diagnostic indicators of early prediabetic stages. Recently, a study was carried out to determine whether there is a difference in Grx1 activity and expression in blood plasma of healthy subjects compared to patients with abnormal glucose tolerance. 14 such patients diagnosed with IFG, IGT or diabetes type 2 according to fasting blood glucose or OGTT (median age 66.6 years, median fasting blood glucose $6\,\mathrm{mmol\,l^{-1}}$) who had not received antidiabetic medication previously or during the study were compared to ten healthy subjects (median age 25.5, median fasting blood glucose $4.9\,\mathrm{mmol\,l^{-1}}$). A Grx1 activity assay kit was used in order to measure Grx1 activity after over night fasting and after OGTT. Baseline Grx1 activity was significantly higher among patients. However, healthy subjects presented a significant increase in Grx1 activity two hours after OGTT while a slight decrease was found in the patients. Grx1 was further analysed using ELISA and Western blot, again revealing significantly lower levels in healthy subjects in comparison to patients. No influence of OGTT could be detected here (Du et al., 2013). Our data suggest that heterozygote animals express homozygotes' pathologies in an attenuated and delayed form, especially in terms of body weight, blood glucose level, INS-1 expression and mean islet area. Thus, heterozygote db/+ mice might be compared to patients suffering from abnormal glucose tolerance in the previously mentioned study, presenting an explanation for their elevated Grx levels in comparison to diabetic homozygotes. Furthermore, similar results were found for Trx in a study including 174 patients diagnosed with type 2 diabetes (87 males, age 61 ± 13; 87 females, age 61 ± 14 years) and 16 healthy subjects (9 males, 7 females, age

60 ± 16 years). Trx levels in participants' serum measured by ELISA were found to be significantly higher in patients (Kakisaka et al., 2002).
In order to find out at what developmental stage redoxin levels show measurable differences between diabetic and non-diabetic animals investigation should begin earlier. While we started observing mice when they had already displayed distinct pathologies, the involved mechanisms causing and following altered redoxin levels should become clearer in specimen with less advanced phenotypical changes. Abnormally high plasma insulin levels for example can already be observed in 10 to 14 days old animals (Coleman and Hummel, 1974). We consider a Pdx1 knockout model to be a revealing approach. Homozygote animals lacking Pdx1 do not develop a pancreas and die days after birth (Jonsson et al., 1994). In adult animals, Pdx1 is known to transactivate the insulin promotor (Ohlsson et al., 1993). Mice with a heterozygote inactivation of Pdx1 ($Pdx1^{+/-}$), similar to human MODY type 4, were previously compared to healthy littermates ($Pdx1^{+/+}$). There were no significant differences in body weight between both groups of mice. Heterozygotes presented elevated blood glucose levels after glucose tolerance tests and lower insulin secretion when pancreata were perfused with glucose in situ. Furthermore, isolated islets from heterozygote animals were of lower count and appeared damaged, which was confirmed by a histologically corrupted islet architecture. Nonetheless, islets did not show impaired insulin release in reaction to glucose, but exhibited a significant increase in apoptosis when cultured in 5 mM or 10 mM glucose for 72 hours which resulted in significantly lowered islet count and mass from 5 weeks of age onwards (Johnson et al., 2003). Taken together, $Pdx1^{+/-}$ animals displayed obvious pathologies of glucose homeostasis which were not yet notable in single beta-cells apart from elevated apoptosis. Therefore, such an animal model should provide an insight into early defects of redox signalling, especially if inactivation of Pdx1 was achieved after birth, for example on day 5. Thereby prenatal influences of defective Pdx1 function were neutralised and the experiment would focus on the beginning of the previously described pathologies.

As Trx family proteins increasingly gain attention by many researchers, their role in diabetes mellitus will certainly be untangled in the near future. Research will hopefully lead to new therapeutical strategies on the basis of those findings. The role of adipose-derived hormones like leptin, in combination with Trx family proteins, might provide new options for future therapy, in the complex and yet not fully explored pathophysiology of adipositas and diabetes mellitus type 2, as well.

5 Summary

Over 300 million people worldwide are suffering from diabetes mellitus type 2, a complex disease consisting of decreased insulin sensitivity, increased gluconeogenesis, impaired insulin secretion and apoptosis of the beta-cells. Furthermore, chronic inflammation and oxidative stress caused by free radicals play a major role in its pathophysiology.
A defence mechanism against oxidative stress is redox signalling. Major actors in re-establishing redox homeostasis are thioredoxin family proteins, including glutaredoxins, peroxiredoxins and thioredoxins. They modify their substrates by oxidation and reduction reactions.
We studied the consequences of an obese phenotype on the Islets of Langerhans in comparison to the lean one. As an animal model we chose db mice. Homozygotes of this strain of C57BL/6 mice suffer from leptin resistance due to a mutation in the transmembrane leptin receptor Ob-Rb, developing an obese, diabetic phenotype. We delineated homozygotes to their heterozygote counterparts and explored the distinctions in vital parameters, islets and redoxin expression.
Blood glucose and body weight were measured weekly from 6 to 18 weeks, confirming obesity and diabetic metabolism in homozygotes. Islet isolation and pancreatectomy were carried out at 6, 12 and 18 weeks. Homozygotes held significantly more islets, larger mean islet area and higher proliferation rates as well as increased apoptosis and failing secretion of insulin. Heterozygotes in contrast had higher insulin expression together with stable blood glucose and body weight.
Trx family proteins distinguished both groups of animals further, displaying varying expression patterns in immunohistology. We analysed gene and protein expression of Grx1 and 5 more detailed and found significantly higher expression in heterozygote animals. Grx1 levels fluctuated together with the metabolic situation in homozyotes while Grx5 decreased with age.
Formation of reactive oxygen species was measured in isolated islets and revealed elevated ROS in db/db specimens.
To investigate the role of leptin, we cultivated non-gluco-responsive MIN6 cells with this adipose-derived hormone using different concentrations. Protein levels for Grx1 and 5 as well as insulin measured by ELISA turned out to be not influenced by leptin without glucose as a cofactor.
We demonstrated that Trx family protein expression differs significantly between the obese, diabetic and the lean phenotype and that they are connected to the progress of obesity and hyperglycaemia. In conclusion, we propose that the redox state of the beta-cell and Grx1 and 5 are closely connected to impaired insulin secretion and destruction of the islets of Langerhans.

6 Zusammenfassung

Über 300 Millionen Menschen weltweit leiden an Diabetes mellitus Typ 2. Die komplexe Pathophysiologie besteht aus verringerter Insulinsensitivität, erhöhter Glukoneogenese, gestörter Insulinsekretion sowie Apoptose der Beta-Zellen. Zudem spielen eine chronische Entzündung sowie oxidativer Stress eine entscheidende Rolle. Ein Verteidigungsmechanismus gegen letzteren stellen redox-regulierte Signalwege dar. Proteine der Thioredoxin-Familie, welche Gluta-, Peroxi- und Thioredoxine beinhaltet, stellen die redox-Homeostase der Zelle wieder her, indem sie ihre Substrate reduzieren. Wir untersuchten die Auswirkungen des adipösen Phänotyps auf die Langerhansschen Inseln im Vergleich zum schlanken anhand der db-Maus. Homozygote dieses Stammes von C57BL/6 Mäusen sind aufgrund einer Mutation im transmembranösen Leptinrezeptor Ob-Rb durch Leptinresistenz gekennzeichnet und entwickeln einen adipösen, diabetischen Phänotyp. Wir beschrieben die Unterschiede zwischen diesen und ihren heterozygoten Artgenossen in Bezug auf Vitalparameter, Langerhans-Inseln und Expression der Redoxine. Blutzucker und Körpergewicht wurden wöchentlich im Alter von 6 bis 18 Wochen gemessen. Die Resultate bestätigten Adipositas und diabetische Stoffwechsellage der Homozygoten. Mit 6, 12 und 18 Wochen wurden Inselisolation und Pankreatektomie durchgeführt. Homozygote Tiere besaßen signifikant mehr Inseln, eine größere mittlere Inselfläche und höhere Proliferationsraten sowie eine gesteigerte Apoptose. Die Heterozygoten hatten die größere Insulinexpression sowie stabilen Nüchternblutzucker und Gewicht. Die unterschiedliche Expression der Redoxine unterschied beide Gruppen weiter. Wir analysierten die Gen- und Proteinexpression von Grx1 und 5 im Detail und fanden eine signifikant höhere Expression in heterozygoten Tieren. In den Homozygoten schwankte das Niveau von Grx1 mit der Stoffwechsellage, während Grx5 mit dem Alter korrelierte. Die Entstehung von reaktiven Sauerstoffspezies wurde in isolierten Inseln gemessen und zeigte eine vermehrte Bildung in db/db-Tieren. Um die Rolle des Leptins zu untersuchen, kultivierten wir glukoseunempfindliche MIN6-Zellen mit verschiedenen Konzentrationen dieses Proteohormons. Die Proteinlevel von Grx1 und 5 sowie Insulin, gemessen per ELISA, zeigten keinen erkennbaren Einfluß des Leptins ohne Glukose als Kofaktor. Wir demonstrierten, dass die Expression von Proteinen der Thioredoxin-Familie den adipösen, diabetischen und den schlanken Phänotyp signifikant unterscheidet, und dass sie mit dem Voranschreiten der Adipositas und der Hyperglykämie in Verbindung stehen. Zusammenfassend postulieren wir, dass der redox-Status der Beta-Zelle sowie Grx1 und 5 eng mit einer gestörten Insulinsekretion und einer Zerstörung der Langerhansschen Inseln zusammenhängen.

