Dextrorphan uncovers a role of the serine-linked mitochondrial one-carbon metabolism in pancreatic islet cell survival at the expense of secretory function

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Table of Contents

1 Summary

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by elevated blood glucose (BG) levels and progressive beta cell failure. Although various medications are available, currently there is no treatment that can halt or reverse the progressive loss of beta cell functionality and cell death. Due to its positive effects on pancreatic islet cell functionality and survival, the N-Methyl-D-Aspartate receptor (NMDAR) antagonist dextrorphan (DXO) is considered a potential antidiabetic drug. However, the effects of DXO strikingly depend on the treatment's duration and concentration. While acute treatment increases glucose-stimulated insulin secretion (GSIS), chronic high-dose treatment induces islet dysfunction and enhances cell survival against cytokine-induced cell death and the beta cell toxin streptozotocin (STZ). Previous studies uncovered a correlation between DXO-induced islet cell dysfunction and the upregulation of ER stress marker *activating transcription factor 4* (*Atf4*) and serine-linked mitochondrial one-carbon metabolism (*mt*OCM) genes, suggesting a metabolic adaptation, which improves the islet cell redox state at the expense of the GSIS.

In this study, the dual effects of acute and chronic DXO treatment on GSIS and cell survival in pancreatic islets were investigated. Through glycolytic flux analyses, a previously hypothesized metabolic pathway underlying the DXO-induced effects was validated, revealing a shift of the glycolytic flux from the tricarboxylic acid (TCA) cycle towards serine synthesis upon chronic high-dose treatment. Gain-of-function (GoF) and Loss-of-function (LoF) experiments demonstrate that ATF4 acts as key regulator of the glucose flux and serine-linked *mt*OCM gene expression levels. Finally, this study demonstrates that the uncovered metabolic pathway serves as a general protective molecular mechanism of islet cells in response to insulin hypersecretion and that its activation is not limited to DXO treatment only.

In summary, this study reveals that islet cells prioritize cellular survival over secretory function by enhancing serine-linked *mt*OCM activity, to mitigate stress-induced damage. This novel insights about the interplay between islet cell protection and functionality contribute to a better understanding of islet cell physiology and point to the possibility that halting and reversing the progression of islet cell demise is generally possible by attenuating insulin hypersecretion and islet cell stress. These findings potentially pave the way for preventive or curative treatments of the pathophysiology of DM.

2 Zusammenfassung

Diabetes mellitus (DM) ist eine chronische Stoffwechselstörung, die sich durch erhöhte Blutzuckerspiegel und fortschreitenden Funktionsverlust der Beta-Zellen auszeichnet. Obwohl verschiedene Medikamente verfügbar sind, gibt es derzeit kein Mittel, das den fortschreitenden Funktionsverlust und das Absterben der Beta-Zellen aufhalten oder rückgängig machen könnte. Aufgrund seiner positiven Wirkungen auf die Funktion und das Überleben der pankreatischen Langerhans Inselzellen gilt der N-Methyl-D-Aspartat-Rezeptor (NMDAR)-Antagonist Dextrorphan (DXO) als potenzielles Antidiabetikum. Die Wirkungen von DXO sind jedoch von der Behandlungsdauer abhängig. Während eine akute Behandlung die Glukose-stimulierte Insulinausschüttung (GSIS) erhöht, führt eine chronische Hochdosisbehandlung zu einer Fehlfunktion der Beta-Zellen und verbessertem Überleben der Zellen gegenüber Zytokin-ausgelöstem Zelltod und dem Beta-Zellgift Streptozotocin (STZ). Vorherige Studien zeigten eine Korrelation zwischen der DXO-induzierten Inselzelldysfunktion und der Hochregulierung des ER-Stress-Markers ***activating transcription factor 4*** (***Atf4***), sowie der Serin-verknüpften mitochondriellen Ein-Kohlenstoff-Stoffwechsel-(*mt*OCM)-Gene. Diese deuten auf eine metabolische Anpassung hin, die den Redox-Status der Inselzellen auf Kosten der Glukose-induzierten Insulinausschüttung (GSIS) verbessert.

In dieser Studie wurden die dualen Effekte der akuten und chronischen DXO-Behandlung auf die GSIS und das Zellüberleben der pankreatischen Inselzellen untersucht, wobei auf vorherige Erkenntnisse aufgebaut wurde. Analysen des glykolytischen Flusses konnten einen zuvor hypothesierten Stoffwechselweg validieren, über den DXO seine Effekte induzieren könnte, wobei eine Verschiebung des glykolytischen Flusses vom Tricarbonsäurezyklus (TCA) zur Serinsynthese festgestellt wurde. GoF- und LoF-Experimente zeigten, dass ATF4 als Schlüsselregulator für den Glukosefluss und die Expression der Serin-verknüpften *mt*OCM-Gene wirkt. Schließlich zeigt diese Studie, dass der aufgedeckte Stoffwechselweg als allgemeiner Schutzmechanismus der Inselzellen vor der Hypersekretion von Insulin fungiert und nicht allein auf die Behandlung mit DXO beschränkt ist.

Zusammenfassend zeigt diese Studie, dass Inselzellen das zelluläre Überleben gegenüber der sekretorischen Funktion priorisieren, indem sie die Aktivität des *mt*OCM steigern, um stressbedingte Schäden bewältigen zu können. Diese neuen Erkenntnisse über das Zusammenspiel zwischen Inselzell-Schutz und deren Funktionalität tragen zu einem besseren

Verständnis der Inselzell-Physiologie bei und deuten auf die Möglichkeit hin, dass das fortschreitende Absterben der Inselzellen durch das Verringern der Insulin-Hypersekretion und des Inselzellstresses grundsätzlich zu stoppen oder umzukehren ist. Dies könnte möglicherweise den Weg für präventive oder kurative Behandlungen der Pathophysiologie des DM ebnen.

3 Introduction

3.1 Pancreas: The key organ in metabolic fate

The pancreas is a vital glandular organ located in the abdomen, responsible for crucial metabolic functions. Structurally, it consists of two main tissue units with distinct physiological functions. On the one hand, the exocrine tissue makes up 95% of the pancreas mass and secretes digestive enzymes into the small intestine. After food intake, digestive enzymes and ions are secreted by exocrine acinar cells into the duodenum through the pancreatic duct system.[1] On the other hand, the highly vascularized endocrine unit covers only 1 – 2% of the pancreas and is formed by cell clusters, called pancreatic islets or islets of Langerhans (see Figure 1).[2,3] These islets are composed of different cell types, including alpha cells, beta cells, and others, each producing and secreting specific hormones with distinct roles in the metabolic regulation.[2] Alpha cells secrete glucagon, which promotes the release of stored glucose from the liver, to maintain blood glucose (BG) levels during fasting or phases of low BG (hypoglycemia).[4,5] In turn, beta cells, which comprise 50 – 75% of human islets, produce and release the polypeptide hormone insulin, acting as an antagonist of glucagon.[6] Insulin plays a pivotal role in BG regulation, as it facilitates glucose uptake by peripheral tissue cells including neurons, myocytes and hepatocytes.[2,7-9] Additional cell types forming the pancreatic islets are somatostatin-secreting delta cells, pancreatic polypeptide-synthesizing PP cells, and ghrelin-producing epsilon cells.[10-14]

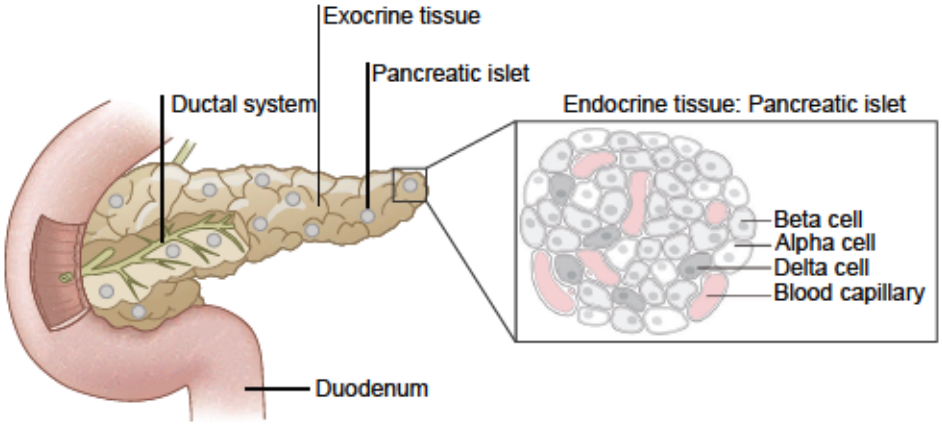

Figure 1: Macro- and microscopic architecture of the pancreas and pancreatic islets.
The pancreas consists of exocrine and endocrine parts, each with distinct structures and functions. The exocrine tissue releases digestive enzymes into the duodenum through the ductal system. The endocrine tissue is organized into pancreatic islets, containing different secretory cell types. Each cell type produces and secretes specific hormones, responsible for the regulation of metabolic processes, including glucose homeostasis. Illustration adapted from Lammert & Zeeb and Otter *et al.*[15,16]

The interactions of pancreatic hormones are of vital importance and keep the body glucose homeostasis in a stable balance, preserving normal BG concentrations (euglycemia) between 3.9 – 5.6 mmol/L (70 – 100 mg/dL) after fasting and below 11 mmol/L (<200 mg/dL) 2 hours *postprandial*.[17,18] Disruptions in these intricate hormonal interactions can lead to metabolic disorders, such as impaired glucose homeostasis, insulin resistance, and other dysregulations, contributing to the development of diabetes mellitus.[2,4,7-9] Therefore, understanding the structures and functionalities of the pancreas is fundamental in understanding metabolic disorders and developing targeted interventions for their management.

3.2 The dynamic role of beta cells: Transforming sensing into metabolic action

Pancreatic beta cells play a fundamental role in the regulation of BG levels through a highly coordinated process of insulin secretion. This mechanism involves a series of molecular events that respond to changes in BG concentration. Through the islet vascularization, glucose enters the beta cells via the glucose transporter 2 (GLUT-2). Once intracellular, glucose undergoes glycolysis, tricarboxylic acid (TCA) cycle, and electron transport chain (ETC) leading to adenosine triphosphate (ATP) generation.[19] The elevated ratio of ATP/adenosine diphosphate (ADP) inhibits ATP-sensitive potassium (K_{ATP}) channels, causing membrane depolarization and the opening of voltage-dependent calcium channels (VDCC), facilitating calcium influx into the beta cell.[20-22] The resulting increase in cytosolic Ca^{2+} concentration plays a crucial role in the exocytosis of mature insulin-containing granules.[23] Calcium stimulates the fusion of insulin granules with the plasma membrane, resulting in the release of insulin into the blood circulation.[24,25]

In addition to glucose, various factors modulate insulin secretion. Incretin hormones, such as glucagon-like peptide 1 (GLP-1), potentiate insulin secretion upon nutrient ingestion.[26-28] GLP-1 acts through the GLP-1 receptor (GLP-1R) on beta cells, activating adenylate cyclase and elevating cyclic adenosine monophosphate (cAMP) levels[29]. Increased cAMP levels stimulate protein kinase A (PKA), which phosphorylates proteins involved in insulin exocytosis, thereby enhancing the release of insulin.[29,30] However, the delicate balance of glucose regulation is disrupted when beta cells experience impairment in insulin secretion such as beta cell dysfunction, de- or trans-differentiation, and apoptosis, causing beta cell demise which in turn contributes to the development of DM and various complications that affect multiple organ systems.[31,32] Therefore, understanding the role of beta cell functionality

and its physiology is vital in comprehending the complexities of diabetes and finding strategies to prevent or treat beta cell functionality within the context of diabetes management.[32]

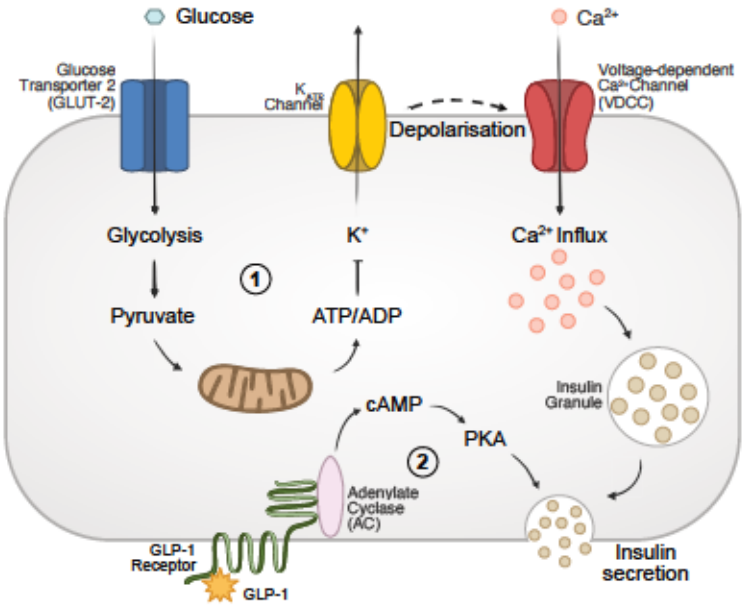

Figure 2: Schematic illustration of glucose-stimulated insulin secretion (GSIS) by pancreatic beta cells.
1, K_{ATP}-dependent GSIS: Glucose enters the beta cell via the glucose transporter 2 (GLUT-2) and is metabolized into adenosine triphosphate (ATP). Increasing ATP/adenosine diphosphate (ADP) ratio levels lead to closure of ATP-sensitive potassium (K_{ATP}) channels and consequently to membrane depolarisation and the opening of voltage-dependent calcium channels (VDCC). The influx of Ca^{2+} triggers the exocytosis of mature insulin into the blood stream. **2**, GLP-1R induced GSIS: Glucagon-like peptide-1 agonists activate the GLP-1 receptor (GLP-1R) and trigger the generation of cyclic adenosine monophosphate (cAMP) by adenylate cyclase (AC). Enhanced cAMP levels activate the protein kinase A (PKA), which in turn causes K_{ATP}-closure, Ca^{2+} influx, and enhance the exocytosis of insulin granules. Illustration generated by A. Pelligra, based on Wollheim *et al.* and Holter *et al.*[33,34]

Recent RNA-sequencing (RNA-seq) analyses of islets deriving from diabetic patients and mice revealed the existence of various beta cell populations with distinct gene expression patterns, exhibiting different characteristics in functionality and resilience.[35-37] These studies suggest that dysfunctional beta cells exhibit increased resistance to apoptosis and extended lifespan as compensatory adaptations against reduced responsiveness.[35,38] Further, these observations hold significant implications for diabetes research and treatment and challenges the conventional belief that dysfunctional islet cells are inevitably destined for failure. Therefore, understanding the underlying mechanisms behind the coexistence of dysfunction and enhanced survival in beta cells from diabetic individuals might offer novel strategies to target islet dysfunction or promote islet cell viability.

3.3 Diabetes mellitus: A chronic battle for patients and society

Diabetes mellitus is a progressive metabolic disease characterized by chronically elevated blood glucose levels, resulting from a dysregulation of hormonal glucose homeostasis. This hormonal dysregulation mainly results from impaired insulin production and secretion, insulin resistance of peripheral tissues, or a combination of both.[32,39-41] In addition to hyperglycemia, and the onset of characteristic concomitant symptoms such as polyphagia (increased appetite), polydipsia (excessive thirst), polyuria (frequent urination), weight loss, and fatigue, diabetes can be diagnosed by various tests.[42,43] These tests include for example the measurement of plasma glucose levels during an oral glucose tolerance test (OGTT) or the determination of glycated hemoglobin value (HbA1c), which reflects the glycation level of hemoglobin (Hb), commonly used as indicator for chronic hyperglycemia (≥6.5% or ≥48 mmol/mol).[44-46] Untreated diabetes is a serious risk factor for severe secondary long-term complications, including cardiovascular diseases, retinopathy, neuropathies, nephropathy, increased susceptibility to infections, and cancer, or can even induce life-threatening conditions like stupor, coma or ketoacidosis, finally leading to death.[45,47-49] To prevent these comorbidities and maintain a stable life quality, early diagnosis and effective management of diabetes, through constant BG monitoring and regular intake of appropriate BG-lowering medications or insulin injections, are crucial.

The etiology of DM is multifactorial and a combination of genetic predisposition, environmental factors, nutrition, and a sedentary lifestyle are considered to be the main trigger of this metabolic disorder, which show different phenotypes and pathomechanisms.[22,32,41] Type 1 DM (T1DM) is an autoimmune disease resulting from the immune-mediated destruction of insulin-secreting beta cells, accounting for about 5 – 10% of all diabetes cases, commonly observed in childhood or adolescence.[22,50] The breakdown of self-tolerance leads to the infiltration of pancreatic islets by autoreactive immune cells, initiating inflammatory processes and progressive beta cell demise.[51] In contrast to common notion, several *post-mortem* analyses of pancreas from individuals with T1DM revealed no consistent reduction of beta cell mass and volume, suggesting that persistent islet dysfunction rather than reduced beta cell mass, plays a fundamental role in the onset of T1DM.[38,52-55] Since endogenous insulin production is dramatically low or not present in T1DM, this DM subtype requires life-long exogenous insulin supplementation via injection and is therefore considered insulin-dependent.[50] Moreover, environmental factors, including virus infection during

pregnancy and childhood, foreign antigens in infant nutrition, and childhood vaccination, have been implicated as potential triggers for T1DM.[56,57]

Type 2 DM (T2DM) is the most common DM form, accounting for approximately 90 – 95% of cases.[18] In addition to beta cell dysfunction and demise, a key characteristic of T2DM involves the progressive development of insulin resistance in peripheral tissues, such as liver, skeletal muscles, and adipose tissue, which is thought to arise from the excessive accumulation of fat (hyperlipidemia) and its lipotoxic metabolites.[22,39,58-60] Therefore, the incidence of T2DM increases with age, but physical inactivity and environmental factors play a significant role in its etiology as well, with genetic factors also contributing.[22,39] Longitudinal cohort studies, such as the German Diabetes Study (GDS), revealed great variability in the severeness of beta cell dysfunction and peripheral insulin resistance, as well as in the heterogeneity of manifested secondary diseases in T2DM patients.[61-63] These differences are thought to arise from the intricate interplay of various risk factors, leading the categorization of following T2DM clusters:[62-64] severe insulin-deficient diabetes (SIDD), mild obesity-related diabetes (MOD), mild age-related diabetes (MARD), and severe insulin-resistant diabetes (SIRD).[62] However, T2DM is considered non-insulin-dependent, since lifestyle interventions such as diet and exercise or oral medications are initially effective in its management or reversibility.[18,65,66] Injection of basal or bolus insulin are only required in very severe or late stages of T2DM, due to the progressive nature of the pathophysiology.[67] Notably, recent studies indicate that in early-onset T2DM and insulin-dependent cases, drastic interventions such as bariatric surgery or highly restricted caloric diets can offer partial reversal of functional impairment, challenging the notion of T2DM's irreversibility.[66,68-71]

Other, better defined forms of diabetes, are relatively rare (1 – 2% of all cases) and more specific DM subtypes, which are either associated with hyperglycemia in pregnancy (HIP), arise from specific monogenic defects in beta cell functionality, such as neonatal diabetes mellitus (NDM) and maturity-onset diabetes of the young (MODY), or result from secondary causes such as diseases of the exocrine pancreas, intake of medications, and viral infections.[22,72-75] HIP comprises gestational diabetes mellitus in pregnancy (DIP) and gestational diabetes mellitus (GDM), which are both diagnosed during pregnancy for the first time. DIP is mostly associated with pre-existing DM or diagnosed within early pregnancy, GDM, which is the most common HIP form (84% of all HIP cases), is diagnosed in the second and third trimester of pregnancy.[76,77] On the other hand, MODY is an early-onset, non-insulin-

dependent form of DM and manifests before the age of 25, while ND is defined as diabetes that is diagnosed before six months of age.[72,78,79]

Taken together DM is a global health concern, affecting more than 500 millions of diagnosed patients and hundreds of millions of undiagnosed individuals (aged 20 – 79 years) worldwide, representing a massive burden on healthcare systems.[18,80,81] According to the International Diabetes Federation (IDF), this number is projected to rise to 780 million by 2045 with estimated health expenditures of USD 1.05$ trillion in that year.[18] The economic burden of diabetes is substantial, including direct medical costs, productivity losses due to disability or premature death, and the costs associated with managing complications.[18] In the last decades, the number of children and adolescents affected by obesity and DM has increased dramatically, emphasizing the escalating nature of this health crisis.[82-86] Therefore, diabetes imposes a significant burden on individuals, families, and society, which requires urgent support and interventions to halt and prevent its progression into an escalating global pandemic.

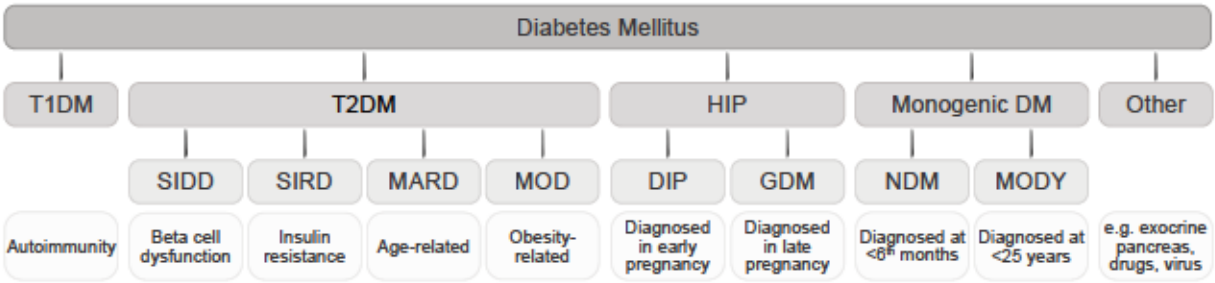

Figure 3: Overview of Diabetes Mellitus (DM) subtypes and clusters, delignated by distinct etiological factors and pathophysiological characteristics. Type 1 DM (T1DM) is mainly characterized by autoimmunity; Type 2 DM (T2DM) encloses a variety of etiological factors, resulting in different characteristics and clusters, including severe insulin deficient diabetes (SIDD), severe insulin-resistant diabetes (SIRD), mild obesity-related diabetes (MOD), and mild age-related diabetes (MARD); Hyperglycemia in pregnancy (HIP) comprise diabetes in pregnancy (DIP) and gestational DM (GDM), both related to hyperglycemia diagnosed during pregnancy but at different gestation stages; Monogenic DM reflect genetic-related forms of DM, which occur in newborns such as neonatal DM (NDM) or in adolescence such as maturity-onset diabetes of the young (MODY); Other forms of DM result from secondary causes including disorders of the exocrine tissue, drug-induced, and viral infections. Illustration generated by A. Pelligra, partially based on Herder *et al.*[62]

3.4 The domino effect: The role of cell stress in triggering metabolic disorders

Besides a genetic predisposition, exogenous factors such as nutrition, physical inactivity, and stress play a pivotal role in the development and progression of metabolic disorders. It is widely recognized that in the long-term, these factors correlate with the onset of comorbidities like elevated cholesterol levels, hypertension, and hyperlipidemia, culminating in an increased risk for obesity and T2DM.[87-89] Notably, obesity, which is defined as a body mass index (BMI) above 30 kg/m², exhibits a profound pathophysiology associated with T2DM, termed "diabesity".[90-93] Excessive weight gain and the expansion of visceral adipose tissue (VAT) were shown to trigger the release of pro-inflammatory adipokines and cytokines, creating a pro-inflammatory microenvironment that compromises insulin sensitivity in peripheral tissues as well as pancreatic beta cell functionality and survival.[94,95] In addition to lipotoxicity, stress-induced release of glucocorticoids inhibits glucose uptake by muscles and adipose tissue, disrupting the glucose homeostasis and promoting insulin resistance.[87,88] Upon the induction of islet dysfunction and insulin resistance, beta cells strive to compensate for increased insulin demand through mechanisms such as enhanced proliferation and expansion of beta cell mass.[93,96] However, this compensatory state is transient as prolonged exposure to adverse conditions leads to beta cell exhaustion, dysfunction, and ultimately demise.[31,93,96] The sustained metabolic stress caused by chronic exposure to elevated glucose levels, lipotoxicity, and pro-inflammatory cytokines, induces a cascade of detrimental events, as these stressors trigger the activation of endoplasmic reticulum (ER) stress and oxidative stress, which play pivotal roles in beta cell failure and metabolic disorders.[97-100] Hence, understanding the central mechanisms that lead to beta cell demise is crucial for effective T2DM prevention and management strategies.

3.4.1 ER stress: From survival to demise

ER stress plays a crucial role in the pathophysiology of T2DM.[101-103] The accumulation of misfolded or unfolded proteins within the ER leads to ER stress, which is known to be a major contributor to islet dysfunction.[102-104] Beta cells heavily rely on proper ER activity for correct folding, *post*-translational modification, and maturation of insulin molecules.[97,98,105] Chaperones, which are located in the ER lumen ensure the correct protein folding and prevent protein aggregations during protein biosynthesis.[106,107] However, persistent insulin demand, such as in conditions like hyperglycemia, overstrain the ER's capacity, disrupting ER homeostasis and triggering ER stress.[32,98,108] This stress activates the unfolded protein response (UPR), a cellular mechanism aimed at restoring ER homeostasis. The initial trigger of the UPR cascade is the accumulation of misfolded or unfolded proteins, leading to the dissociation of BiP (binding immunoglobulin protein), also known as GRP78 (78 kDa glucose-regulated protein) encoded by the *Heat Shock Protein Family A Member 5* gene (*Hspa5*), from the luminal domains of the three main transmembrane UPR sensors: inositol-requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6), and protein kinase RNA-like ER kinase (PERK).[97,106] These UPR branches work cooperatively to alleviate ER stress but prolonged and unresolved ER stress can lead to islet dysfunction, dedifferentiation, oxidative stress, and ultimately, beta cell apoptosis.[98,102,109,110] PERK phosphorylates eukaryotic initiation factor 2 (eIF2α), leading to the attenuation of protein translation and activation of activating transcription factor 4 (ATF4). Notably, ATF4 plays a critical role in maintaining cellular integrity by regulating the expression of key genes involved in amino acid (AA) metabolism, redox homeostasis and apoptosis.[105,111-114] However, studies indicate that the activation of the ATF4-associated UPR branch contributes to islet dysfunction and the pathogenesis of diabetes.[101,105,115] In fact, enhanced ER stress and elevated expression levels of ATF4-associated members have been observed in beta cells of pancreatic sections from T2DM patients and islets from mice of the T2DM mouse model strain *db/db*.[103,116,117] This indicates a direct association between ER stress, ATF4, and the development of T2DM, as imbalanced ER homeostasis is also linked to decreased glucose-stimulated insulin secretion (GSIS), providing further evidence of its involvement in T2DM pathophysiology.[118-120] Thus, elucidating the intricate interplay between ER stress, apoptosis, oxidative stress regulation and insulin secretion of beta cells might be crucial for developing therapeutic interventions to preserve beta cell viability and function in health and diabetes.

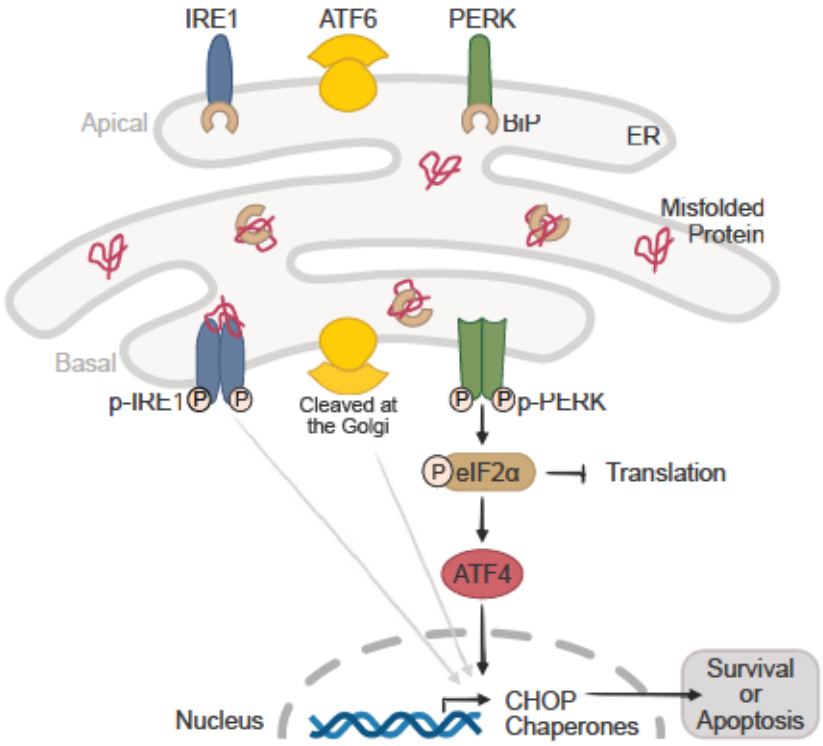

Figure 4: Schematic illustration of endoplasmic reticulum (ER) stress and the ATF4-related unfolded protein response. Apical, binding immunoglobulin protein (BiP) interacts with the luminal domains of transmembrane UPR sensors inositol-requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6), and protein kinase RNA-like ER kinase (PERK). BiP dissociation, resulting from sensed misfolded proteins, triggers phosphorylation of IRE1 and PERK or cleavage for ATF6. Basal, phosphorylated PERK (p-PERK) phosphorylates eukaryotic initiation factor 2 (eIF2α), which inhibits the biosynthesis of new proteins and activates the transcription factor ATF4. In turn, ATF4 induces the gene expression of CCAAT/-enhancer-binding protein homologous protein (CHOP) to initiate apoptosis, or chaperones and pro-survival genes to enhance cell survival. Illustration generated by A. Pelligra, adapted from Biden *et al.* (modified).[102]

3.4.2 Oxidative Stress in beta cells: From necessity to excess

In recent decades, it has been discovered that diabetic stress such as hyperglycemia, glucolipotoxicity, and inflammation lead to an imbalance between the generation of reactive oxygen species (ROS) and the cellular antioxidant defense system, resulting in the onset of oxidative stress.[121-123] ROS are highly reactive molecules such as superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-), which are considered to play a crucial role in development of beta cell dysfunction.[121,124,125] Due to low expression of antioxidant enzymes and high endogenous ROS generation, pancreatic beta cells are particularly vulnerable to oxidative stress.[126,127] This susceptibility is further exacerbated by the interconnected relationship between ER stress and oxidative stress, which creates a vicious cycle that further contributes to beta cell damage, dysfunction, apoptosis, and insulin resistance in peripheral tissues, ultimately accelerating the progression of T2DM.[98,128-131] Therefore, oxidative stress is widely considered a significant factor in the development and advancement of T2DM.