7 List of abbreviations

Ala	Alanine
AOK	Allgemeine Ortskrankenkasse
ASK1	apoptosis signaling kinase 1
ATP	adenosine triphosphate
BMI	body mass index
BSA	bovine serum albumin
Cys	cysteine
DCFH-DA	2,7-dichlorofluorescein diacetate
DDG	Deutsche Diabetes Gesellschaft (German diabetes association)
DDP-4	dipeptidyl peptidase-4
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FOXO1	Forkhead box protein O1
GDA	German diabetes association
GLP-1	glucagon-like peptide-1
Gln	Glutamine
GLUT2	Glucose transporter 2
Gly	Glycine
GR	Glutathione reductase
Grx	glutaredoxin
GSH	Glutathione (reduced)
GSSG	Glutathione (oxidised)
HRP	Horseadish peroxidase conjugated
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IL1	Interleukin 1
JNK	c-Jun N-terminal kinase
IL-1	interleukin-1
IL-1Ra	IL-1 receptor antagonist
MAP	Mitogen-activated protein
MIN6	Mouse insulinoma, 6th subclone
MODY	maturity onset diabetes of the young
NAPDH	nicotinamide adenine dinucleotide phosphate
NRX	Nuceloredoxin

NZO	New Zealand Obese
OGTT	oral glucose tolerance test
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pdx1	Pancreatic duodenal homeobox-1
Phe	Phenylalanine
Pro	Proline
Prx	peroxiredoxin
qRT-PCR	quantitative real-time polymerase chain reaction
RNS	reactive nitrogen species
ROS	reactive oxygen species
Ser	Serine
TNF-alpha	tumor necrosis factor-alpha
TRIS	tris(hydroxymethyl)aminomethane
Trx	thioredoxin
TrxR	thioredoxin reductase
TXN	human thioredoxin gene
Txnip	Thioredoxin-interacting protein
Tyr	Tyrosine
VEGF	vascular endothelial growth factor
WAT	White adipose tissue

8 List of figures

9 List of tables

10 References

American Diabetes Association. Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care.*, 26:Suppl 1:S5–20, Jan 2003.

Anello M., Lupi R., Spampinato D., Piro S., Masini M., Boggi U., Del Prato S., Rabuazzo A. M., Purrello F., Marchetti P.. Functional and morphological alterations of mitochondria in pancreatic beta cells from type 2 diabetic patients. *Diabetologia.*, 48(2):282–9, Feb 2005.

Banting F. G., Best C. H., Collip J. B., Campbell W. R., A F. A.. Pancreatic extracts. 1922. *Can Med Assoc J.*, 12(3):141146, March 1922.

Beer S. M., Taylor E. R., Brown S. E., Dahm C. C., Costa N. J., Runswick M. J., Murphy M. P.. Glutaredoxin 2 catalyzes the reversible oxidation and glutathionylation of mitochondrial membrane thiol proteins: implications for mitochondrial redox regulation and antioxidant DEFENSE. *J Biol Chem.*, 279(46):47939–51, Nov 2004.

Bell R. H., Hye R. J.. Animal models of diabetes mellitus: physiology and pathology. *J Surg Res.*, 35(5):433–60, Nov 1983.

Berndt C., Hudemann C., Hanschmann E. M., Axelsson R., Holmgren A., Lillig C. H.. How does iron-sulfur cluster coordination regulate the activity of human glutaredoxin 2? *Antioxid Redox Signal.*, 9(1):151–7, Jan 2007.

Boveris A.. Mitochondrial production of superoxide radical and hydrogen peroxide. *Adv Exp Med Biol.*, 78:67–82, 1977.

Bushweller J. H., Aslund F., Wüthrich K., Holmgren A.. Structural and functional characterization of the mutant Escherichia coli glutaredoxin (C14—-S) and its mixed disulfide with glutathione. *Biochemistry.*, 31(38):9288–93, Sep 1992.

Butler A. E., Janson J., Bonner-Weir S., Ritzel R., Rizza R. A., Butler P. C.. Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. *Diabetes.*, 52(1): 102–10, Jan 2003.

Camaschella C., Campanella A., De Falco L., Boschetto L., Merlini R., Silvestri L., Levi S., Iolascon A.. The human counterpart of zebrafish shiraz shows sideroblastic-like microcytic anemia and iron overload. *Blood.*, 110(4):1353–8, Aug 2007.

Carlsson C., Borg L. A., Welsh N.. Sodium palmitate induces partial mitochondrial uncoupling and reactive oxygen species in rat pancreatic islets in vitro. *Endocrinology.*, 140(8): 3422–8, Aug 1999.

Cha H., Kim J. M., Oh J. G., Jeong M. H., Park C. S., Park J., Jeong H. J., Park B. K., Lee Y. H., Jeong D., Yang D. K., Bernecker O. Y., Kim D. H., Hajjar R. J., Park W. J.. PICOT is a critical regulator of cardiac hypertrophy and cardiomyocyte contractility. *J Mol Cell Cardiol.*, 45(6):796–803, Dec 2008.

Chen H., Charlat O., Tartaglia L. A., Woolf E. A., Weng X., Ellis S. J., Lakey N. D., Culpepper J., Moore K. J., Breitbart R. E., Duyk G. M., Tepper R. I., Morgenstern J. P.. Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice. *Cell.*, 84(3):491–5, Feb 1996.

Cheng K., Delghingaro-Augusto V., Nolan C. J., Turner N., Hallahan N., Andrikopoulos S., Gunton J. E.. High passage MIN6 cells have impaired insulin secretion with impaired glucose and lipid oxidation. *PLoS One.*, 7(7):e40868, Jul 2012.

Chrestensen C. A., Starke D. W., Mieyal J. J.. Acute cadmium exposure inactivates thioltransferase (Glutaredoxin), inhibits intracellular reduction of protein-glutathionyl-mixed disulfides, and initiates apoptosis. *J Biol Chem.*, 275(34):26556–65, Aug 2000.

Coleman D. L.. Obese and diabetes: two mutant genes causing diabetes-obesity syndromes in mice. *Diabetologia.*, 14(3):141–8, Mar 1978.

Coleman D. L., Hummel K. P.. Hyperinsulinemia in pre-weaning diabetes (db) mice. *Diabetologia.*, 10(1):Suppl:607–10, Nov 1974.