Commonly, ROS are generated as a by-product of the ETC in mitochondria during cellular ATP production.[132] In fact, they are mainly generated by electron leaking at numerous sites within the ETC.[133] Under healthy physiological conditions, the cells maintain a delicate balance between the production and elimination of ROS through the cellular antioxidant defense system. Antioxidant enzymes, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx), work together to neutralize ROS and prevent oxidative damage.[132] In contrast to common notion, ROS is not only a harmful by-product which necessitates to be rapidly neutralized, but plays functional roles as well. Indeed, ROS also serve as signaling molecules for various cellular processes including oxidative modification of numerous protein types, such as enzymes, ion channels, and transcription factors, stabilisation of processes during proliferation, and cell fate regulation.[133] Particularly noteworthy is their essential role and intriguing feature in triggering the GSIS in beta cells.[125,134] However, excessive ROS, arising for example from dysfunctional mitochondria, overstrains the antioxidant defense system, causing oxidative stress.[132] Similar effects were shown to be induced by hyperglycemia and the resulting GSIS, in insulinoma cells of mice, rats, and in human pancreatic islets, with H_2O_2 directly deriving from the ETC.[124,125]

In T2DM, chronic hyperglycemia, increased fatty acid levels, and the activation of inflammatory processes are considered the main triggers for elevated ROS production and beta cell demise.[99,121,135,136] Additionally, these factors lead to the accumulation of advanced glycation end (AGE) products, which are compounds deriving from non-enzymatic interaction of glucose with proteins, lipids, or nucleic acids.[137,138] AGEs directly generate reactive ROS and activate receptor-mediated pathways that lead to further inflammation, ultimately worsening oxidative stress in T2DM.[137,138] Due to the highly reactive nature of ROS, they initiate chain reactions which can further propagate the oxidative damage to cellular components such as lipids, proteins, and DNA, leading to disruption of their structures and functionalities.[99,121,135] In particular, it was observed that ROS can interfere with the regulation of calcium levels and compromise the integrity and fluidity of beta cell membranes by lipid peroxidation, affecting the insulin release in response to glucose stimulation.[139,140] Indeed, increased by-products of lipid peroxidation and oxidative stress markers have been observed in individuals with obesity and T2DM.[141,142] Further, ROS can react with proteins, causing oxidative modifications of amino acid residues, formation of protein adducts, and cross-linking of proteins.[124] Such modifications impair protein structures, disrupt enzymatic activity, and interfere with protein

interactions, ultimately affecting the insulin biosynthesis and proper cell function.[124,143] Moreover, ROS can also directly attack DNA, generating base modifications, DNA strand breaks, and DNA-protein cross-links, compromising genomic integrity and leading to the accumulation of mutations.[135,144] These have detrimental consequences for cell functionality and cell survival, contributing to cell dysfunction and progressive beta cell demise.[135,143] A similar mechanism is also triggered by the beta cell toxin Streptozotocin (STZ), a beta cell specific genotoxic agent, which is widely utilized as diabetogenic stress inducer in the field of diabetes research.[145,146]

In summary, excessive ROS production and oxidative stress play a critical role in beta cell functionality, survival, and overall in the development and progression of T2DM. Therefore, understanding their generation, regulation, and containment, i.e. through enhanced antioxidant defense systems, might help to prevent or even reverse the pathomechanisms underlying T2DM.

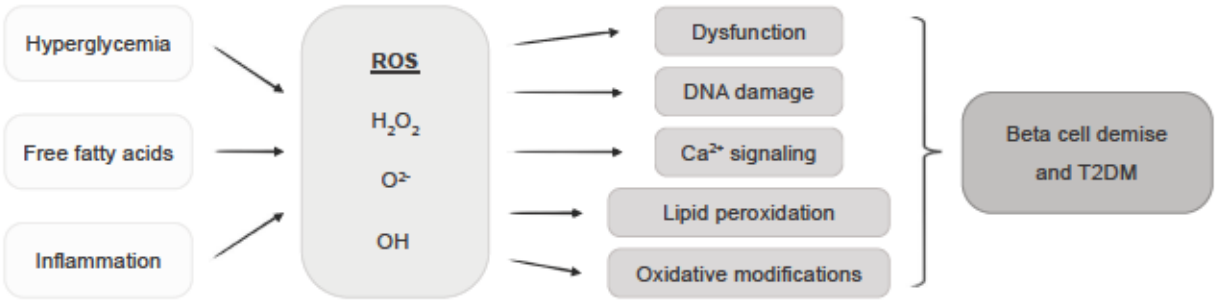

Figure 5: Overview of reactive oxygen species (ROS) induction and their implication in beta cell demise and T2DM. Diabetic stressors such as hyperglycemia, enhanced free fatty acid levels and inflammation trigger the excessive generation of various ROS species, including superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-). Highly reactive ROS initiate oxidative chain reactions, disrupting cellular structures and functions, causing secretory dysfunction, DNA strand breaks, disruption of intracellular Ca^{2+} signaling, and inducing lipids peroxidation and oxidative modifications. These processes ultimately culminate in beta cell demise, contributing to the development of T2DM. Illustration generated by A. Pelligra.

3.5 The one-carbon metabolism: Complexities of redox regulation and cell fate

Among all metabolic pathways, the one-carbon metabolism (OCM) stands out as one of the most crucial mechanisms for safeguarding cellular physiology in health and alleviating stress during cellular stress conditions. The OCM is a biochemical network of crucial importance for the vitality and functionality of many cell types.[147-149] It is intricately involved in multiple cellular roles, most notably cell proliferation and antioxidant defense mechanisms.[147-149] This versatile pathway operates across different cellular compartments, including cytoplasm and mitochondria, regulating a range of functions which are crucial for cellular homeostasis.[150-152] The key metabolic processes regulated in the OCM span from redox control and amino acid recycling to nucleotide synthesis and methylation reactions, even extending to epigenetic regulations.[150-152] Furthermore, it is fundamental for the generation and recycling of amino acids such as glycine and methionine. Ultimately, the OCM plays an essential role in cellular redox regulation, providing essential redox equivalents and antioxidants such as the glutathione, to safeguard cells against oxidative stress.[153,154]

This complex pathway is further subdivided into three primary sub-pathways, namely the folate cycle, methionine cycle, and transsulfuration pathway, each with distinct yet interlinked reactions.[155] To exert its proper function, the OCM requires folate, as main donor of one-carbon (C1)-units, which are pivotal for the synthesis of purine and thymidine, the regeneration of methionine, and the re-methylation of the harmful homocysteine.[156,157] To this end, the essential co-factor folate, commonly known as vitamin B9 and whose synthetic form is referred to as folic acid, is taken up by nutrition and converted into its metabolic functional form tetrahydrofolate (THF).[158] Notably, the folate cycle consists of a cytosolic and a mitochondrial branch comprising isoenzymes encoded by distinct genes, which catalyze homologous reactions.[151,159,160] In both branches THF is gradually metabolized, using serine as C1-unit donor.[150,153,161] These reactions are carried out, among others, by the OCM enzymes serine hydroxy methyltransferase (SHMT1/2), methyl-tetrahydrofolate dehydrogenase (MTHFD1/2), and aldehyde dehydrogenase 1 family member L (ALDH1L1/2), generating glycine and nicotinamide adenine dinucleotide (NADPH) as by-products.[153,156]

The methionine cycle is closely interlinked with the folate cycle through the re-methylation of homocysteine.[155] During these reactions the amino acid methionine and S-adenosyl methionine (SAM), an additional key C1-unit donor, are generated. SAM is a critical

substrate, influencing gene expression patterns but most importantly regulating epigenetic and differentiation processes.[158]

Alternatively to methionine, homocysteine can also be converted into cysteine within the transsulfuration pathway.[154,158] Cysteine is a fundamental amino acid, serving for protein synthesis but also for redox balance in the generation of the potent antioxidant glutathione (GSH).[154] As this pathway is only restricted to liver, brain, lymphoid cells, and pancreas, the role of OCM is thought to be mainly focused in maintaining the cellular homeostasis in non-proliferative cells.[154,155]

In summary, the OCM is indispensable for the vitality of cells, orchestrating functions ranging from nucleotide and amino acid biosynthesis to redox homeostasis. The interplay between the folate cycle, methionine cycle, and transsulfuration pathway underscores the complex yet harmonious nature of this metabolic network. A comprehensive understanding of OCM not only enriches our insights into fundamental cellular processes but also unveils potential avenues for therapeutic interventions targeting cellular dysfunctions.

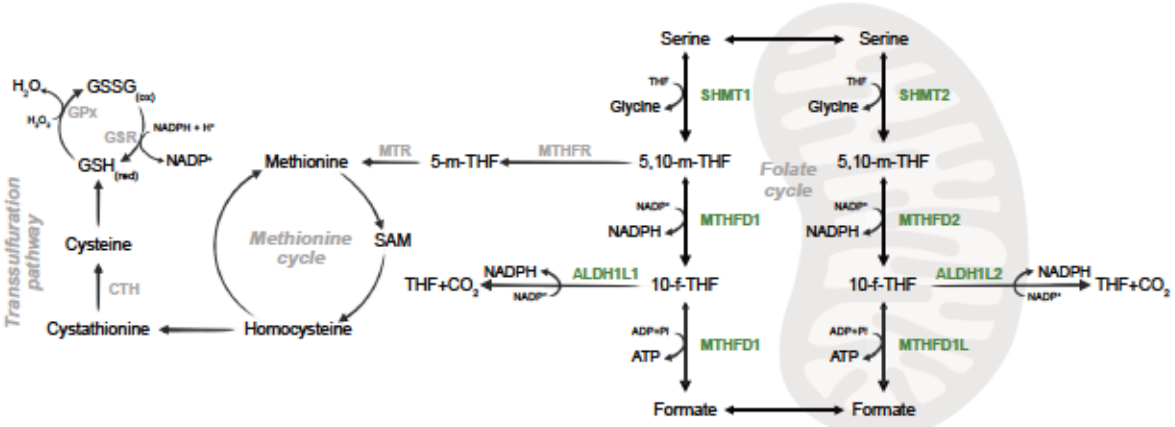

Figure 6: Schematic illustration of the one-carbon metabolism (OCM) pathways.
The OCM consists of intercompartmental reactions between mitochondria and cytoplasm, subdivided in folate cycle, methionine cycle and transsulfuration pathway. All pathways are interlinked, each playing a distinct but crucial role in cell viability and healthy redox homeostasis. In the folate cycle, tetrahydrofolate (THF) is gradually converted into methyl-THF (5,10-m-THF) and formyl-THF (10-f-THF) by the cytosolic and mitochondrial enzymes serine hydroxy methyltransferase (SHMT1/2), Methyl-Tetrahydrofolate dehydrogenase 1 (MTHFD1/2), and aldehyde dehydrogenase 1 family member L (ALDH1L1/2). This process generates glycine, nicotinamide adenine dinucleotide (NADPH), adenosine triphosphate (ATP) and carbon dioxide (CO_2). 5,10-m-THF is further processed by methyltetrahydrofolate reductase (MTHFR) and methionine synthase (MTR) into methionine, serving for the methionine cycle. Methionine in utilized for generating S-adenosyl methionine (SAM) and recycling of homocysteine. Alternatively, homocysteine undergoes the transsulfuration pathway mediated by the enzyme cystathionine gamma-lyase (CTH) and serving for the synthesis of cysteine and glutathione. The transsulfuration pathway is directly linked to the recycling process of oxidized glutathione (GSSH) into its reduced form (GSR) glutathione reductase (GSR). Enzymes of the folate cycle are highlighted in green. Illustration generated by A. Pelligra, based on Quevedo-Ocampo *et al.* and Newman *et al*. (modified), inspired by Biorender.[156,158]

3.6 Current T2DM treatments and limitations

Treatment and management of diabetes have made notable advancements in recent years, improving the life quality of affected patients. Current treatment strategies for T2DM often focus on symptom management and the prevention of complications, rather than targeting the underlying causes of the disease. However, the heterogeneity in etiology, pathomechanisms and clinical characteristics of T2DM patients often remain unconsidered in drug prescription.[162] One major traditional intervention in the initial management of T2DM consists of lifestyle modification, such as caloric restriction, improved physical activity, and the intake of oral medications. To date, a vast variety of glucose-lowering medications is available, each differing in their mechanism of action (MOA) and adverse effects.[163] As a first-line therapy, Metformin, belonging to the class of biguanides, is widely prescribed as an oral medication for T2DM. It effectively lowers blood sugar levels by reducing liver glucose production (gluconeogenesis) and enhancing insulin sensitivity.[164,165] Metformin might cause gastrointestinal side effects, such as nausea or diarrhea and is contraindicated in subjects with kidney impairment or conditions that might lead to lactic acidosis.[166] Despite these limitations, metformin remains a valuable and commonly prescribed medication, especially because initial approaches with first-line medications are particularly effective in the early disease stages.[164,167] However, first-line medications are not able to halt the progressive nature of DM and their long-term efficacy is usually observed to diminish over time, necessitating higher dosage or the addition of second-line treatments[168-170].

The second-line medication usually comprises of a dual therapy often including sulfonylureas, such as gliclazide, glimepiride and glibenclamide (Glib), in addition to incretin-based medications such as GLP-1R agonists or dipeptidyl peptidase 4 (DPP-4) inhibitors.[22] However, due to its MOA, which consists of blocking ATP-sensitive channels, sulfonylureas contribute to hypoglycemia by inducing glucose-independent insulin release.[22,171] In contrast, GLP-1R agonists, such as exendin-4 (Ex-4), exert their effects in a glucose-dependent manner by mimicking the endogenous incretin effect, which stimulates the secretion of insulin.[172,173] The duration of this effect is regulated by DPP-4's action in GLP-1 degradation and can be extended by the use of DPP-4 inhibitors.[165,174] However, the administration of GLP-1R agonists has a high incidence of gastrointestinal impairments, including nausea and vomiting.[172] Furthermore, long-term use of sulfonylureas and GLP-1R agonists can further worsen islet

functionality in individuals with type 2 diabetes, necessitating insulin injections to compensate for the impaired secretory functionality.[175-178]

The most recently released anti-diabetic drug for the treatment of T2DM is tirzepatide, an innovative anti-diabetic drug that acts as a dual agonist for the glucose-dependent insulinotropic polypeptide (GIP) and GLP-1R's.[179,180] Tirzepatide distinguishes itself from other anti-diabetic drugs by its ability to not only enhance insulin secretion but also significantly reduce body weight and HbA1c levels.[179,180] Moreover, it exhibits favorable cardiovascular outcomes and low incidence of adverse effects, underscoring its therapeutic potential in improving overall clinical outcomes for individuals with T2DM.[179,180] In addition, the gastrointestinal-related side effects are very infrequent among users.[181] However, no long-term side effects are yet reported about tirzepatide.

In the last decade, the N-methyl-D-aspartate receptor (NMDAR) antagonist dextromethorphan (DXM) and its active metabolite dextrorphan (DXO), have been suggested as potential novel anti-diabetic drugs.[182-184] In fact, DXO was found to act as insulin secretagogue, enhancing the GSIS of human and mouse pancreatic islets and additionally promote islet cell survival upon cytokine-induced cell death.[184-186] Studies have demonstrated that DXM reduces ***postprandial*** BG excursions in individuals with diabetes, to improve their long-term outcomes and to delay the onset of T2DM in ***db/db*** mice.[183,184,186] Preliminary data even indicate that DXM and DXO might possibly reduce inflammatory processes in pancreatic islets of a non-obese diabetic (NOD) mouse model, suggesting their potential also as a preventive treatment for T1DM.[187] However, the utilization of these NMDAR antagonists as therapeutic agents has been limited due to their side effects on the central nervous system (CNS), including fatigue, nausea, and dizziness.[183-185] Even though, novel derivatives of DXM and DXO have been developed to minimizing CNS-related side effects, while maintaining or enhancing the desired anti-diabetic effects, further structural modifications are required to synthesize novel and potentially preventive anti-diabetic derivates with an optimal safety profile.[185,188,189]

Besides drug development, current research is shifting its focus towards restoring endogenous insulin secretion and comprehending the molecular mechanisms underlying dysfunctional insulin secretion.[190] Promising approaches, such as the transplantation of insulin-producing islets into the anterior chamber of the eye or the hepatic vein of diabetic patients, are close to being established.[191,192] These techniques involve the transplantation of

autologous islets, as well as ***in vitro*** generated islets derived from differentiated pluripotent stem cells.[190,192] Furthermore, there is ongoing investigation into small-molecule compounds that aim to stimulate controlled beta cell proliferation, preserve beta cell functionality, and/or protect beta cells from cell death e.g. by suppressing inflammatory processes or glucolipotoxicity.[190,193,194]

Despite advancements in treatment options, a cure for diabetes remains elusive, as current treatments do not prevent, halt, or reverse the progressive nature of T2DM. This underscores the urgent need for research to understand the complex development of this disease. Further, the vast heterogeneity behind the root causes and mechanisms of T2DM highlights the necessity for a more personalized approach to disease management. To address this, research efforts must prioritize the identification of novel therapeutic targets and understand the underlying molecular mechanisms of dysfunctional insulin secretion and triggers of insulin resistance. Ultimately, the goal must be to translate these findings into a preventive treatment or a cure for the pathophysiology of T2DM in humans.