Conrad M., Jakupoglu C., Moreno S. G., Lippl S., Banjac A., Schneider M., Beck H., Hatzopoulos A. K., Just U., Sinowatz F., Schmahl W., Chien K. R., Wurst W., Bornkamm G. W., Brielmeier M.. Essential role for mitochondrial thioredoxin reductase in hematopoiesis, heart development, and heart function. *Mol Cell Biol.*, 24(21):9414–23, Nov 2004.

Daily D., Vlamis-Gardikas A., Offen D., Mittelman L., Melamed E., Holmgren A., Barzilai A.. Glutaredoxin protects cerebellar granule neurons from dopamine-induced apoptosis by activating NF-kappa B via Ref-1. *J Biol Chem.*, 276(2):1335–44, Jan 2001.

DDG. Epidemiologie und Verlauf des Diabetes mellitus in Deutschland., May 2004.

DDG. Medical antihyperglycaemic treatment of type 2 diabetes mellitus: update of the evidence-based guideline of the German Diabetes Association. *Exp Clin Endocrinol Diabetes.*, 117(9):522–557, Oct 2009.

DDG. S3-Leitlinie Therapie des Typ-1-Diabetes - Version 1.0; September / 2011, 2011.

DeFronzo R. A.. Pathogenesis of type 2 diabetes mellitus. *Med Clin North Am.*, 88(4):787–835, Jul 2004.

Del Zotto H., Gómez Dumm C. L., Drago S., Fortino A., Luna G. C., Gagliardino J. J.. Mechanisms involved in the beta-cell mass increase induced by chronic sucrose feeding to normal rats. *J Endocrinol.*, 174(2):225–31, Aug 2002.

Di Simplicio P., de Giorgio L. A., Cardaioli E., Lecis R., Miceli M., Rossi R., Anichini R., Mian M., Seghieri G., Franconi F.. Glutathione, glutathione utilizing enzymes and thioltransferase in platelets of insulin-dependent diabetic patients: relation with platelet aggregation and with microangiopatic complications. *Eur J Clin Invest.*, 25(9):665–9, Sep 1995.

Ding Y., Yamada S., Wang K. Y., Shimajiri S., Guo X., Tanimoto A., Murata Y., Kitajima S., Watanabe T., Izumi H., Kohno K., Sasaguri Y.. Overexpression of peroxiredoxin 4 protects against high-dose streptozotocin-induced diabetes by suppressing oxidative stress and cytokines in transgenic mice. *Antioxid Redox Signal.*, 13(10):1477–90, Nov 2010.

Dröge W.. Free Radicals in the Physiological Control of Cell Function. *Physiol Rev.*, 82(1): 47–95, Jan 2002.

Du Y., Zhang H., Montano S., Hegestam J., Ekberg N. R., Holmgren A., Brismar K., Ungerstedt J. S.. Plasma glutaredoxin activity in healthy subjects and patients with abnormal glucose levels or overt type 2 diabetes. *Acta Diabetol.*, 9, Jul 2013.

Eckel R. H., Kahn S. E., Ferrannini E., Goldfine A. B., Nathan D. M., Schwartz M. W., Smith R. J., Smith S. R.. Obesity and type 2 diabetes: what can be unified and what needs to be individualized? *J Clin Endocrinol Metab.*, 96(6):1654–63, Jun 2011.

Enoksson M., Fernandes A. P., Prast S., Lillig C. H., Holmgren A., Orrenius S.. Overexpression of glutaredoxin 2 attenuates apoptosis by preventing cytochrome c release. *Biochem Biophys Res Commun.*, 327(3):774–9, Feb 2005.

Evans J. L., Goldfine I. D., Maddux B. A., Grodsky G. M.. Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes. *Endocr Rev.*, 23(5): 599–622, Oct 2002.

Evans J. L., Goldfine I. D., Maddux B. A., Grodsky G. M.. Are oxidative stress-activated signaling pathways mediators of insulin resistance and beta-cell dysfunction? *Diabetes.*, 52 (1):1–8, Jan 2003.

Fain J. N., Madan A. K., Hiler M. L., Cheema P., Bahouth S. W.. Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. *Endocrinology.*, 145(5):2273–82, May 2004.

Farooqi I. S., Matarese G., Lord G. M., Keogh J. M., Lawrence E., Agwu C., Sanna V., Jebb S. A., Perna F., Fontana S., Lechler R. I., DePaoli A. M., O'Rahilly S.. Beneficial effects of leptin on obesity, T cell hyporesponsiveness, and neuroendocrine/metabolic dysfunction of human congenital leptin deficiency. *J Clin Invest.*, 110(8):1093–103, Oct 2002.

Fenton H. J. H.. LXXIII.Oxidation of tartaric acid in presence of iron. *J. Chem. Soc., Trans.*, 65:899–910, 1894.

Fleury C., Mignotte B., Vayssière J. L.. Mitochondrial reactive oxygen species in cell death signaling. *Biochimie.*, 84(2-3):131–41, Feb-Mar 2002.

Flier J. S., Cook K. S., Usher P., Spiegelman B. M.. Severely impaired adipsin expression in genetic and acquired obesity. *Science.*, 237(4813):405–8, Jul 1987.

Friedman J. M., Halaas J. L.. Leptin and the regulation of body weight in mammals. *Nature.*, 395(6704):763–70, Oct 1998.

Fujii J., Ikeda Y.. Advances in our understanding of peroxiredoxin, a multifunctional, mammalian redox protein. *Redox Rep.*, 7(40):123–30, Feb 2002.

Garris D. R., Garris B. L.. Cytochemical analysis of pancreatic islet hypercytolipidemia following diabetes (db/db) and obese (ob/ob) mutation expression: influence of genomic background. *Pathobiology.*, 71(5):231–40, 2004.

Geiss L. S., Gowie C. C.. Type 2 diabetes and persons at high risk of diabetes. *Diabetes Public Health: From Data to Policy*, pages 15–32, 2010.

Guariguata L., Whiting D., Weil C., Unwin N.. The International Diabetes Federation Diabetes Atlas methodology for estimating global and national prevalence of diabetes in adults. *Diabetes Res Clin Pract.*, 94(3):322–32, Dec 2011.

Halaas J. L., Gajiwala K. S., Maffei M., Cohen S. L., Chait B. T., Rabinowitz D., Lallone R. L., Burley S. K., Friedman J. M.. Weight-reducing effects of the plasma protein encoded by the obese gene. *Science.*, 269(5223):543–6, Jul 1995.

Halaas J. L., Boozer C., Blair-West J., Fidahusein N., Denton D. A., Friedman J. M.. Physiological response to long-term peripheral and central leptin infusion in lean and obese mice. *Proc Natl Acad Sci U S A.*, 94(16):8878–83, Aug 1997.

Hanschmann E. M.. *Thioredoxin family proteins in physiology and disease.* PhD thesis, Philipp University of Marburg, 2011.

Hasan N. M., Longacre M. J., Stoker S. W., Boonsaen T., Jitrapakdee S., Kendrick M. A., Wallace J. C., MacDonald M. J.. Impaired anaplerosis and insulin secretion in insulinoma cells caused by small interfering RNA-mediated suppression of pyruvate carboxylase. *J Biol Chem.*, 283(42):28048–59, Oct 2008.

Hauner H., Köster I., Schubert I.. Prevalence of Diabetes mellitus and Quality of Care in Hesse, Germany, 1998-2004. *Dtsch Arztebl.*, 104(41):A 2799805, 2007.

Haunhorst P., Berndt C., Eitner S., Godoy J. R., Lillig C. H.. Characterization of the human monothiol glutaredoxin 3 (PICOT) as iron-sulfur protein. *Biochem Biophys Res Commun.*, 394(2):372–6, Apr 2010.

Haunhorst P., Hanschmann E., Bräutigam L., Stehling O., Hoffmann B., Mühlenhoff U., Lill R., Berndt C., Lillig C.. Crucial function of vertebrate glutaredoxin 3 (PICOT) in iron homeostasis and hemoglobin maturation. *Mol Biol Cell.*, 24(12):1895–903, Jun 2013.