3.7 The dual effects of DXO on islet cells: Between functionality and survival

In the last decade, studies have demonstrated that deletion or pharmacologic inhibition of islet cell NMDAR promotes GSIS and cell viability.[195,196] Moreover, NMDAR antagonists, such as DXM and DXO, have shown potential therapeutic effects as anti-diabetic drugs.[16,182] Studies conducted by the Institute of Metabolic Physiology (Heinrich-Heine University, Düsseldorf) have shown that DXM and DXO enhance the insulin secreting effects in a glucose dependent manner already at 10 mM glucose concentration, by increasing the frequency and duration of beta cell membrane depolarization and Ca^{2+} influx.[184,185,197] However, recent ***in vitro*** experiments have demonstrated that prolonged and excessive insulin secretion induced by DXO also has a negative impact on islet cell functionality.[186] Similar was also observed during a small clinical trial, where few patients developed a reversible insulin-dependent diabetes mellitus (IDDM) as a consequence of chronic and high-dose administration of DXM.[198] These effects suggest that the beneficial GSIS enhancing effects of DXO are strictly dependent on the treatments duration and dose. Conversely, chronic high-dose DXO pre-treated and dysfunctional pancreatic islets display enhanced cell survival against cytokine treatment, suggesting the activation of genes, which might prevent islet cell demise upon diabetogenic stress.[186]

To identify potential candidate genes which are responsible for the DXO-induced islet cell protection, an RNA-seq analysis was conducted using chronic high-dose DXO-treated pancreatic mouse islets.[186] Among all differentially expressed genes, ***Phosphoglycerate dehydrogenase*** (***Phgdh***), ***Phosphoserine aminotransferase*** (***Psat1***), ***Phosphoserine phosphatase*** (***Psph***), ***Shmt2***, ***Mthfd2,*** and ***Aldh1l2*** were identified, encoding enzymes of a specific metabolic pathway, namely the serine-linked *mt*OCM folate cycle.[186] Notably, these enzymes are well known to play a pivotal role in the pro-survival pathway of the OCM and have been previously found to be highly upregulated in various cancer cell types.[199-205] Furthermore, the RNA-seq analysis revealed the upregulation of ***Atf4***, which is not only associated with ER stress, but also known to regulate expression levels of previously listed serine-linked *mt*OCM genes.[114,206-208]

In order to associate the observed induction of islet cell dysfunction and enhanced cell survival by chronic high-dose DXO treatment to the upregulation of ***Atf4*** and serine-linked *mt*OCM genes, J. Mrugala hypothesized a metabolic mechanism (Figure 7).[186] According to this hypothesis, persistent DXO treatment leads to the activation of ATF4, thus the upregulation of the serine-linked *mt*OCM pathway.[186] The enhancement of this pathway consequently shifts the glycolytic flux into the *mt*OCM pathway, leading to a reinforced antioxidant defense, which allows to alleviate cell stress and survive cytokine treatment.[186] On the other hand, the activation of the OCM deprives glucose intermediates from the TCA cycle, leading to reduced intracellular ATP levels, thus impairing the GSIS.[186]

Taken together, previous studies suggest that DXO enhances islet cell survival at the expense of secretory function following chronic high-dose treatment. Notably, this treatment correlates with the upregulation of serine-linked *mt*OCM genes, encoding enzymes which potentially mediate the cell-protective effects of DXO. However, the hypothesized metabolic model which includes these enzymes and their specific roles required validation, to assess their importance in islet cell physiology.

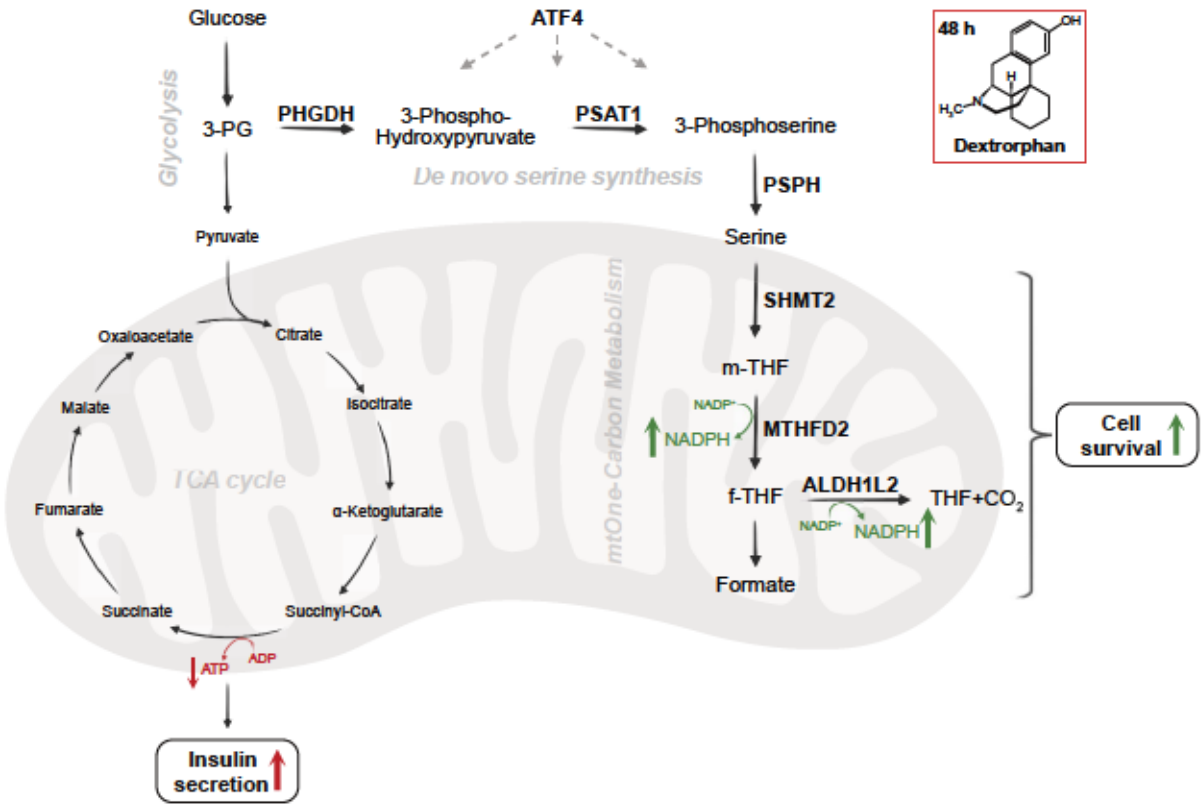

Figure 7: Metabolic mechanism proposed for enhanced beta cell survival at the expense of secretory function.
Schematic model proposal of dextrorphan (DXO) inducted serine-linked mitochondrial (*mt*) OCM at the expense of tricarboxylic acid (TCA) cycle activity. Chronic high-dose DXO treatment activates ATF4 which in turn induces the upregulation of the serine-linked *mt*OCM and the generation of redox equivalents, to enhance cell survival, indicated by the bold arrows This causes the deviation of the glycolytic flux into the upregulated metabolic pathway, depriving glucose intermediated from the TCA cycle, resulting in decreased ATP levels and impaired insulin secretion, indicated by thin arrows. Abbreviations: ATF4: activating transcription factor 4; 3-PG, 3-phosphoglycerate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; NADH/NAD^+, ratio of nicotinamide adenine dinucleotide; NADPH/$NADP^+$, ratio of nicotinamide adenine dinucleotide phosphate; mt, mitochondrial; TCA cycle, tricarboxylic acid cycle; Succinyl-CoA, Succinyl-Coenzyme A; PHGDH, Phosphoglycerate dehydrogenase; PSAT1, Phosphoserine aminotransferase 1; PSPH, Phosphoserine phosphatase; SHMT2, Serine hydroxy methyltransferase 2; MTHFD2, Methylenetetrahydrofolate dehydrogenase 2; ALDH1L2, Aldehyde dehydrogenase 1 family member L2; m-THF, methyl-THF; f-THF, formyl-THF; THF, tetrahydrofolate; CO_2, carbon dioxide. Illustration generated by A. Pelligra, adapted from J. Mrugala (modified) and inspired by Biorender.[186]

3.8 Aim of the study

Diabetes is a global pandemic characterized by a decline in islet cell functionality and viability, with no available preventable or reversal cure. Hence, understanding the mechanisms of islet dysfunction and survival is crucial in diabetes research for developing effective treatments. Recent studies have demonstrated that the NMDAR antagonist DXO promotes islet cell survival under diabetogenic stressors, suggesting its potential as a therapeutic approach for mitigating islet cell demise in diabetes. However, its mechanisms underlying islets cell protection against cell stress induction were not yet understood and remained to be elucidated.

The primary objective of this study consisted in validating or falsifying the previously hypothesized metabolic model, thus investigating the potential causal link between the upregulation of serine-linked *mt*OCM genes and the observed enhanced islet cell survival upon chronic high-dose DXO treatment. To this end, the potential deviation of the glycolytic flux towards the OCM, as well as alterations in mitochondrial NADH/NAD^+, ATP/ADP, and NADPH/$NADP^+$ ratios, induced by DXO, were analyzed.

An additional goal of this study consisted in determining the role of ER stress marker ATF4 and in the manipulation of the potential molecular mechanism underlying islet cell dysfunction and/or enhanced survival. Therefore, gain-of-function (GoF) experiments targeting ***Atf4***, as well as loss-of-function (LoF) experiments additionally targeting serine-linked *mt*OCM genes ***Phgdh***, ***Mthfd2***, and ***Shmt2***, were aimed to be performed.

Since the serine-linked *mt*OCM and its effects were not yet analyzed in primary pancreatic islets, this study finally aimed to scrutinize its metabolic role in islet cell physiology, upon other insulin secreting agents and secretagogues. Therefore, the effects induced by chronic treatment with NMDAR antagonist MK-801, high-palmitate + high-glucose induced glucolipotoxicity, and insulin secretagogues glibenclamide (Glib) and exendin-4 (Ex-4) were analyzed regarding GSIS, viability, and gene expression levels of pancreatic islets.

Overall, this study aimed to contribute to a deeper understanding of islet cell physiology, potentially paving the way for preventive treatments in the pathophysiology of DM.

4 Material and Methods

Most of the methods were used in the study of J. Mrugala and published in Pelligra *et al.*[186,209]

4.1 Mouse Models

All analyses within this study were conducted on pancreatic islets from male C57BL/6J mice (Janvier) aged between 9 to 11 weeks, C57BL/6NTac (Taconic) aged between 9 to 11 weeks, and mice with conditional expression of red fluorescence in beta cells (***Ins1^{Cre}*** Beta-***tdTomato; obtained by intercrossing*** B6(Cg)-***Ins1$^{tm1.1(Cre)Thor}$***/J (available from Jackson Laboratories/JAX, #026801) with B6.Cg-Gt(ROSA)26Sor$^{tm9(CAG-tdTomato)Hze}$/J (JAX #007909))[210-212], at the age of 9 months. All mice were housed at the animal facilities of the Heinrich-Heine-University (HHU) Düsseldorf or the Deutsches Diabetes Zentrum (DDZ) under constant monitoring of housing conditions (22°C, 55% humidity, 12:12 h light:dark cycle) and fed with standard laboratory chow and water ***ad libitum***. All experiments and organ extractions within this study were approved by the local Animal Ethics Committee of the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (LANUV North Rhine-Westphalia, Germany) and conducted in accordance with the German Animal Protection Laws.

4.2 Isolation and pharmacologic pre-treatment of mouse pancreatic islets

All ***in vitro*** experiments conducted in this study were performed on pancreatic islets, isolated from C57BL/6J mice. Pancreatic islets were isolated according to a modified protocol based on Yesil ***et al.***[213] Briefly, after occlusion of the papilla vateri, pancreata were perfused with Dulbecco's Modified Eagle Medium (DMEM) + GlutaMAX (1 g/ml glucose) (Gibco® by Life Technologies) containing LiberaseTM TL Research Grade (Roche) through the bile duct. Immediately after pancreas excision, the enzymatic digestion took place at 37°C for 16.5 min and stopped by adding DMEM containing 15% heat-inactivated Fetal Bovine Serum (FBS, Gibco® by Life Technologies). The pancreas lysate was then washed, filtered through a 40-mesh sieve, and subjected to gradient centrifugation at 1,200x g for 25 min using Lymphoprep (STEMCell Technologies) or Histopaque 1077 (Sigma-Aldrich) and DMEM + GlutaMAX. After centrifugation, the islets were collected from the interphase and transferred into islet medium. The islet medium used in this study was composed of Connaught Medical Research Laboratories (CMRL) 1066 medium, supplemented with following additives:

Table 4.1: Composition of CMRL islet medium

Reagent	Final concentration	Supplier
heat-inactivated FBS	15%	Gibco® by Life Technologies
Penicillin	100 U/ml	Gibco® by Life Technologies
Streptomycin	100 µg/ml	Gibco® by Life Technologies
β-Mercaptoethanol	50 µM	Gibco® by Life Technologies
Sodium Bicarbonate	0.15%	Gibco® by Life Technologies
Glucose	10 mM	Sigma-Aldrich

Finally, the islets were washed twice in islet medium and cultured in a humidified incubator at 37°C and 5% CO_2 overnight before conducting all treatments and functional assays.

Within this study, pancreatic islet cells were treated at 37°C and 5% CO_2 as indicated in the figure legends for 24 – 72 h with different agents, namely 0.1 – 10 µM dextrorphan tartrate (DXO, Sigma-Aldrich, #D127, dissolved in H_2O), 1 µM glibenclamide (Glib, Abcam, #ab120267, dissolved in DMSO), 100 nM exendin-4 (Ex-4, Sigma-Aldrich, E7144), 1 µM Thapsigargin (Thapsi, Sigma Aldrich, #T9033, dissolved in DMSO), 10 µM MK-801 hydrogen maleate (Sigma-Aldrich, #M107), or 500 µM Na-palmitate (Sigma Aldrich, #P9767, dissolved in 10 mM NaOH + 0,1% BSA) coupled to 10% FFA-free BSA (Sigma Aldrich, #A7030) + 25 mM glucose.

4.3 Glucose-stimulated insulin secretion (GSIS) assay

In this study, islet cell functionality was analyzed in un- and pre-treated islets, as indicated in the figure legends. To assess basal and glucose-stimulated insulin secretion (GSIS), an insulin secretion assay was conducted. Prior to stimulation with low and high glucose concentrations, 8 islets were washed twice and starved for 1 h in 2 mM glucose containing Krebs Ringer HEPES (KRH) buffer, whose components are listed in following Table 4.2.

Table 4.2: Composition of KRH buffer

Reagent	Final concentration	Supplier
HEPES	15 mM	Gibco® by Life Technologies
KCl	5 mM	Chemsolute by Th.Geyer
NaCl	120 mM	Carl Roth
$CaCl_2$	2 mM	Sigma-Aldrich
Glycine	10 µM	Sigma-Aldrich
$NaHCO_3$	24 mM	Sigma-Aldrich
Bovine Serum Albumin	0.1%	Sigma-Aldrich
Glucose	2 mM	Sigma-Aldrich

All incubation steps were performed at 37°C and 5% CO_2. For basal and GSIS measurements, the starved islets were incubated for 1 h in KRH buffer containing 2 mM (low glucose) and then for an additional hour in 20 mM (high glucose). After each stimulation, supernatants containing the secreted insulin were collected, and islets were lysed in Radioimmunoprecipitation assay (RIPA) buffer, composed as listed in following Table 4.3, to measure the total islets insulin content. The amount of secreted insulin and islet insulin content was determined using an ultra-sensitive rat insulin ELISA kit (Crystal Chem) following the manufacturer's instructions. Therefore, low glucose supernatants were used undiluted, while high glucose and lysate samples were respectively used at 1:10 and 1:400 dilutions. The colorimetric readout was carried out by using either the Infinite M200 NanoQuant reader (Tecan) or the GloMax® Discover Microplate Reader (Promega).