Hayashi T., Ueno Y., Okamoto T.. Oxidoreductive regulation of nuclear factor kappa B. Involvement of a cellular reducing catalyst thioredoxin. *J Biol Chem.*, 268(15):11380–8, May 1993.

Hekerman P., Zeidler J., Korfmacher S., Bamberg-Lemper S., Knobelspies H., Zabeau L., Tavernier J., Becker W.. Leptin induces inflammation-related genes in RINm5F insulinoma cells. *BMC Mol Biol.*, 8:article 41, May 2007.

Hirota K., Matsui M., Murata M., Takashima Y., Cheng F. S., Itoh T., Fukuda K., Yodoi J.. Nucleoredoxin, glutaredoxin, and thioredoxin differentially regulate NF-kappaB, AP-1, and CREB activation in HEK293 cells. *Biochem Biophys Res Commun.*, 274(1):177–82, Jul 2000.

Ho Y. S., Xiong Y., Ho D. S., Gao J., Chua B. H., Pai H., Mieyal J. J.. Targeted disruption of the glutaredoxin 1 gene does not sensitize adult mice to tissue injury induced by ischemia/reperfusion and hyperoxia. *Free Radic Biol Med.*, 43(9):1299–312, Nov 2007.

Hofmann M. A., Schiekofer S., Isermann B., Kanitz M., Henkels M., Joswig M., Treusch A., Morcos M., Weiss T., Borcea V., Abdel Khalek A. K., Amiral J., Tritschler H., Ritz E., Wahl P., Ziegler R., Bierhaus A., Nawroth P. P.. Peripheral blood mononuclear cells isolated from patients with diabetic nephropathy show increased activation of the oxidative-stress sensitive transcription factor NF-kappaB. *Diabetologia.*, 42(2):222–32, Feb 1999.

Holmgren A.. Thioredoxin. 6. The amino acid sequence of the protein from escherichia coli B. *Eur J Biochem.*, 6(4):475–84, Dec 1968.

Holmgren A.. Hydrogen donor system for Escherichia coli ribonucleoside-diphosphate reductase dependent upon glutathione. *Proc Natl Acad Sci U S A.*, 73(7):2275–9, Jul 1976.

Holmgren A.. Glutathione-dependent enzyme reactions of the phage T4 ribonucleotide reductase system. *J Biol Chem.*, 253(20):7424–30, Oct 1978.

Holmgren A.. Glutathione-dependent synthesis of deoxyribonucleotides. Purification and characterization of glutaredoxin from Escherichia coli. *J Biol Chem.*, 254(9):3664–71, May 1979.

Holmgren A.. Thioredoxin. *Annu Rev Biochem.*, 54:237–71, 1985.

Holmgren A.. Thioredoxin and glutaredoxin systems. *J Biol Chem.*, 264(24):13963–6, Aug 1989.

Hügl S. R., White M. F., Rhodes C. J.. Insulin-like growth factor I (IGF-I)-stimulated pancreatic beta-cell growth is glucose-dependent. Synergistic activation of insulin receptor substrate-mediated signal transduction pathways by glucose and IGF-I in INS-1 cells. *J Biol Chem.*, 273(28):17771–9, Jul 1998.

Huh J. Y., Kim Y., Jeong J., Park J., Kim I., Huh K. H., Kim Y. S., Woo H. A., Rhee S. G., Lee K. J., Ha H.. Peroxiredoxin 3 is a key molecule regulating adipocyte oxidative stress, mitochondrial biogenesis, and adipokine expression. *Antioxid Redox Signal.*, 16(3):229–243, Feb 2012.

Hummel K. P., Dickie M. M., Coleman D. L.. Diabetes, a new mutation in the mouse. *Science.*, 153(3740):1127–8, Sep 1966.

Hummel K. P., Coleman D. L., Lane P. W.. The influence of genetic background on expression of mutations at the diabetes locus in the mouse. I. C57BL-KsJ and C57BL-6J strains. *Biochem Genet.*, 7(1):1–13, Aug 1972.

Ikegami H., Ono M., Fujisawa T., Hiromine Y., Kawabata Y., Yamato E.. Molecular scanning of the gene for thioredoxin, an antioxidative and antiapoptotic protein, and genetic susceptibility to type 1 diabetes. *Ann N Y Acad Sci.*, 1150:103–5, Dec 2008.

International Diabetes Federation. IDF Diabetes Atlas, Brussels, Belgium, 2003.

International Diabetes Federation. IDF Diabetes Atlas, 5th edn. Update 2012 Brussels, Belgium, 2012. URL http://www.idf.org/diabetesatlas.

Islam M. S., Berggren P. O., Larsson O.. Sulfhydryl oxidation induces rapid and reversible closure of the ATP-regulated K+ channel in the pancreatic beta-cell. *FEBS Lett.*, 319(1-2): 128–32, Mar 1993.

Iuchi Y., Okada F., Tsunoda S., Kibe N., Shirasawa N., Ikawa M., Okabe M., Ikeda Y., Fujii J.. Peroxiredoxin 4 knockout results in elevated spermatogenic cell death via oxidative stress. *Biochem J.*, 419(1):149–58, Apr 2009.

Ivarsson R., Quintens R., Dejonghe S., Tsukamoto K., in 't Veld P., Renström E., Schuit F. C.. Redox control of exocytosis: regulatory role of NADPH, thioredoxin, and glutaredoxin. *Diabetes.*, 54(7):2132–42, Jul 2005.

Jakupoglu C., Przemeck G. K., Schneider M., Moreno S. G., Mayr N., Hatzopoulos A. K., de Angelis M. H., Wurst W., Bornkamm G. W., Brielmeier M., Conrad M.. Cytoplasmic thioredoxin reductase is essential for embryogenesis but dispensable for cardiac development. *Mol Cell Biol.*, 25(5):1980–8, Mar 2005.

Jeong D., Cha H., Kim E., Kang M., Yang D. K., Kim J. M., Yoon P. O., Oh J. G., Bernecker O. Y., Sakata S., Le T. T., Cui L., Lee Y. H., Kim D. H., Woo S. H., Liao R., Hajjar R. J., Park W. J.. PICOT inhibits cardiac hypertrophy and enhances ventricular function and cardiomyocyte contractility. *Circ Res.*, 99(3):307–14, Aug 2006.

Johnson J. D., Ahmed N. T., Luciani D. S., Han Z., Tran H., Fujita J., Misler S., Edlund H., Polonsky K. S.. Increased islet apoptosis in Pdx1+/- mice. *J Clin Invest.*, 111(8):1147–60, Apr 2003.

Johnson J. H., Ogawa A., Chen L., Orci L., Newgard C. B., Alam T., Unger R. H.. Underexpression of beta cell high Km glucose transporters in noninsulin-dependent diabetes. *Science.*, 250(4980):546–9, Oct 1990.

Jones P. M., Persaud S. J.. *Islet Function and Insulin Secretion - Textbook of Diabetes*. Wiley-Blackwell, 4th edition, Aug 2010.

Jonsson J., Carlsson L., Edlund T., Edlund H.. Insulin-promoter-factor 1 is required for pancreas development in mice. *Nature.*, 371(6498):606–9, Oct 1994.

Kahn C. R., Vicent D., Doria A.. Genetics of non-insulin-dependent (type-II) diabetes mellitus. *Annu Rev Med.*, 47:509–31, 1996.

Kakisaka Y., Nakashima T., Sumida Y., Yoh T., Nakamura H., Yodoi J., Senmaru H.. Elevation of serum thioredoxin levels in patients with type 2 diabetes. *Horm Metab Res.*, 34 (3):160–4, Mar 2002.

Karin M., Ben-Neriah Y.. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu Rev Immunol.*, 18:621–63, 2000.

Kato N., Motohashi S., Okada T., Ozawa T., Mashima K.. PICOT, protein kinase C theta-interacting protein, is a novel regulator of FcepsilonRI-mediated mast cell activation. *Cell Immunol.*, 251(1):62–7, Jan 2008.