Table 4.3: Composition of RIPA buffer

Reagent	Final concentration	Supplier
Tris-HCl (pH 7.4)	50 mM	Sigma-Aldrich
Na-deoxycholate	0.25%	AppliChem
IGEPAL	1%	Sigma-Aldrich
NaCl	150 mM	Carl Roth
EDTA	1 mM	Ambion

4.4 Measurement of islet cell viability in pseudo-islets via flow cytometry

For flow cytometric analyses of cell viability, 80 – 100 pseudo-islets per condition were treated for 24 h with high doses of STZ at the concentration of 2.5 mM in a 6-well dish. Afterwards, islet cell media and dispersed islets were transferred to FACS tubes, centrifuged for 5 min at 400x g and washed with phosphate-buffered saline (PBS). Islet cells were then stained with FVS660 (1:1,000 in PBS, BD Biosciences) for 15 min at room temperature (RT) in the dark. After staining, the cells were washed in PBS or fixed with 4% paraformaldehyde (PFA) for 30 min at RT in case of previous adenovirus transduction, and the amount of FVS660-positive (FL4-positive, dead) and FVS660-negative (FL4-negative, living) cells was determined using the FACSCalibur (BD Biosciences) CytoFlexS (Beckmann Coulter). The quantification of cell viability was performed using FlowJo software version 10 (BD Biosciences).

4.5 Measurement of islet cell viability in islets via microscopy

Since previous analyses point to the possibility that DXO improves the antioxidant defense system of pancreatic islet cells, all viability assays within this study were performed upon oxidative stress induction, using the beta cell toxin STZ. Islet cell viability of mouse pancreatic islets and pseudo-islets was examined after 1.5 mM STZ treatment for 24 h using the LIVE-DEAD Viability-Cytotoxicity Kit (Thermo Fisher Scientific). Subsequently to treatment, whole islets were incubated at 37°C and 5% CO_2, protected from light, for 1 h, with a staining solution consisting of 10 µg/ml Hoechst 33342 (DNA stain), 2 µM Calcein AM (live cells), and 4 µM Ethidium homodimer-1 (EthD-1, dead cells) in KRH buffer supplemented with 0.1% BSA (Sigma-Aldrich) and 10 mM glucose (Sigma-Aldrich). This staining solution allowed for the co-staining of cell nuclei, viable cells, and dead cells. Afterwards, z-stack images of the stained islets were captured using a Zeiss ApoTome (Carl Zeiss MicroImaging GmbH) equipped with a Plan-Apochromat 10x/0.45 objective. All acquired images were analyzed using Fiji (ImageJ) image analysis software and cell death was quantified by applying a semi-automated quantification macro. The code for pancreatic islet analyses of cell viability has been deposited on the Zenodo link: https://doi.org/10.5281/zenodo.5820007. The area of dead cells (EthD-1-positive) within each islet was normalized to the total islet cell area (Hoechst-positive).

4.6 Western blot

For the analyses of protein expression levels, a total of 20 – 80 pancreatic islets were collected and washed with ice-cold PBS. The islets were then homogenized in RIPA buffer containing a cocktail of protease inhibitors (Roche). After a 10-minute cell disruption step at 4°C, 4x Laemmli buffer (Bio-Rad) supplemented with 40 mM NaF (Sigma-Aldrich) and 4% β-Mercaptoethanol (Carl Roth) was added to the samples. Subsequently, the samples were boiled for 5-10 min at 95°C. Polypeptides and a protein ladder (Precision Plus Protein Dual Color from Bio-Rad or PageRuler™ Pre-stained from Thermo Fischer Scientific) were separated using 4 – 15% pre-cast Mini-Protean TGX Stain-free Protein Gels (Bio-Rad). The separated proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes using the Trans-Blot® Turbo™ Transfer System (Bio-Rad). The membranes were blocked for 1 h at RT using either 5% bovine serum albumin (BSA) (AppliChem) or 5% non-fat dried milk (Carl Roth), dissolved in PBS with 0.1% Tween-20 (AppliChem). Afterwards, the membranes

were incubated overnight at 4°C with specific rabbit or mouse monoclonal/polyclonal primary antibodies. Following Table 4.4 provides an overview of the primary antibodies used in the study. After the primary antibody incubation, the membranes were washed 3 times with PBS containing 0.1% Tween-20 and incubated for 1 h at RT with an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody. Specifically, donkey anti-rabbit HRP (Jackson ImmunoResearch #711-035-152) at a dilution of 1:1,000, or donkey anti-mouse HRP (Jackson ImmunoResearch #715-035-150) at a dilution of 1:1,000 in 5% BSA or Non-Fat Dried Milk (NFDM), was used as the secondary antibody. For detection, the Clarity Western ECL substrate (Bio-Rad) was used following manufacturer's instructions. The images of the membranes were acquired using the ChemiDoc XRS or ChemiDoc MP Imaging System (Bio-Rad), while the quantification of protein levels was performed using the Image Lab analysis software (Bio-Rad). The protein content in each sample was normalized to the housekeeping protein Beta-actin, or in the case of adenoviral experiments, to GFP expression. All western blot images presented within this study are representatives for the analyses. Fully uncropped and unedited gel images are depicted in Supplement 10.2.

Table 4.4: Primary antibodies used for western blot analyses

	Species	Antibodies	Supplier	Identifier	Dilution	Diluent
Monoclonal	Mouse	anti-Beta-actin	Sigma-Aldrich	Cat#A5441; RRID: AB_476744	1:5,000	BSA
	Mouse	anti-GFP	Roche	Cat#11814460001; RRID: AB_390913	1:1,000	BSA
	Mouse	total OXPHOS rodent antibody cocktail	Abcam	Cat#ab110413; RRID: AB_2629281	1:1,000	NFDM
	Rabbit	anti-ALDH1L2	Thermo FS	Cat#PA5-48161; RRID: AB_2633619	1:1,000	BSA
	Rabbit	anti-ATF4 (D4B8)	Cell signaling	Cat#11815S; RRID: AB_2616025	1:1,000	BSA
Polyclonal	Rabbit	anti-GLUT-2	Santa Cruz	Cat#Sc-9117; RRID: AB_641068	1:750	BSA
	Rabbit	anti-MTHFD2	Thermo FS	Cat#PA5-28169; RRID: AB_2545645	1:1,000	NFDM
	Rabbit	anti-PHGDH	Cell signaling	Cat#13428S; RRID: AB_2750870	1:1,000	BSA
	Rabbit	anti-SHMT2	Thermo FS	Cat#PA5-32228; RRID: AB_2549701	1:1,000	BSA

4.7 GC-MS flux analysis of [U-^{13}C]-labeled metabolites

The metabolic flux analysis was performed as described in Pelligra *et al.*[209] For each condition, 100 – 300 pseudo-islets or pancreatic mouse islets were initially pre-incubated for 48 h in normal islet media or islet media containing 10 mM uniformly labeled [^{13}C]-Glucose ([U-^{13}C]-Glucose) (Sigma, 389374). Subsequently, islets were transferred to the respective islet media with or without supplementation of 10 µM DXO for an additional 1 to 48 h. Afterwards, islet cells were washed three times with ice-cold isotonic NaCl solution (Fresenius Kabi), and metabolites were extracted using the method described by Arrivault *et al.*[214] with minor modifications. Metabolite analysis was conducted using a 7890B gas chromatography system connected to a 7200 QTOF mass spectrometer (Agilent Technologies), as described previously.[215] The MassHunter Qualitative software (v b08, Agilent Technologies) was used for compound identification by comparing mass spectra to an in-house library of authentic standards and to the NIST14 Mass Spectral Library (https://www.nist.gov/srd/nist-standard-reference-database-1a-v14). Peak areas were integrated using MassHunter Quantitative software (v b08, Agilent Technologies) and normalized to the internal standard ribitol (Sigma, A5502). To determine the [U-^{13}C] incorporation, the fragment *m/z* 204 (C_2 fragment) was used for serine, and the fragment *m/z* 273 (C_5 fragment) for citrate. For both fragments, the potential isotopologues were evaluated. The normalized peak areas were corrected for the natural abundance using the R package IsoCorrectoR.[216]

4.8 Ultra-Performance Liquid Chromatography (UPLC)

Quantification of intra-mitochondrial analytes was performed as previously described in Pelligra *et al.*[209] Therefore Ultra-Performance Liquid Chromatographic (UPLC) analyses were performed to determine NADH/NAD$^+$, ATP/ADP, and NADPH/NADP$^+$ ratios. To carry out these analyses, 400 pancreatic mouse islets were pre-treated with or without 10 µM DXO for 48 h. Subsequently, the islets were washed in 1 ml ice-cold 0.9% NaCl solution and fractionated using the Mitochondria/Cytosol Fractionation Kit (Abcam, ab65320). Mitochondrial purine extraction was achieved by lysing the mitochondria in 100 µl ice-cold lysis buffer (22.25% ddH2O, 22.25% chloroform, 55.5% methanol). After a centrifugation step of 5 min, at 21,000x g and 4°C, the supernatant containing mitochondrial analytes was collected, vacuum-dried, and then reconstituted in 50 µl of HBSS (Hanks' Balanced Salt Solution) at RT. UPLC analyses

were performed using the Waters Acquity Ultra Performance Liquid Chromatographic System Bio H class, following the protocol described by Aplak *et al.*[217] For purine analysis, a sample volume of 40 µl was injected onto a Cortecs C18+ UPLC column (3.0x150 mm, 1.6 µM) (Waters Corp.; Milford, MA, USA) using a cooling autosampler. Separation was achieved by employing a linear gradient of buffer A (200 mM KH2PO4/200 mM KCl, pH 6) and buffer B (200 mM KH2PO4/200 mM KCl/7.5% acetonitrile (v/v), pH 6) at a flow rate of 0.340 ml/min, following the following profile: initially 100% A; at 0.03 min, 96% A and 4% B; at 4.53 min, 91% A and 9% B; at 22.63 min, 5% A and 95% B; at 26.10 min, 5% A and 95% B; and at 26.50 min, 100% A. The analytes were detected using a Waters TUV Detector Module at 254 nm. After each run, the column was washed and re-equilibrated using 100% buffer A at a flow rate of 0.34 ml/min for 8 min.

4.9 Gain- and Loss-of-Function experiments in pancreatic pseudo-islet

For Gain-of-function (GoF) and Loss-of-function (LoF) experiments, si-RNA mediated knockdown (KD) or adenovirus-induced overexpression (OE) in pancreatic islets was performed on single-cell suspension of overnight-cultured isolated mouse islets. For this purpose, islets were washed multiple times in Dulbecco's Phosphate Buffered Saline (DPBS, Gibco® by Life Technologies) and digested with 2 ml 0.05% Trypsin-EDTA (Gibco® by Life Technologies) for 5 – 10 min. Once the islets were fully dissociated, the enzymatic digestion was stopped using 3 ml islet medium, and the number of viable cells was determined by staining with trypan blue (0.4%, Gibco® by Life Technologies). Subsequently, the cell suspension was either transduced with adenovirus (see chapter 4.9.1) or transfected with a mixture of si-RNA and lipofectamine (see chapter 4.9.2). Pseudo-islets were generated using the hanging drop method, following the protocol by Cavallari *et al.*[218] with minor modifications. Briefly, single cells were resuspended in islet medium at a density of 1500 cells/30 µl and pipetted onto the lids of petri dishes in 30 µl drops. The lids were inverted and placed on petri dishes containing 25 ml DPBS, and the hanging drops were incubated for 3 days in a humidified incubator at 37°C and 5% CO_2 allow pseudo-islet formation. Afterwards, the pseudo-islets were collected in islet medium and used for functional analyses.

4.9.1 GoF: Adenovirus mediated gene overexpression in pancreatic islet cells

To achieve efficient overexpression of *ATF4* in mouse pancreatic islets, dispersed islet cells were transduced with adenoviruses for 6 h at a multiplicity of infection (MOI) of 200. This process was carried out in a humidified incubator at 37°C and 5% CO_2. The used adenoviruses contained overexpression plasmids with either a *Green Fluorescent Protein* (*GFP*) reporter gene alone (ViraQuest Inc., VQAd CMV eGFP, referred to as Ad-*GFP*), serving as control, or a combination of *GFP* and human *ATF4* (Vector Biolabs, #ADV-201618, referred to as Ad-*ATF4*). According to "BLASTX 2.12.0+ BLAST" tool by Ensemble, which relies on the "Gappes BLAST and PSI-BLAST" method, the protein encoded by the human *ATF4* gene sequence (GenID: 468, RefSeq: BC011994) shares 85% sequence identity with the C57BL/6 mouse protein (Suppl. Fig. 12).[219,220] All genes were under the control of strong cytomegalovirus promoter (P_{CMV}). Following transduction, pseudo-islets were generated using the hanging drop technique as previously described in chapter 4.3. After successful pseudo-islet generation, the pseudo-islets were washed multiple times with fresh islet medium to remove any infectious particles, prior to functional assays. Furthermore, a polymerase chain-reaction (PCR) analysis targeting the adenovirus plasmid construct was conducted, using the supernatant medium to confirm the absence of infectious viral particles.

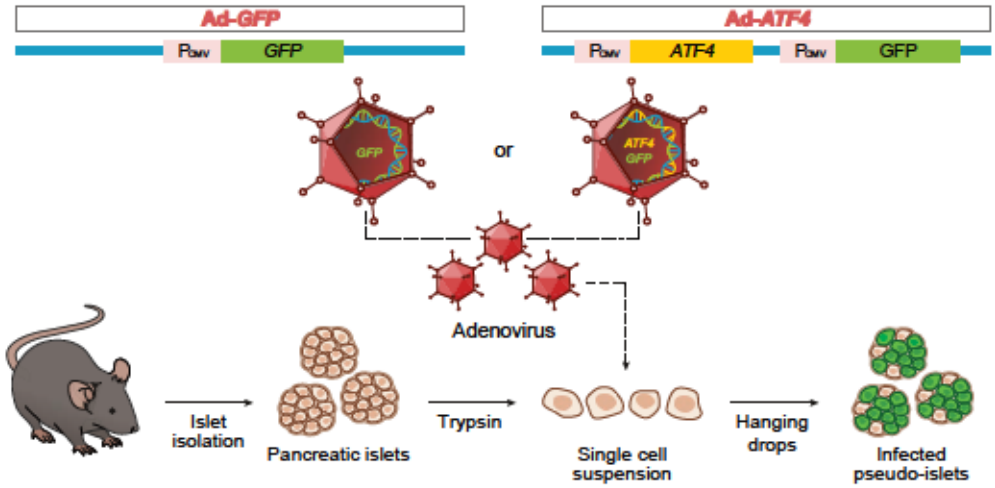

Figure 8: Methodical illustration of adenoviral transduction of disperser pancreatic mouse islet cells.
Single cell suspensions of trypsinized primary islets were infected with adenovirus carrying either a *GFP* control or *ATF4* + *GFP* plasmid construct. The formation of pseudo-islets was achieved by hanging-drop technique. Successful transduced islet cells show protein expression of the GFP fluorescent reporter. Illustration generated by Y. Koh, adapted from J. Mrugala (modified)[186].

4.9.2 LoF: si-RNA-mediated gene knockdown in pancreatic islet cells

Specific gene knockdown (KD) in mouse pancreatic islets was achieved by lipofection. To summarize the procedure, control si-RNA (si-*Ctrl*) or si-RNA targeting specific mouse genes of interest (si-*Gol*) (Horizon Discovery) was mixed with Lipofectamine RNAiMax Transfection Reagent (Thermo Fisher Scientific) and OptiMEM (Gibco® by Life Technologies). The si-RNAs used in this study are listed in following Table 4.5.

Table 4.5: si-RNAs for specific gene KD in pancreatic islet cells

si-RNA-ID	Supplier	Identifier
ON-TARGETplus Mouse *Atf4* si-RNA-SMARTpool	Horizon D.	L-042737-01-0010
ON-TARGETplus Mouse *Mthfd2* si-RNA-SMARTpool	Horizon D.	L-042690-01-0005
ON-TARGETplus Mouse *Phgdh* si-RNA-SMARTpool	Horizon D.	L-045115-00-0010
ON-TARGETplus Mouse *Shmt2* si-RNA-SMARTpool	Horizon D.	L-057586-01-0005
ON-TARGETplus Non-targeting Pool (Control)	Horizon D.	D-001810-10-50

After 5 min of incubation at room temperature (RT), the mixture was added to the islet cell suspension at a final si-RNA concentration of 50 nM and a density of 1500 cells/30 µl. The single cells were then reaggregated into pseudo-islets using the hanging drop technique. After 3 days of formation, pseudo-islets were collected for subsequent treatments and assays.