Kawasaki F., Matsuda M., Kanda Y., Inoue H., Kaku K.. Structural and functional analysis of pancreatic islets preserved by pioglitazone in db/db mice. *Am J Physiol Endocrinol Metab.*, 288(3):E510–8, Mar 2005.

Kieffer T. J., Habener J. F.. The adipoinsular axis: effects of leptin on pancreatic beta-cells. *Am J Physiol Endocrinol Metab.*, 278(1):E1–E14, Jan 2000.

Kilimnik G., Kim A., Jo J., Miller K., Hara M.. Quantification of pancreatic islet distribution in situ in mice. *Am J Physiol Endocrinol Metab.*, 297(6):E1331–E8, Dec 2009.

Kluth O., Mirhashemi F., Scherneck S., Kaiser D., Kluge R., Neschen S., Joost H. G., Schürmann A.. Dissociation of lipotoxicity and glucotoxicity in a mouse model of obesity associated diabetes: role of forkhead box O1 (FOXO1) in glucose-induced beta cell failure. *Diabetologia.*, 54(3):605–16, Mar 2011.

Köster I., Schubert I., Huppertz E.. Follow up of the CoDiM-Study: Cost of diabetes mellitus 2000-2009. *Dtsch Med Wochenschr.*, 137(19):1013–6, May 2012.

Krippeit-Drews P., Lang F., Häussinger D., Drews G.. H2O2 induced hyperpolarization of pancreatic B-cells. *Pflugers Arch.*, 426(6):552–4, Apr 1994.

Kulkarni R. N., Wang Z. L., Wang R. M., Hurley J. D., Smith D. M., Ghatei M. A., Withers D. J., Gardiner J. V., Bailey C. J., Bloom S. R.. Leptin rapidly suppresses insulin release from insulinoma cells, rat and human islets and, in vivo, in mice. *J Clin Invest.*, 100(11): 2729–36, Dec 1997.

Laferrère B., Caixas A., Fried S. K., Bashore C., Kim J., Pi-Sunyer F. X.. A pulse of insulin and dexamethasone stimulates serum leptin in fasting human subjects. *Eur J Endocrinol.*, 146(6):839–45, Jun 2002.

Lai Y., Schneider D., Kidszun A., Hauck-Schmalenberger I., Breier G., Brandhorst D., Brandhorst H., Iken M., Brendel M. D., Bretzel R. G., Linn T.. Vascular endothelial growth factor increases functional beta-cell mass by improvement of angiogenesis of isolated human and murine pancreatic islets. *Transplantation.*, 79(11):1530–6, Jun 2005.

Langerhans P.. *Beitrage zur mikroscopischen anatomie der bauchspeichel druse.* PhD thesis, Berlin, 1869.

Laurent T. C., Moore E. C., Reichard P.. Enzymatic synthesis of deoxyribonucleotides. IV. ISOLATION AND CHARACTERIZATION OF THIOREDOXIN, THE HYDROGEN DONOR FROM ESCHERICHIA COLI B. *J Biol Chem.*, 239:3436–44, Oct 1964.

Lee T. H., Kim S. U., Yu S. L., Kim S. H., Park D. S., Moon H. B., Dho S. H., Kwon K. S., Kwon H. J., Han Y. H., Jeong S., Kang S. W., Shin H. S., Lee K. K., Rhee S. G., Yu D. Y.. Peroxiredoxin II is essential for sustaining life span of erythrocytes in mice. *Blood.*, 101(12): 5033–8, Jun 2003.

Lekli I., Mukherjee S., Ray D., Gurusamy N., Kim Y. H., Tosaki A., Engelman R. M., Ho Y. S., Das D. K.. Functional recovery of diabetic mouse hearts by glutaredoxin-1 gene therapy: role of Akt-FoxO-signaling network. *Gene Ther.*, 17(4):478–85, Apr 2010.

Lenzen S., Drinkgern J., Tiedge M.. Low antioxidant enzyme gene expression in pancreatic islets compared with various other mouse tissues. *Free Radic Biol Med.*, 20(3):463–6, 1996.

Li L., Shoji W., Takano H., Nishimura N., Aoki Y., Takahashi R., Goto S., Kaifu T., Takai T., Obinata M.. Increased susceptibility of MER5 (peroxiredoxin III) knockout mice to LPS-induced oxidative stress. *Biochem Biophys Res Commun.*, 355(3):715–22, Apr 2007.

Lillig C. H., Berndt C., Vergnolle O., Lönn M. E., Hudemann C., Bill E., Holmgren A.. Characterization of human glutaredoxin 2 as ironsulfur protein: A possible role as redox sensor. *Proc Natl Acad Sci U S A.*, 102(23):8168–73, Jun 2005.

Loffreda S., Yang S. Q., Lin H. Z., Karp C. L., Brengman M. L., Wang D. J., Klein A. S., Bulkley G. B., Bao C., Noble P. W., Lane M. D., Diehl A. M.. Leptin regulates proinflammatory immune responses. *FASEB J.*, 12(1):57–65, Jan 1998.

Lönn M. E., Hudemann C., Berndt C., Cherkasov V., Capani F., Holmgren A., Lillig C. H.. Expression pattern of human glutaredoxin 2 isoforms: identification and characterization of two testis/cancer cell-specific isoforms. *Antioxid Redox Signal.*, 10(3):547–57, Mar 2008.

Lundberg M., Johansson C., Chandra J., Enoksson M., Jacobsson G., Ljung J., Johansson M., Holmgren A.. Cloning and expression of a novel human glutaredoxin (Grx2) with mitochondrial and nuclear isoforms. *J Biol Chem.*, 276(28):26269–75, Jul 2001.

Lundberg M., Fernandes A. P., Kumar S., Holmgren A.. Cellular and plasma levels of human glutaredoxin 1 and 2 detected by sensitive ELISA systems. *Biochem Biophys Res Commun.*, 319(3):801–9, Jul 2004.

Maedler K., Sergeev P., Ehses J. A., Mathe Z., Bosco D., Berney T., Dayer J. M., Reinecke M., Halban P. A., Donath M. Y.. Leptin modulates beta cell expression of IL-1 receptor antagonist and release of IL-1beta in human islets. *Proc Natl Acad Sci U S A.*, 101(21): 8138–43, May 2004.

Maffei M., Fei H., Lee G. H., Dani C., Leroy P., Zhang Y., Proenca R., Negrel R., Ailhaud G., Friedman J. M.. Increased expression in adipocytes of ob RNA in mice with lesions of the hypothalamus and with mutations at the db locus. *Proc Natl Acad Sci U S A.*, 92(15): 6957–60, Jul 1995.

Makino Y., Yoshikawa N., Okamoto K., Hirota K., Yodoi J., Makino I., Tanaka H.. Direct association with thioredoxin allows redox regulation of glucocorticoid receptor function. *J Biol Chem.*, 274(5):3182–8, Jan 1999.

Martin J. L.. Thioredoxin–a fold for all reasons. *Structure.*, 15(3):245–50, Mar 1995.

Matsui M., Oshima M., Oshima H., Takaku K., Maruyama T., Yodoi J., Taketo M. M.. Early embryonic lethality caused by targeted disruption of the mouse thioredoxin gene. *Dev Biol.*, 178(1):179–85, Aug 1996.

Maxwell S. R., Thomason H., Sandler D., Leguen C., Baxter M. A., Thorpe G. H., Jones A. F., Barnett A. H.. Antioxidant status in patients with uncomplicated insulin-dependent and non-insulin-dependent diabetes mellitus. *Eur J Clin Invest.*, 27(6):484–90, Jun 1997.

Mohamed A. K., Bierhaus A., Schiekofer S., Tritschler H., Ziegler R., Nawroth P. P.. The role of oxidative stress and NF-kappaB activation in late diabetic complications. *Biofactors.*, 10(2-3):157–67, 1999.

Moreno-Aliaga M. J., Stanhope K. L., Havel P. J.. Transcriptional regulation of the leptin promoter by insulin-stimulated glucose metabolism in 3t3-l1 adipocytes. *Biochem Biophys Res Commun.*, 283(3):544–8, May 2001.