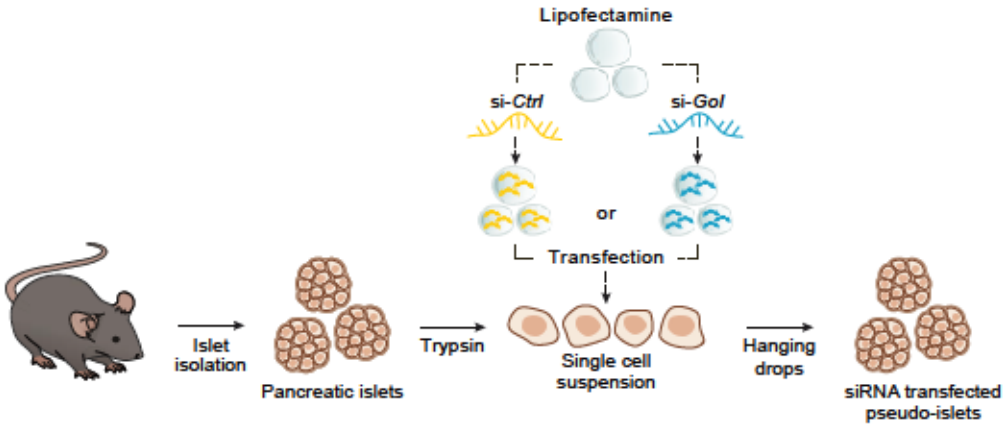

Figure 9: Methodical illustration of si-RNA lipofection of disperser pancreatic mouse islet cells.
Single cell suspensions of trypsinized primary islets were transfected with lipofectamine vehicles containing either si-*Ctrl* or si-*Gol*. The formation of pseudo-islets was achieved by hanging-drop technique. Illustration generated by Y. Koh and A. Pelligra.

4.10 RNA isolation from mouse pancreatic islets

To quantify gene expression levels and perform RNA-seq analyses with mouse pancreatic islets, total RNA was extracted using the RNeasy Mini Kit (Qiagen). 80 – 100 cultured islets were first washed in ice-cold PBS and lysed in RLT lysis buffer (Qiagen) using a cell disrupter for 10 min at 4°C. The lysates were then transferred in RNAeasy columns and washed several times with RW1 buffer (Qiagen) according to manufacturer's instruction. To remove genomic DNA contamination, an on-column DNase digestion was performed using the RNase-Free DNase Set (Qiagen). Finally, the RNA was eluted in 20 µL RNase-free H_2O (Qiagen).

4.11 Reverse transcriptase PCR and quantitative real-time PCR

For the quantification of mRNA levels using quantitative real-time PCR (qPCR), isolated RNA was transcribed into complementary DNA (cDNA). The reverse transcription (RT) step was performed using either the SuperScript® II RT Kit (Invitrogen by Thermo Fisher Scientific) or High-Capacity cDNA RT Kit (Invitrogen by Thermo Fisher Scientific) with 10 µL RNA, according to manufacturer's instructions. The qPCR was carried out by TRIO thermocycler (Biometra Analytic Jena) according to following thermocycler setting profile:

Table 4.6: Thermocycler setting profile for cDNA synthesis

Settings	Step 1	Step 2	Step 3	Step 4
Temperature	25°C	37°C	85°C	4°C
Time	10 min	120 min	5 min	∞

The resulting cDNA samples were then subjected to qPCR analysis, using either the FastStart Essential Green Master-Mix (Roche) or LUNA® Universal (RT)-qPCR reagent (NEB). The oligonucleotide sequences used for qPCR are listed in the following Table 4.7. The qPCR was carried out and analyzed by the LightCycler® Nano Device (Roche) with the LightCycler® Nano software 1.1.2, the QuantStudio 1 system (Applied Biosystems), or the QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems) with the Quantstudio Design Analysis Desktop Software. Finally, expression levels of the gene of interest were calculated relative to the reference housekeeping genes *Beta actin* and *Hprt*, using the comparative C_T method, with the result expressed as $2^{-\Delta CT}$. All data were normalized to their respective control samples.

Table 4.7: Forward and reverse primer sequences, used for qPCR analyses. FP: Forward Primer, RP: Reverse Primer

Gene		Sequence 5' – 3'	Marker for
Mouse *Beta-actin*	FP	*CTA AGG CCA ACC GTG AAA AG*	Housekeeper
	RP	*ACC AGA GGC ATA CAG GGA CA*	
Mouse *Hprt*	FP	*GCT GGT GAA AAG GAC CTC T*	Housekeeper
	RP	*CAC AGG ACT AGA ACA CCT GC*	
Mouse *Atf4*	FP	*GGG TTC TGT CTT CCA CTC CA*	ER stress
	RP	*AAG CAG CAG AGT CAG GCT TTC*	
Human *ATF4*	FP	*CTG TGG ATG GGT TGG TCA GT*	ER stress
	RP	*GGC ATC CAA GTC GAA CTC CT*	
Mouse *Phgdh*	FP	*GCA GGT GGT GGA GAA GCA*	DNSS
	RP	*TCC ACA TTG TCC ACA CCT GT*	
Mouse *Psat1*	FP	*GGA ACG GTG AAC ATT GTC CA*	DNSS
	RP	*CAC AGT CTC GTT TGC ACA GA*	
Mouse *Psph*	FP	*GAA GGA ATC GAT GAG CTG GC*	DNSS
	RP	*ATG CCA GGA GTC AGA TGT GG*	
Mouse *Shmt2*	FP	*TGG CAA GAG ATA CTA CGG AGG*	*mt*OCM
	RP	*AGA TCC GCT TGA CAT CAG ACA*	
Mouse *Mthfd2*	FP	*GAT CCT GTC ACT GCA AAG CC*	*mt*OCM
	RP	*CGG GAG TGA TAT AAC CAG CTT*	
Mouse *Aldh1l2*	FP	*TTG AGG ACT CCA CGG ACT TC*	*mt*OCM
	RP	*ACC ACC ATC TCT GCC TCT TC*	
Mouse *Ins1*	FP	*TCA GAG ACC ATC AGC AAG CA*	Beta cells
	RP	*CCC ACA CAC CAG GTA GAG AG*	
Mouse *Ins2*	FP	*GGA GCG TGG CTT CTT CTA CA*	Beta cells
	RP	*CAG TGC CAA GGT CTG AAG GT*	
Mouse *Slc2a2*	FP	*TCT CAT TGA CTG GAG CCC TC*	Beta cells
	RP	*GAG TGT GGT TGG AGC GAT CT*	
Mouse *MafA*	FP	*ACC ATC ACC ATC ACC ACC AT*	Beta cells
	RP	*TGA CCT CCT CCT TGC TGA AG*	
Mouse *Pdx1*	FP	*CAG TGG GCA GGA GGT GCT TA*	Beta cells
	RP	*AGT TCA ACA TCA CTG CCA*	
Mouse *Nkx6-1*	FP	*ATC TTC TGG CCC GGA GTG AT*	Beta cells
	RP	*AAA GTC TTC TCC AGG GCG AA*	

4.12 Statistical analysis

All statistical analyses were performed with GraphPad Prism 9 software and conducted according to the information provided in the figure legends. Different statistical tests were conducted, depending on the experimental design. For comparisons between two related groups, an unpaired two-tailed student's t-test or a paired two-tailed student's t-test was used. Multiple group conditions were analyzed using one- or two-way ANOVA, followed by Dunnett's, Šidák's, or Tukey's comparison analysis as recommended by Prism 9 software. Unless stated otherwise, data are presented as mean ± standard error of the mean (SEM). Microscopical analyses of cell viability, flux- and UPLC analyses were conducted under blinded conditions. In insulin secretion and cell viability assays, single significant outliers (p-value ≤ 0.05) identified by Grubbs' test for outliers were excluded. For both methods, only experiments showing either significant GSIS and DXO exhaustion, or cell death induction in control treatments were included in the analyses.

4.13 Personal contributions

A. Pelligra, J. Mrugala and K. Griess were primarily responsible for conducting and designing most of the experiments. A. Pelligra was supervised by E. Lammert.

J. Mrugala initiated the research on the DXO-mediated protective mechanism and instructed A. Pelligra with common pancreatic islet-methodologies in December 2018. The project was subsequently handed over to A. Pelligra in January 2020. Both performed and prepared all samples serving for the glycolytic flux analyses.

J. Mrugala performed all RNA-seq analyses in collaboration with the Functional Genomics Center in Zurich (FGCZ, ETH Zurich, Switzerland), which also conducted the statistical analyses. The procedures followed the protocol published in J. Mrugala's study and in Pelligra *et al.*[186,209]

A. Pelligra prepared all samples for the UPLC analyses and established si-RNA-mediated gene-KD on primary islet cells with pseudo-islet generation, with the help of O. Nortmann. A. Pelligra and K. Griess performed all si-RNA-mediated gene-KD, with the help of O. Nortmann and P. Kirschner.

K. Griess performed the adenoviral overexpression experiments, established the flow cytometric analyses and conducted most of the related experiments. P. Kirschner was also introduced to this technique and conducted some flow experiments as well. All adenoviral experiments and flow cytometric analyses were carried out at the DDZ.

All other experiments, including insulin secretion assays, microscopic viability assays, qPCR, and western blot analyses were performed by A. Pelligra, K. Griess, J. Mrugala, O. Nortmann, and P. Kirschner, as indicated in the figure legends.

In collaboration, P. Westhoff (Institute of Plant Biochemistry and Cluster of Excellence on Plant Science (CEPLAS), HHU, Düsseldorf, Germany) conducted the GC-MS flux analyses for the quantification of incorporated [U-^{13}C]. B. Steckel (Department of Molecular Cardiology, HHU, Düsseldorf, Germany) performed all UPLC analyses. P. Westhoff and B. Steckel respectively contributed to writing the experimental procedure sections "GC-MS flux analysis of [U-^{13}C]-labeled metabolites" and "Ultra-Performance Liquid Chromatography (UPLC)."

A. Pelligra created most figures or partially modified elements from original figures, as indicated in the legends. Y. Koh created Figure 8 and parts of Figure 9, guided by A. Pelligra's ideas and design.

5 Results

Most of the here presented results were published in Pelligra *et al.*[209]

Studies have demonstrated that chronic high-dose treatment (48 h) with DXO and its pro-drug DXM, enhances cell survival in the presence of cytokine treatment and oxidative stress.[184-186,189] In particular, J. Mrugala provided evidence that DXO exerts time dependent effects, ranging from 1 – 48 h of treatment, which correlated with the activation of the *mt*OCM.[186] Within this study, these findings were extended by elucidating the role of ATF4 and the serine-linked *mt*OCM in the regulation of pancreatic islet functionality and survival. Further, the effects induced by DXO were also investigated for other chronic treatments such as NMDAR antagonist MK-801, glucolipotoxicity induced by high-palmitate + high-glucose, and other insulin secretagogues such as Glib and Ex-4. These findings provide new valuable insights into the complex regulation of pancreatic islet functionality and survival, and might have implications for the development of novel therapies for diabetes.

5.1 Chronic high-dose DXO treatment enhances beta cell survival against STZ-induced cell death at the expense of secretory function

To investigate time-dependent effects of DXO, pancreatic islets were exposed to high doses (10 µM) of DXO for acute (1 h) and chronic (48 h) treatments. The islets were then analyzed for GSIS and cell survival in response to STZ, a commonly used pharmacological beta cell toxin for inducing oxidative stress and insulin-dependent diabetes mellitus.[127,221] Consistent with previous reports, acute DXO treatment enhanced GSIS at high glucose concentrations and depleted the insulin content (Figure 10A – B).[184-186] However, this DXO pre-treatment did not provide cell protection against STZ-induced cell death, as indicated by the Ethidium homodimer-1 (EthD-1, dead cells) staining (Figure 10C – D). In contrast, chronic exposure of islets to 10 µM DXO induced opposite effects, namely drastically decreased GSIS at high glucose concentrations (Figure 10E), a severe depletion of insulin content (Figure 10F), and full protection of pancreatic islets from STZ-induced cell death (Figure 10G – H). These results demonstrate that the effects of DXO on pancreatic islet cell functionality and survival are depended on the duration of exposure time, hence acute or chronic treatment.

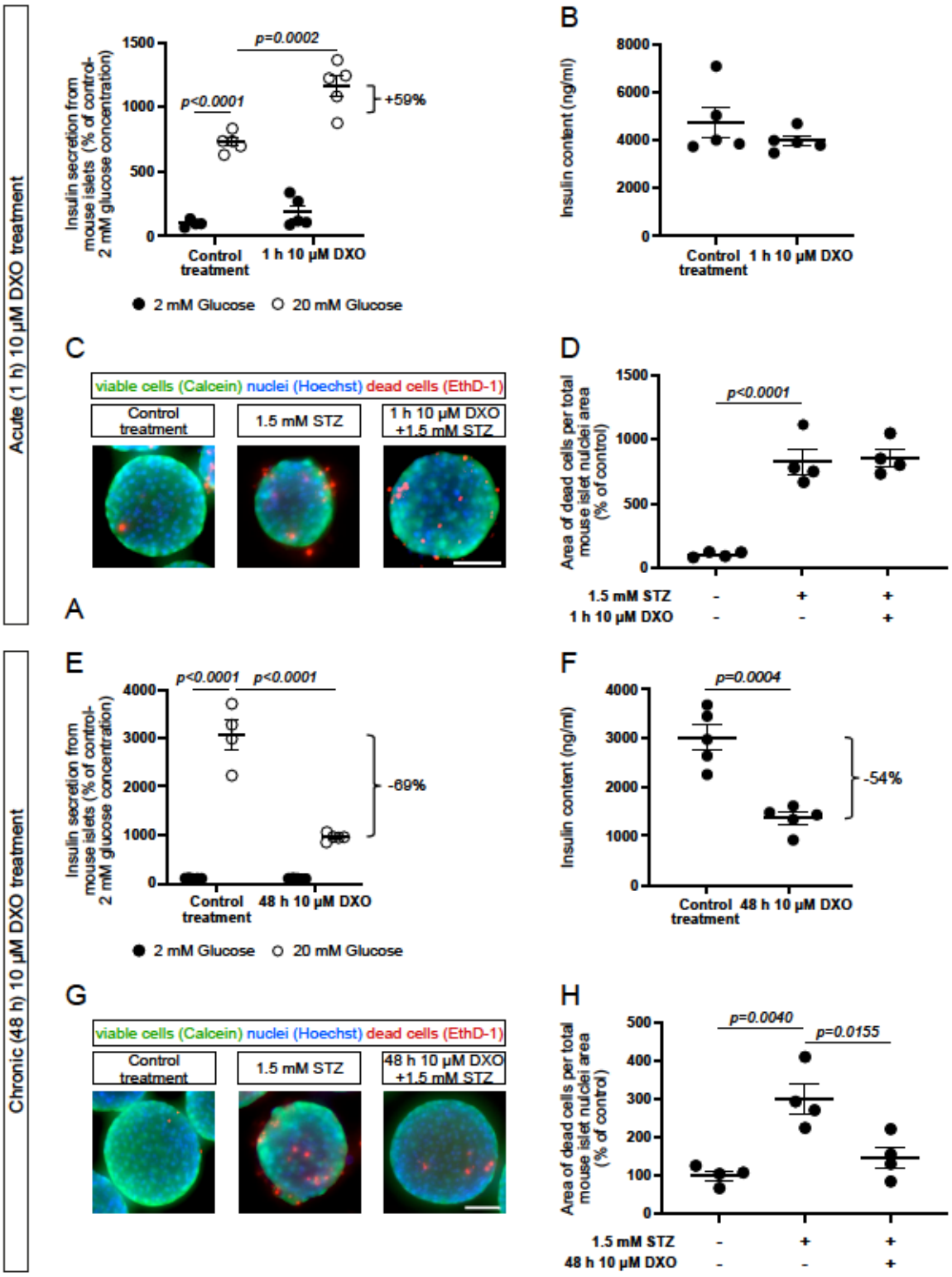

Figure 10: Acute (1 h) DXO treatment enhances insulin secretion, while chronic (48 h) DXO induces islet dysfunction but protects against STZ-induced cell death. A, GSIS and **B**, Insulin content of untreated and 1 h pre-treated pancreatic mouse islets with 10 µM DXO. Insulin secretion was measured at 2 mM and 20 mM glucose (n = 5 wells). **C**, Representative microscopy images and **D**, Quantification of untreated and 24 h 1.5 mM STZ treated pancreatic mouse islets in absence or presence of 10 µM DXO for 1 h (n = 4 stainings). **E**, GSIS and **F**, Insulin content of untreated and 48 h pre-treated pancreatic mouse islets with 10 µM DXO. Insulin secretion was measured at 2 mM and 20 mM glucose (n = 5 wells). **G**, Representative microscopy images and **H**, Quantification of untreated and 24 h 1.5 mM STZ treated pancreatic mouse islets in absence or presence of 10 µM DXO for 48 h (n = 4 stainings). Data are shown as mean ± SEM with corresponding p-values and calculated as percentage of control. Statistical significance was determined by two-way ANOVA followed by Tukey's multiple comparison test (A + E), two-tailed unpaired student's t-test (B + F), and one-way ANOVA followed by Dunnett's multiple comparison test (D + H). Scale bar: 50 µm. All experiments were conducted by A. Pelligra.