Murata H., Ihara Y., Nakamura H., Yodoi J., Sumikawa K., Kondo T.. Glutaredoxin exerts an antiapoptotic effect by regulating the redox state of Akt. *J Biol Chem.*, 278(50):50226–33, Dec 2003.

Narayan K. M. V., Imperatore G., Benjamin S. M., Engelgau M. M.. Targeting people with pre-diabetes. Lifestyle interventions should also be aimed at people with pre-diabetes. *BMJ.*, 325(7361):403–4, Aug 2002.

Neumann C. A., Krause D. S., Carman C. V., Das S., Dubey D. P., Abraham J. L., Bronson R. T., Fujiwara Y., Orkin S. H., Van Etten R. A.. Essential role for the peroxiredoxin Prdx1 in erythrocyte antioxidant defence and tumour suppression. *Nature*, 424(6948):561–5, Jul 2003.

Nonn L., Williams R. R., Erickson R. P., Powis G.. The absence of mitochondrial thioredoxin 2 causes massive apoptosis, exencephaly, and early embryonic lethality in homozygous mice. *Mol Cell Biol.*, 23(3):916–22, Feb 2003.

Nourooz-Zadeh J., Rahimi A., Tajaddini-Sarmadi J., Tritschler H., Rosen P., Halliwell B., Betteridge D. J.. Relationships between plasma measures of oxidative stress and metabolic control in NIDDM. *Diabetologia.*, 40(6):647–53, Jun 1997.

Ohlsson H., Karlsson K., Edlund T.. IPF1, a homeodomain-containing transactivator of the insulin gene. *EMBO J.*, 12(11):4251–9, Nov 1993.

Okuya S., Tanabe K., Tanizawa Y., Oka Y.. Leptin increases the viability of isolated rat pancreatic islets by suppressing apoptosis. *Endocrinology.*, 142(11):4827–30, Nov 2001.

Opara E. C., Abdel-Rahman E., Soliman S., Kamel W. A., Souka S., Lowe J. E., Abdel-Aleemt S.. Depletion of total antioxidant capacity in type 2 diabetes. *Metabolism.*, 48(11): 1414–7, Nov 1999.

Pai H. V., Starke D. W., Lesnefsky E. J., Hoppel C. L., Mieyal J. J.. What is the functional significance of the unique location of glutaredoxin 1 (GRx1) in the intermembrane space of mitochondria? *Antioxid Redox Signal.*, 9(11):2027–33, Nov 2007.

Parikh H., Carlsson E., Chutkow W. A., Johansson L. E., Storgaard H., Poulsen P., Saxena R., Ladd C., Schulze P. C., Mazzini M. J., Jensen C. B., Krook A., Björnholm M., Tornqvist H., Zierath J. R., Ridderstråle M., Altshuler D., Lee R. T., Vaag A., Groop L. C., Mootha V. K.. TXNIP Regulates Peripheral Glucose Metabolism in Humans. *PLoS Med.*, 4(5):e158, May 2007.

Paulescu N. C.. 1. Action de l'extrait pancréatique injecté dans le sang, chez un animal diabétique. 2. Influence du laps de temps écoulé depuis linjection intraveineuse de lextrait pancréatique chez un animal diabétique. 3. Influence de la quantité de pancreas employée pour préparer lextrait injecté dans le sang, chez un animal diabétique. 4. Action de lextrait pancréatique injecté dans le sang chez un animal normal. *C.R. Soc. Biol.*, 85(27):555–559, 1921.

Powis G., Montfort W. R.. Properties and biological activities of thioredoxins. *Annu Rev Pharmacol Toxicol.*, 41:261–95, 2001.

Puff R., Dames P., Weise M., Göke B., Seissler J., Parhofer K. G., Lechner A.. Reduced proliferation and a high apoptotic frequency of pancreatic beta cells contribute to genetically-determined diabetes susceptibility of db/db BKS mice. *Horm Metab Res.*, 43(5):306–11, May 2011.

Reddy S., Bradley J., Ginn S., Pathipati P., Ross J. M.. Immunohistochemical study of caspase-3-expressing cells within the pancreas of non-obese diabetic mice during cyclophosphamide-accelerated diabetes. *Histochem Cell Biol.*, 119(6):451–61, Jun 2003.

Reinbothe T. M., Ivarsson R., Li D. Q., Niazi O., Jing X., Zhang E., Stenson L., Bryborn U., Renström E.. Glutaredoxin-1 mediates NADPH-dependent stimulation of calcium-dependent insulin secretion. *J Mol Endocrinol.*, 23(6):893–900, Jun 2009.

Rhee S. G., Kang S. W., Chang T. S., Jeong W., Kim K.. Peroxiredoxin, a novel family of peroxidases. *IUBMB Life.*, 52(1-2):35–41, Jul 2001.

Rich S. S.. Mapping genes in diabetes. Genetic epidemiological perspective. *Diabetes.*, 39(11): 1315–9, Nov 1990.

Robertson R. P., Harmon J., Tran P. O., Tanaka Y., Takahashi H.. Glucose toxicity in beta-cells: type 2 diabetes, good radicals gone bad, and the glutathione connection. *Diabetes.*, 52 (3):581–7, Mar 2003.

Robertson R. P., Harmon J., Tran P. O., Poitout V.. Beta-cell glucose toxicity, lipotoxicity, and chronic oxidative stress in type 2 diabetes. *Diabetes.*, 53:Suppl 1:S119–24, Feb 2004.

Rodríguez-Manzaneque M. T., Ros J., Cabiscol E., Sorribas A., Herrero E.. Grx5 glutaredoxin plays a central role in protection against protein oxidative damage in Saccharomyces cerevisiae. *Mol Cell Biol.*, 19(12):8180–90, Dec 1999.

Rodríguez-Manzaneque M. T., Tamarit J., Bellí G., Ros J., Herrero E.. Grx5 is a mitochondrial glutaredoxin required for the activity of iron/sulfur enzymes. *Mol Biol Cell.*, 13(4): 1109–21, Apr 2002.

Rubartelli A., Bajetto A., Allavena G., Wollman E., Sitia R.. Secretion of thioredoxin by normal and neoplastic cells through a leaderless secretory pathway. *J Biol Chem.*, 267(34): 24161–4, Dec 1992.

Saitoh M., Nishitoh H., Fujii M., Takeda K., Tobiume K., Sawada Y., Kawabata M., Miyazono K., Ichijo H.. Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *EMBO J.*, 17(9):2596–606, May 1998.

Schipf S., Werner A., Tamayo T., Holle R., Schunk M., Maier W., Meisinger C., Thorand B., Berger K., Mueller G., Moebus S., Bokhof B., Kluttig A., Greiser K. H., Neuhauser H., Ellert U., Icks A., Rathmann W., Völzke H.. Regional differences in the prevalence of known Type 2 diabetes mellitus in 45-74 years old individuals: results from six population-based studies in Germany (DIAB-CORE Consortium). *Diabet Med.*, 29(7):e88–e95, Jul 2012.

Schulze P. C., Yoshioka J., Takahashi T., He Z., King G. L., Lee R. T.. Hyperglycemia promotes oxidative stress through inhibition of thioredoxin function by thioredoxin-interacting protein. *J Biol Chem.*, 279(29):30369–74, Jul 2004.

Seo M. S., Kang S. W., Kim K., Baines I. C., Lee T. H., Rhee S. G.. Identification of a new type of mammalian peroxiredoxin that forms an intramolecular disulfide as a reaction intermediate. *J Biol Chem.*, 275(27):20346–54, Jul 2000.

Seufert J.. Leptin effects on pancreatic beta-cell gene expression and function. *Diabetes*, 53 Suppl 1:S152–8, Feb 2004.

Seufert J., Kieffer T. J., Habener J. F.. Leptin inhibits insulin gene transcription and reverses hyperinsulinemia in leptin-deficient ob/ob mice. *Proc Natl Acad Sci U S A.*, 96(2):674–9, Jan 1999.

Shimabukuro M., Wang M. Y., Zhou Y. T., Newgard C. B., Unger R. H.. Protection against lipoapoptosis of beta cells through leptin-dependent maintenance of Bcl-2 expression. *Proc Natl Acad Sci U S A.*, 95(16):9558–61, Aug 1998.

Söderberg A., Sahaf B., Rosén A.. Thioredoxin reductase, a redox-active selenoprotein, is secreted by normal and neoplastic cells: presence in human plasma. *Cancer Res.*, 60(8): 2281–9, Apr 2000.

Swenne I.. The role of glucose in the in vitro regulation of cell cycle kinetics and proliferation of fetal pancreatic B-cells. *Diabetes.*, 31(9):754–60, Sep 1982.

Takashima Y., Hirota K., Nakamura H., Nakamura T., Akiyama K., Cheng F. S., Maeda M., Yodoi J.. Differential expression of glutaredoxin and thioredoxin during monocytic differentiation. *Immunol Lett.*, 68(2-3):397–401, Jun 1999.

Talchai C., Xuan S., Lin H. V., Sussel L., Accili D.. Pancreatic β cell dedifferentiation as a mechanism of diabetic β cell failure. *Cell.*, 150(6):1223–34, Sep 2012.

Tartaglia L. A., Dembski M., Weng X., Deng N., Culpepper J., Devos R., Richards G. J., Campfield L. A., Clark F. T., Deeds J., Muir C., Sanker S., Moriarty A., Moore K. J., Smutko J. S., Mays G. G., Wool E. A., Monroe C. A., Tepper R. I.. Identification and expression cloning of a leptin receptor, OB-R. *Cell.*, 83(7):1263–71, Dec 1995.

The Diabetes Control and Complications Trial Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med.*, 329(14):977–86, Sep 1993.

Thorens B., Wu Y. J., Leahy J. L., Weir G. C.. The loss of GLUT2 expression by glucose-unresponsive beta cells of db/db mice is reversible and is induced by the diabetic environment. *J Clin Invest.*, 90(1):77–85, Jul 1992.

Tretter L., Adam-Vizi V.. Inhibition of Krebs Cycle Enzymes by Hydrogen Peroxide: A Key Role of a-Ketoglutarate Dehydrogenase in Limiting NADH Production under Oxidative Stress. *J Neurosci.*, 20(24):8972–9, Dec 2000.

Tuman R. W., Doisy R. J.. The influence of age on the development of hypertriglyceridaemia and hypercholesterolaemia in genetically diabetic mice. *Diabetologia*, 13(1):7–11, Jan 1977.

Tyrberg B., Eizirik D. L., Hellerström C., Pipeleers D. G., Andersson A.. Human pancreatic beta-cell deoxyribonucleic acid-synthesis in islet grafts decreases with increasing organ donor age but increases in response to glucose stimulation in vitro. *Endocrinology.*, 137(12): 5694–9, Dec 1996.

UK Prospective Diabetes Study (UKPDS) Group. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). *Lancet.*, 352(9131):837–53, Sep 1998.

Wabitsch M., Hauner H., Hertrampf M., Muche R., Hay B., Mayer H., Kratzer W., Debatin K. M., Heinze E.. Type II diabetes mellitus and impaired glucose regulation in Caucasian children and adolescents with obesity living in Germany. *Int J Obes Relat Metab Disord.*, 28(2):307–13, Feb 2004.

Wang X., Phelan S. A., Forsman-Semb K., Taylor E. F., Petros C., Brown A., Lerner C. P., Paigen B.. Mice with targeted mutation of peroxiredoxin 6 develop normally but are susceptible to oxidative stress. *J Biol Chem.*, 278(27):25179–90, Jul 2003.

Weir G. C., Bonner-Weir S.. Five stages of evolving beta-cell dysfunction during progression to diabetes. *Diabetes.*, 53(Suppl 3):S16–21, Dec 2004.

Wingert R. A., Galloway J. L., Barut B., Foott H., Fraenkel P., Axe J. L., Weber G. J., Dooley K., Davidson A. J., Schmid B., Paw B. H., Shaw G. C., Kingsley P., Palis J., Schubert H., Chen O., Kaplan J., Zon L. I.. Deficiency of glutaredoxin 5 reveals Fe-S clusters are required for vertebrate haem synthesis. *Nature.*, 436(7053):1035–9, Aug 2005.

Witte S., Villalba M., Bi K., Liu Y., Isakov N., Altman A.. Inhibition of the c-Jun N-terminal kinase/AP-1 and NF-kappaB pathways by PICOT, a novel protein kinase C-interacting protein with a thioredoxin homology domain. *J Biol Chem.*, 275(3):1902–9, Jan 2000.

Wolf G., Aumann N., Michalska M., Bast A., Sonnemann J., Beck J. F., Lendeckel U., Newsholme P., Walther R.. Peroxiredoxin III protects pancreatic β cells from apoptosis. *J Endocrinol.*, 207(2):163–75, Nov 2010.

Wolff S. P., Dean R. T.. Glucose autoxidation and protein modification. The potential role of 'autoxidative glycosylation' in diabetes. *Biochem J.*, 245(1):243–50, Jul 1987.

Wu H., Lin L., Giblin F., Ho Y. S., Lou M. F.. Glutaredoxin 2 knockout increases sensitivity to oxidative stress in mouse lens epithelial cells. *Free Radic Biol Med.*, 51(11):2108–17, Dec 2011.

Yamamoto M., Yamato E., Toyoda S., Tashiro F., Ikegami H., Yodoi J., Miyazaki J.. Transgenic expression of antioxidant protein thioredoxin in pancreatic beta cells prevents progression of type 2 diabetes mellitus. *Antioxid Redox Signal.*, 10(1):43–9, Jan 2008.

Yamashita H., Shao J., Ishizuka T., Klepcyk P. J., Muhlenkamp P., Qiao L., Hoggard N., Friedman J. E.. Leptin administration prevents spontaneous gestational diabetes in heterozygous Lepr(db/+) mice: effects on placental leptin and fetal growth. *Endocrinology.*, 142(7): 2888–97, Jul 2001.

Yamazaki M., Kato A., Kato C., Fujii E., Adachi K., Miyoshi A., Suzuki M.. Segmentation of the Pathophysiological Stages of Diabetic Changes in the db/db Mouse. *J Toxicol Pathol.*, 22(2):133–7, Jun 2009.

Yaqoob M., Patrick A. W., McClelland P., Stevenson A., Mason H., White M. C., Bell G. M.. Relationship between markers of endothelial dysfunction, oxidant injury and tubular damage in patients with insulin-dependent diabetes mellitus. *Clin Sci (Lond).*, 85(5):557–62, Nov 1993.

Ye H., Jeong S. Y., Ghosh M. C., Kovtunovych G., Silvestri L., Ortillo D., Uchida N., Tisdale J., Camaschella C., Rouault T. A.. Glutaredoxin 5 deficiency causes sideroblastic

anemia by specifically impairing heme biosynthesis and depleting cytosolic iron in human erythroblasts. *J Clin Invest.*, 120(5):1749–61, May 2010.

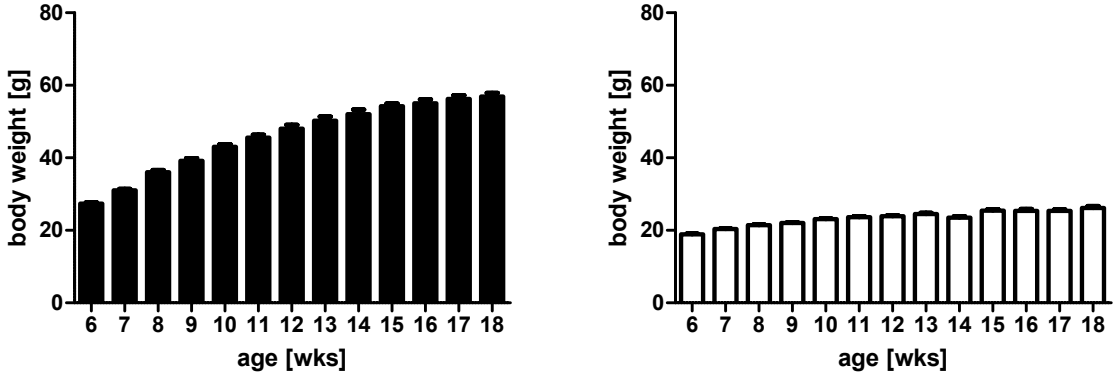
Zelissen P. M., Stenlof K., Lean M. E., Fogteloo J., Keulen E. T., Wilding J., Finer N., Rössner S., Lawrence E., Fletcher C., McCamish M.. Effect of three treatment schedules of recombinant methionyl human leptin on body weight in obese adults: a randomized, placebo-controlled trial. *Diabetes Obes Metab.*, 7(6):755–61, Nov 2005.