The protective effects of DXO on pancreatic islet cells were further confirmed through flow cytometric analysis using fixable viability stain (FSV660) (Figure 11). Consistent with previously shown data (see Figure 10), treatment with DXO for 48 h resulted in a significant reduction of high-dose STZ-induced cell death in pancreatic islet cells, decreasing from 33.6% to 10.8% (Figure 11A). Since pancreatic islets are composed of various endocrine cell types with beta cells being of primary importance in metabolic disorders such as diabetes, it was of particular interest to investigate the protective effects of DXO on this specific cell type.[2,3] Notably, flow cytometric analysis of *tdTomato*-expressing red fluorescent beta cells revealed that beta cells were susceptible to STZ-induced cell death, with cell survival decreasing from 59.9% in control to 41.9% after high-dose STZ treatment (Figure 11B). However, 48 h of DXO treatment provided complete protection of beta cells against the diabetogenic beta cell toxin.

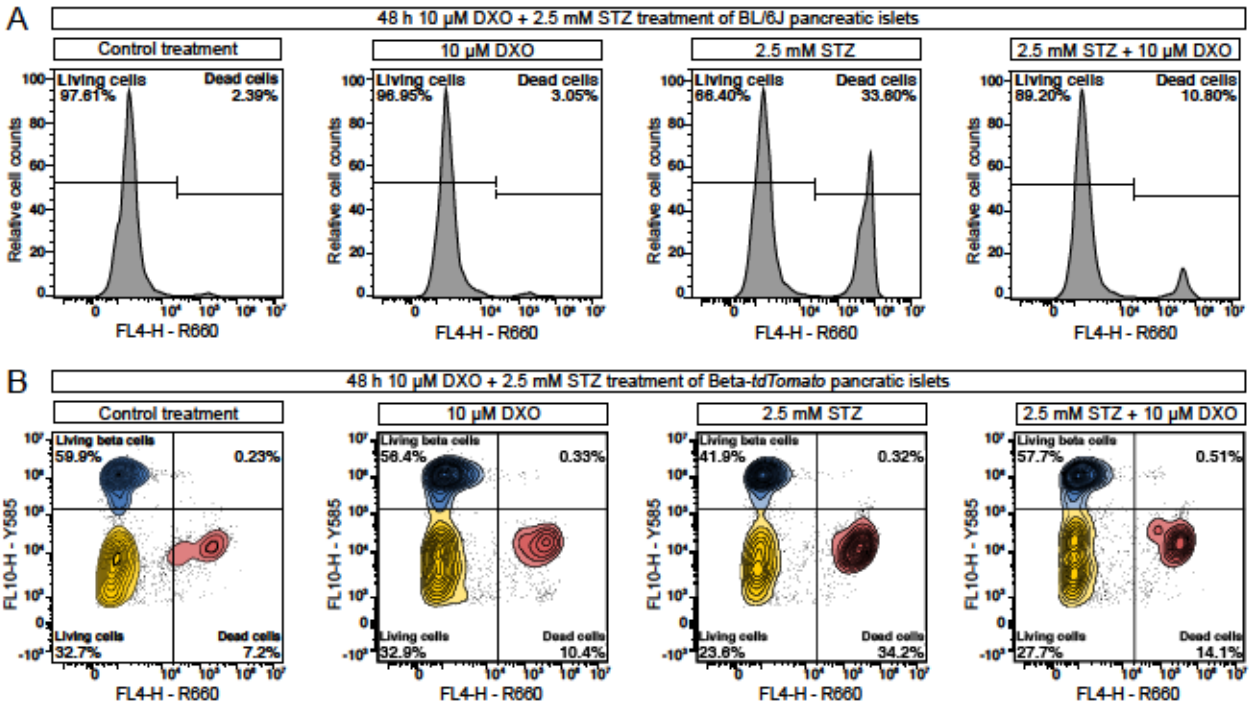

Figure 11: Flow cytometric analyses show that DXO protects pancreatic beta cells from STZ-induced cell death.
A, Representative histograms of untreated and 24 h 2.5 mM STZ treated pancreatic mouse islets, in absence or presence of 10 µM DXO for 48 h. FL4-H negative depict living and FL4-H positive dead cell populations. **B**, Representative contour plots of untreated and 24 h 2.5 mM STZ treated *Ins1*^{Cre} Beta tdTomato expressing islet cells, in absence or presence of 10 µM DXO for 48 h. Blue population (FL4-H negative + FL10-H positive) represent living beta cells, yellow population (FL4-H negative + FL10-H negative) represent living non-beta cells, and red population (FL4-H positive + FL10-H positive) represent dead islet cells. Cell viability was analyzed using FVS660. Pancreatic islet isolations were performed by A. Pelligra, while flow cytometric analyses were performed by K. Griess.

5.2 Chronic and high-dose DXO treatment activates the serine-linked *mt*OCM

To elucidate the molecular mechanisms underlying DXO-mediated islet cell protection at the expense of secretory function, bulk RNA-seq analyses data of J. Mrugala's study were utilized.[186] In support of previous findings collected by J. Mrugala 24 h mouse islet treatment with 10 µM DXO revealed several differentially expressed genes, including those coding for ER stress marker ATF4 and the serine-linked *mt*OCM enzymes.[186] Interestingly, treatment with the insulin secretagogue also enhanced other ER stress marker genes, which are associated with the ATF4 activating UPR branch, such as *DNA damage-inducible transcript 3* (*Ddit3* encoding CHOP) and *Heat shock protein family A (Hsp70) member 5* (*Hspa5* encoding BiP) (Figure 12A). Protein expression analyses further confirmed the upregulation of ATF4 in pancreatic islets after 48 h of DXO treatment (Figure 12B – C), suggesting that DXO-induced activation of the ATF4-associated UPR might play a critical role in protecting pancreatic islet cells from STZ-induced cell death. Additionally, the RNA-seq analysis identified genes related to the *de novo* serine synthesis (*DNSS*) pathway (Figure 12D), which were also validated at protein level through the expression of Phgdh after 48 h of DXO treatment (Figure 12E – F). Among all OCM genes, only *Cth*, encoding a cytosolic enzyme, was upregulated while the other significantly upregulated genes *Shmt2*, *Mthfd2*, and *Aldh1l2* encode for mitochondrial OCM enzymes. The upregulation of the mitochondrial *Aldh1l2* gene was also confirmed on protein level in pancreatic mouse islets treated with DXO for 48 h (Figure 12H – I).

Notably, RNA-seq data revealed the downregulation of beta cell marker genes (Figure 12J), including *Ins1* and *Ins2*, encoding for insulin, *Solute carrier family 2 member 2* (*Slc2a2*), encoding the glucose transporter 2 (GLUT-2), and the genes *MAF bZIP transcription factor A* (*MafA*) and *Pancreatic and Duodenal Homeobox-1* (*Pdx1*) encoding transcription factors. However, downregulation of beta cell marker gene *Slc2a2* was not consistent at protein levels, shown by GLUT-2 transporter expression after 48 h of DXO treatment (Figure 12K – L).

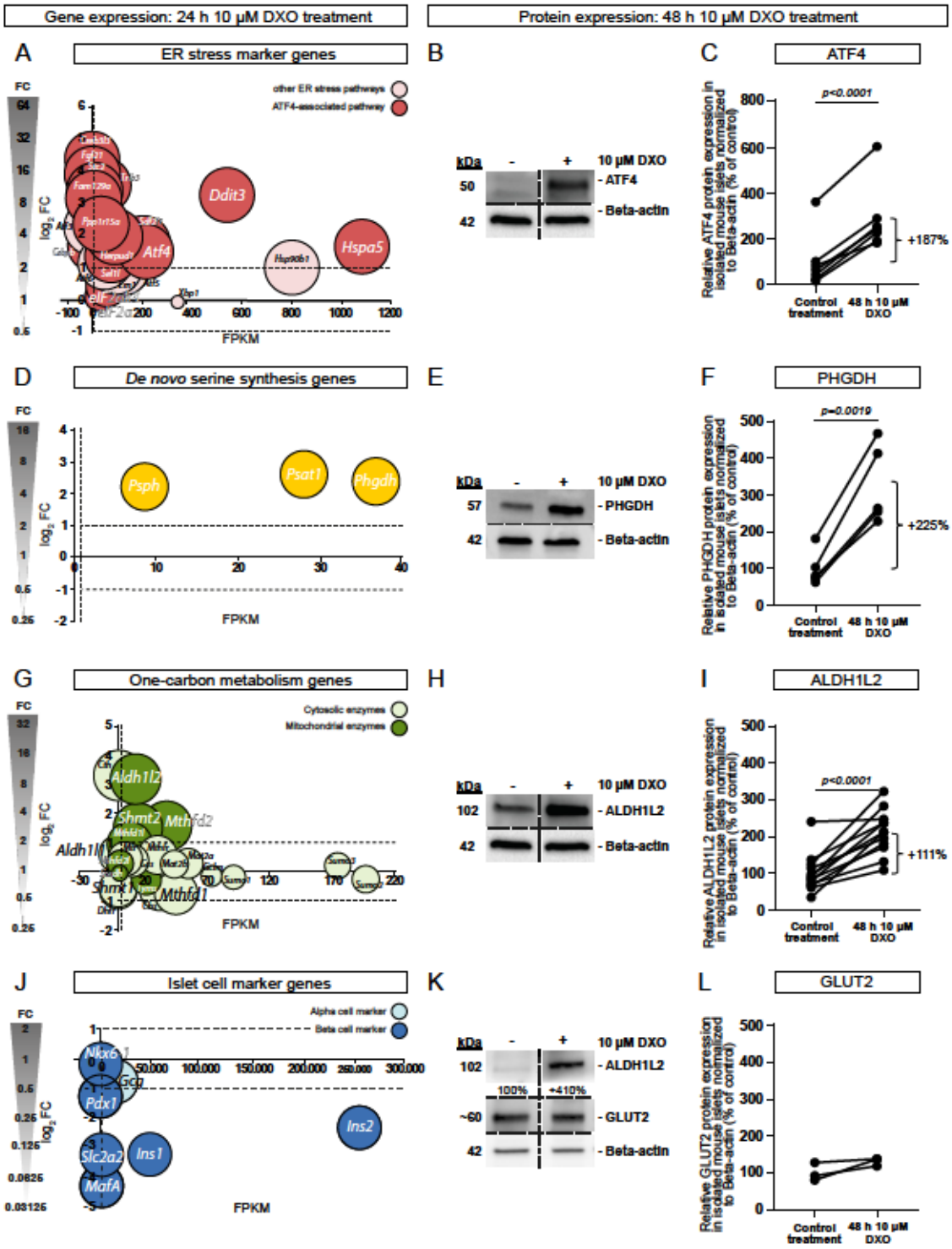

Figure 12: Chronic DXO treatment induces the ATF4-associated pathway and genes of serine-linked *mt*OCM.
A, Transcriptome analysis of ER stress marker genes. **B**, Representative ATF4 immunoblot of mouse pancreatic islets treated with 10 µM DXO for 48 h and **C**, Quantification (n = 7 independent experiments). **D**, Transcriptome analysis of genes encoding for enzymes of *de novo* serine synthesis. **E**, Representative PHGDH immunoblot of mouse islets treated with 10 µM DXO for 48 h and **F**, Quantification (n = 5 independent experiments). **G**, Transcriptome analysis of genes encoding for cytosolic and mitochondria enzymes of the OCM. **H**, Representative ALDH1L2 immunoblot of mouse islets treated with 10 µM DXO for 48 h **I**, Quantification (n = 12 independent experiments). **J**, Transcriptome analysis of islet cell marker genes. **K**, Representative GLUT-2 immunoblot of mouse pancreatic islets treated with 10 µM DXO for 48 h. **L**, Quantification (n = 3 independent experiments). Protein expression was normalized to Beta actin. Graphs show log_2 Fold Change (FC), Fragments Per Kilobase Million (FPKM), and p-values of genes based on RNA-seq data. Dashed lines: cut-off of the FPKM and the log_2 FC. Gradients on the left indicate the FC. Genes of the ATF4-associated branch and the folate cycle in A and G are highlighted by larger font size. All quantifications of blots are shown as percentage of control with corresponding p-values. Significance was determined by two-tailed paired student's t-test (D, G, J). RNA-seq data (A, D, G, J) were collected by J. Mrugala, who together with K. Griess and A. Pelligra (B, H, and K) contributed in protein expression analyses (C, E, F, and I).

To investigate whether the upregulation of serine-linked *mt*OCM genes depends on the DXO treatment duration and/or on the DXO concentration, transcriptome- and qPCR analyses of pancreatic islets were conducted on the one hand with acute, and on the other hand with chronic DXO treatments, at different concentrations (Figure 13). For this purpose, an RNA-seq analysis was conducted with islets, previously treated with 10 µM DXO for 1 h (Figure 13A – D). In contrast to previously shown effects of chronic high-dose DXO treatment (see Figure 12A, D, G, J), acute DXO pre-treatment had minimal or no substantial effects on mRNA levels (Figure 13A – D). However, *Ddit3*, *Herpud1*, *Fgf21* and *Trib3* (Figure 13A) were significantly upregulated also following 1 h DXO treatment. Since these genes are required for the activation of ATF4, their early upregulation might suggest a relationship between the upregulation of this ER stress branch and the effects observed with chronic insulin secretagogue treatment.[222]

To analyze whether chronic DXO treatment induces serine-linked *mt*OCM and islet dedifferentiation also at lower concentrations, expression levels of respective genes were quantified in islets treated with 0, 0.1, 1, and 10 µM DXO for 48 h (Figure 13E – M). Notably, all analyzed genes were highly significantly regulated at the concentration of 10 µM DXO, whereas only few genes, namely, *Atf4*, *Psat1*, *Shmt2*, and *Aldh1l2*, showed significant upregulation at the concentration of 1 µM DXO (Figure 13A, G, I, K). Overall, these results provide strong evidence that the upregulation of *Atf4*, genes of the serine-linked *mt*OCM, and the downregulation of islet cell marker genes are dependent on both, the duration and concentration of DXO treatment.