Zhang Y., Proenca R., Maffei M., Barone M., Leopold L., Friedman J. M.. Positional cloning of the mouse obese gene and its human homologue. *Nature.*, 372(6505):425–32, Dec 1994.

Zhou Y., Kok K. H., Chun A. C., Wong C. M., Wu H. W., Lin M. C., Fung P. C., Kung H., Jin D. Y.. Mouse peroxiredoxin V is a thioredoxin peroxidase that inhibits p53-induced apoptosis. *Biochem Biophys Res Commun.*, 268(3):921–7, Feb 2000.

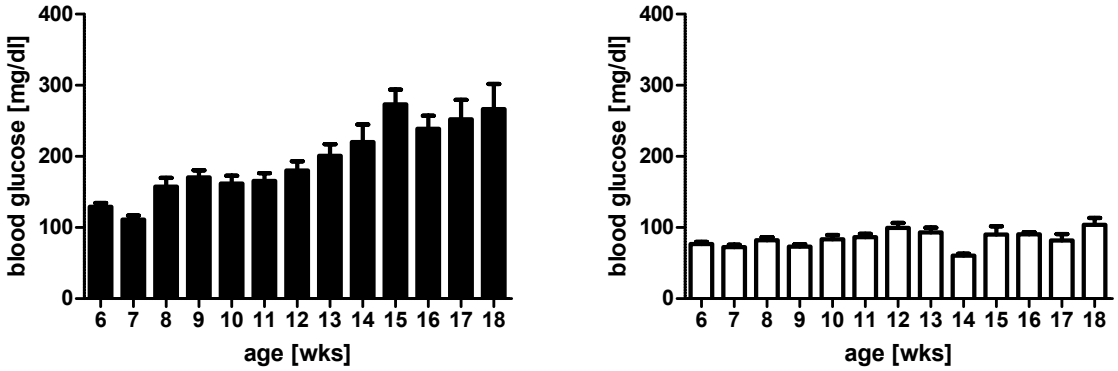
11 Appendix

11.1 Follow-up of the body weight and blood glucose level of db/db and db/+ mice



(a) db/db (b) db/+

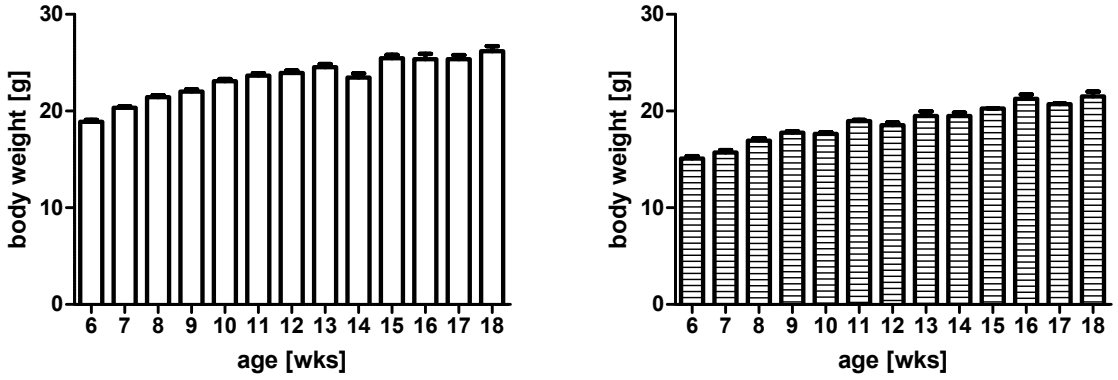
Figure 11.1: Follow-up of the body weight (n = 11 to 40 per timepoint).



(a) db/db (b) db/+

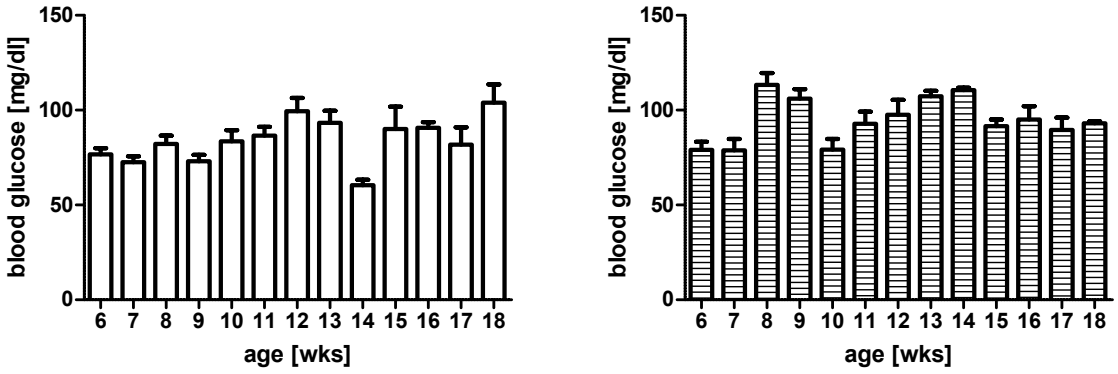
Figure 11.2: Follow-up of the blood glucose level (n = 11 to 36 per timepoint).

11.2 Comparison between db/+ and wild type C57BL/6 mice



(a) db/+ (b) wild type

Figure 11.3: Body weight comparison between db/+ and wild type C57BL/6 mice (n = 2 to 40 per timepoint).



(a) db/+ (b) wild type

Figure 11.4: Blood glucose level comparison between db/+ and wild type C57BL/6 mice (n = 2 to 35 per timepoint).

11.3 qRT-PCR results for Grx2 expression in isolated islets

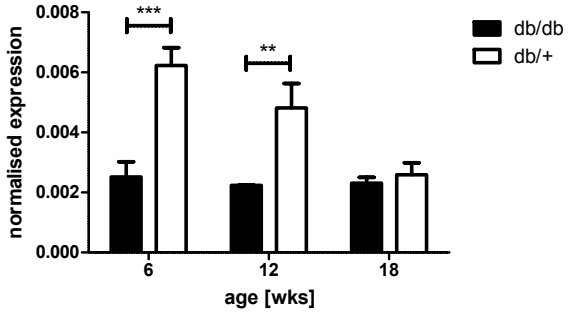


Figure 11.5: Gene expression of Grx2 (relative Grx2 expression normalised with beta-actin, n = 4 to 6 per timepoint). It followed the same pattern as Grx5.

12 Publications

Petry S. F., Linn T.. Islet hypertrophia in db/db mice is accompanied by low glutaredoxin 1 and 5 levels and high ROS production., 12th German Pancreatic Islet Workshop Bremen, March 2015.

Petry S. F., Hanschmann E. M., Rawat N., Eitner S., Lillig C. H., Linn T.. Metabolic stress in pancreatic islets differentially affects cytosolic and mitochondrial members of the Trx family of proteins., Excellence Cluster Cardio-Pulmonary System (ECCPS) - Area G, endogeneous metabolic signals - Retreat 2015 Bad Nauheim, June 2015.

13 Erklärung zur Dissertation

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Ort, Datum	Unterschrift

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15 Curriculum vitae

The curriculum vitae was removed in the published version of the thesis.