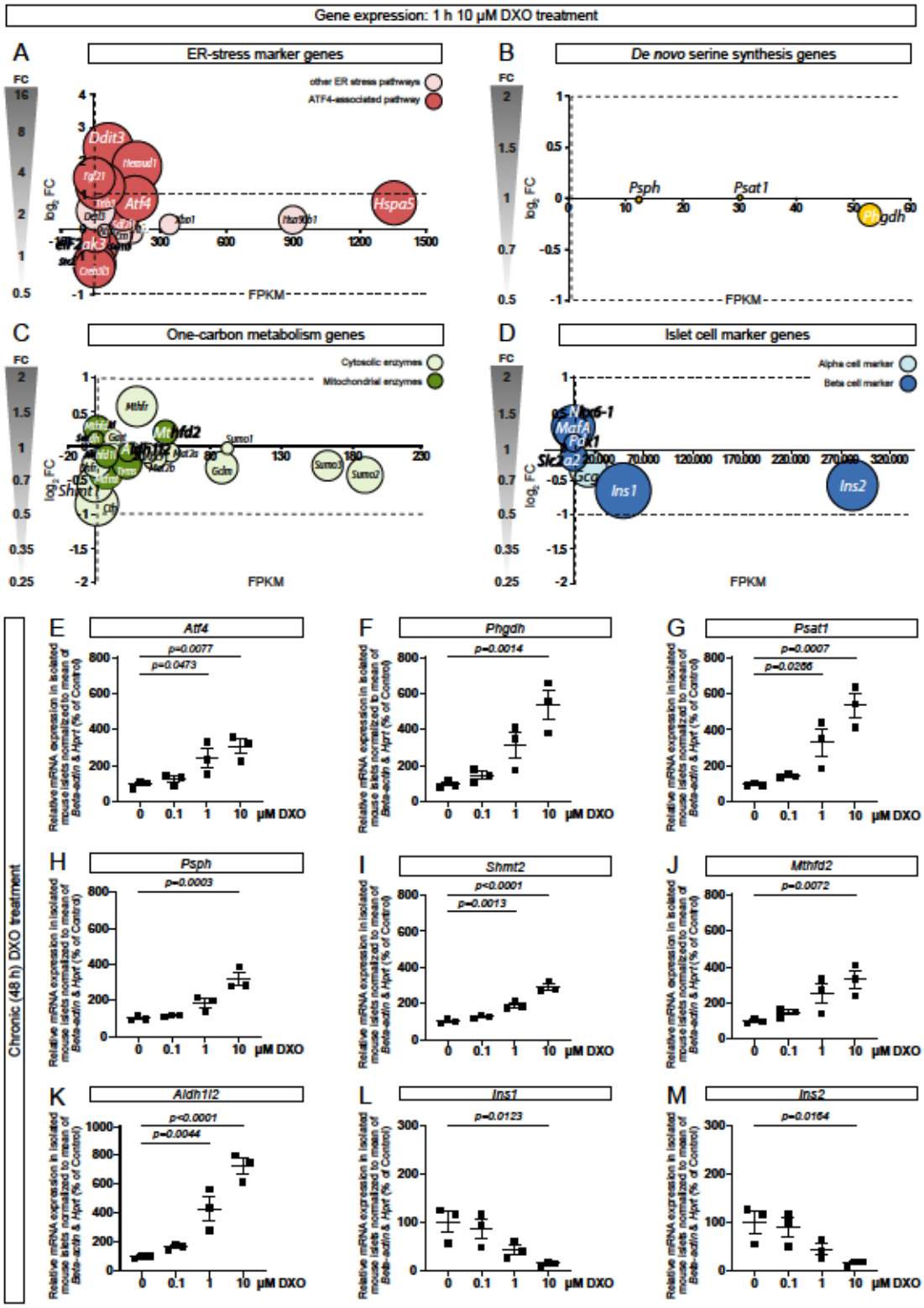

Figure 13: Acute low-dose (1 h 10 µM) and chronic low-dose (48 h ≤ 1 µM) DXO treatment does not induce serine-linked *mt*OCM genes. **A**, Transcriptome analysis of ER stress marker genes, **B**, De novo serine synthesis genes, **C**, OCM genes, and **D**, Islet cell marker genes, after 1 h 10 µM DXO treatment of pancreatic mouse islets. Graphs show log_2 FC, FPKM and p-values of genes based on RNA-seq data. Horizontal axes show FPKM values (Fragments Per Kilobase Million), vertical axes show log_2 FC. Dashed lines: cut-off of the FPKM and the log_2 FC. Gradients on the left indicate the FC. Genes of the ATF4-associated branch and the folate cycle are highlighted by larger font size. **E**, Gene expression levels of *Atf4*, serine-linked *mt*OCM genes **F**, *Phgdh*, **G**, *Psat1*, **H**, *Psph*, **I**, *Shmt2*, **J**, *Mthfd2*, **K**, *Aldh1l2*, and islet cell marker genes **L**, *Ins1* and **M**, *Ins2* after 48 h treatment with 0, 0.1, 1 and 10 µM DXO (n = 3 independent experiments). Data are shown as mean ± SEM with corresponding p-values and calculated as percentage of control. Statistical significance was determined by one-way ANOVA, followed by Dunnett's multiple comparison test (E – M). RNA-seq data were collected by J. Mrugala (A – D), while mRNA expression analyses were performed by K. Griess (E – M).

5.3 Chronic DXO treatment deviates glucose flux into serine-linked OCM

Based on the proposed model shown in Figure 7, chronic high-dose DXO leads to a shift of glycolytic flux towards the serine synthesis pathway into the OCM. To validate this model, isotope tracing experiments were performed on pancreatic islets following acute (1 h, Figure 14A + B) and chronic (48 h, Figure 14C – D) treatment with 10 µM DXO. Specifically, the flux of [U-^{13}C]-labeled glucose was analyzed by measuring the incorporation of [U-^{13}C]-units into citrate and serine as intermediates of the TCA cycle and the ***de novo*** serine synthesis, respectively. The isotope tracing experiments revealed no significant alterations in the incorporation of [U-^{13}C]-units into citrate and serine after 1 h of DXO treatment (Figure 14A + B). Therefore, acute DXO treatment did not deviate the glucose flux in pancreatic islet cells. In contrast, 48 h of DXO treatment led to less incorporation of [U-^{13}C]-units from glucose into citrate across all isotopologues (M+1 to M+5), while levels of unlabeled citrate (M+0) increased (Figure 14C). On the other hand, a higher incorporation of [U-^{13}C]-units from glucose into serine (M+1 and M+2), and decreased levels of unlabeled serine (M+0) were measured after 48 h of DXO treatment (Figure 14D).

To gain further evidences supporting this hypothesized model, ultra-performance liquid chromatography (UPLC) was additionally performed to quantify the levels of NADH/NAD$^+$, ATP/ADP and NADPH/NADP$^+$ ratios in mitochondrial lysates of pancreatic islet cells treated with DXO for 48 h (Figure 14E – G). Consistent with the hypothesized model in Figure 7, mitochondrial NADH/NAD$^+$ ratios (Figure 14E) and ATP/ADP ratios (Figure 14F), as representatives of TCA cycle activity, were significantly decreased by chronic DXO treatment. In contrast, NADPH/NADP$^+$ ratios (Figure 14G) were increased following chronic high-dose DXO treatment, indicating the activation of serine-linked OCM.

Taken together, these results not only suggest that chronic high-dose DXO treatment influences the glucose metabolism which plays a critical role in islet secretory function, but also enhances the serine-linked OCM.

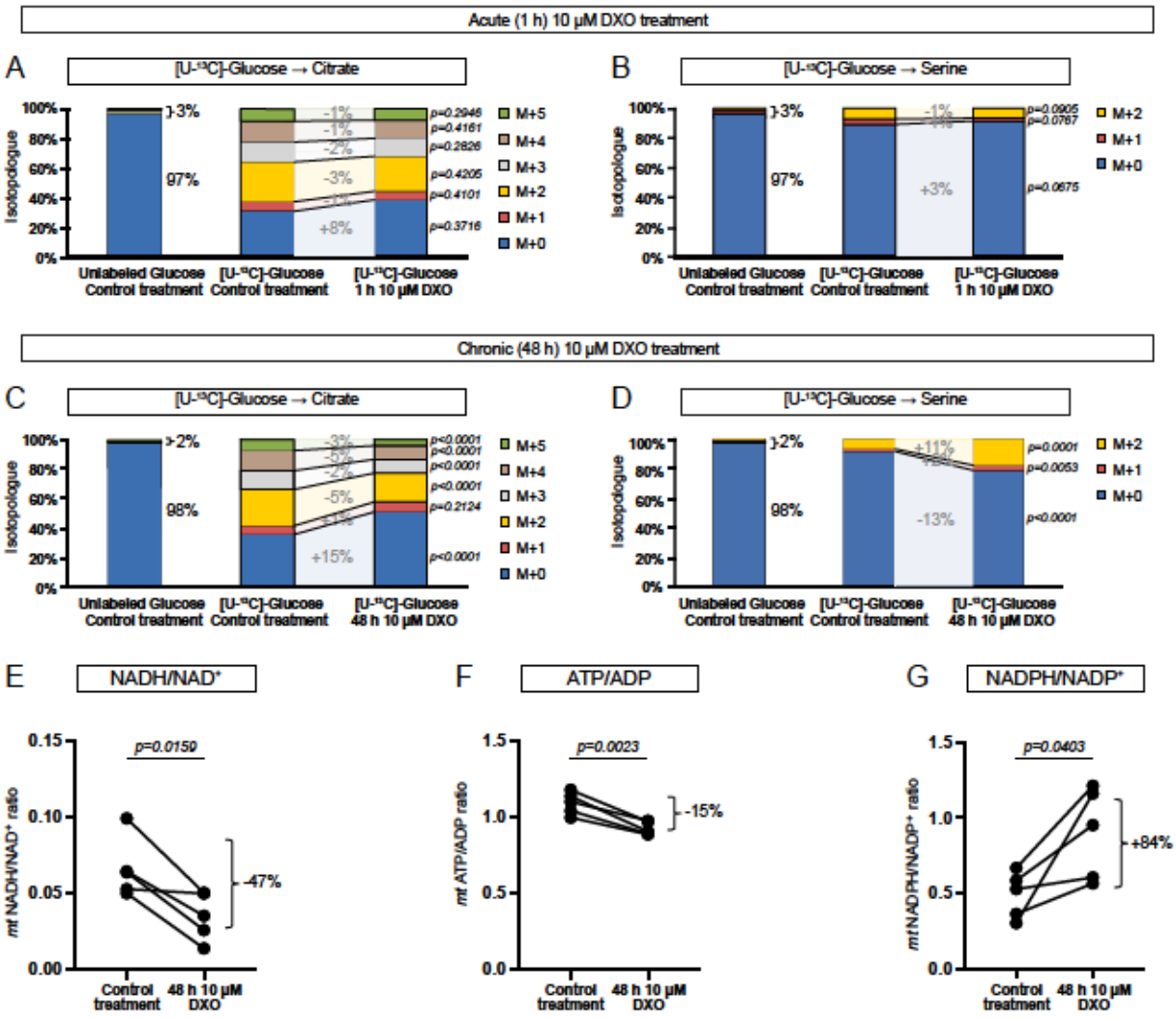

Figure 14: Chronic (48 h) high-dose DXO treatment deviates the glycolytic flux from TCA cycle into the serine-linked OCM. **A – D**, Metabolic tracing of [U-^{13}C]-Units in mouse islets treated for 1 h (A + B) or 48 h (C +D) with or without the insulin secretagogue DXO. Measurement of [U-^{13}C] incorporation into the TCA cycle intermediate citrate (A + C) and the *de novo* serine synthesis product serine (B + D) (n = 3 (A + B) and n = 4 (C + D) independent experiments). **E**, Mitochondrial NADH/NAD$^+$, **F**, ATP/ADP and **G**, NADPH/NADP$^+$ ratios in pancreatic islets treated with 10 µM DXO for 48 h (n = 5 independent experiments). All quantifications are shown as percent of control and with corresponding p-values. Significance was determined by two-tailed unpaired (A – D) and two-tailed paired student's t-test (E – G). Treatments and sample collection for flux analyses upon 1 h and UPLC analyses upon 48 h DXO treatments were performed by A. Pelligra (A, B, E – G). Treatments and sample collection for flux analyses upon 48 h DXO treatment were performed by J. Mrugala (C + D). Flux analyses were performed and evaluated by P. Westhoff (AG Weber) (A – D), while B. Steckel (AG Schrader) performed the UPLC analyses (E – G).

5.4 Chronic DXO effects are reversible after 24 – 48 h washout

To investigate potential relationships between DXO-induced islet dysfunction and pancreatic cell protection, washout experiments were conducted. In these experiments, pancreatic islets which were pre-treated with 10 µM DXO for 48 h, for the induction of islet dysfunction (as shown in Figure 10) were transferred to regular islet media in absence of the insulin secretagogue for an additional 24 – 48 h (Figure 15A). The insulin secretory capacity, which was severely reduced after 72 h of DXO treatment, significantly improved following the washout period, even though the insulin content was not restored (Figure 15B + C). Notably, removal of DXO also caused the vanishing of DXO-mediated islet cell protection (Figure 15D + E). These results indicate an inverse correlation between islet secretory function and islet cell survival.

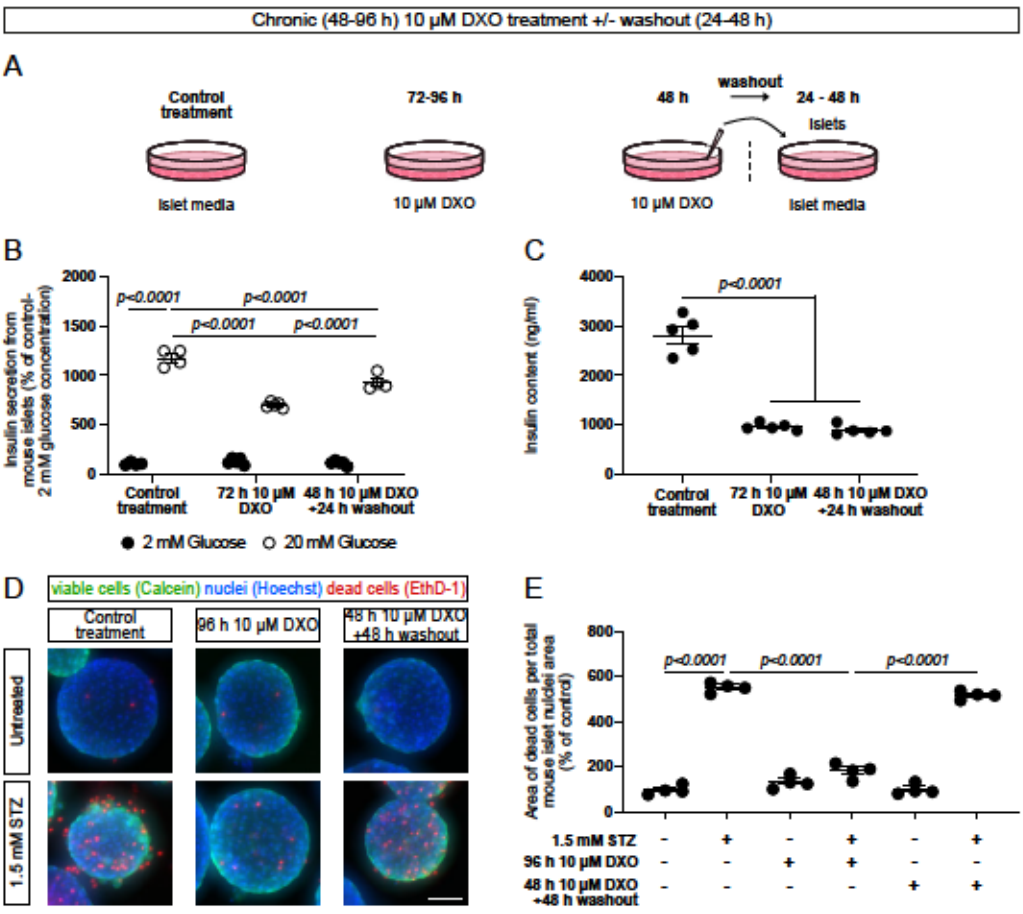

Figure 15: DXO-induced islet dysfunction and cell protection are reversible after 24 – 48 h washout.
A, Schematic illustration of experimental set up of DXO treatment and washout. **B**, GSIS and **C**, Insulin content of untreated, 72 h continuous DXO pre-treated and 24 h washout pancreatic mouse islets after 48 h 10 µM DXO pre-treatment. Insulin secretion was measured at 2 mM and 20 mM glucose (n = 5 wells). **D**, Representative microscopy images and **E**, Quantification of untreated and 24 h 1.5 mM STZ treated pancreatic mouse islets in absence or presence of 10 µM DXO for 72 h or 48 h + 24 h washout (n = 4 staining). Data are shown as mean ± SEM with corresponding p-values and calculated as percentage of control. Statistical significance was determined by two-way ANOVA followed by Tukey's multiple comparison test (A), two-tailed unpaired student's t-test (B), and one-way ANOVA followed by Tukey's multiple comparison test (E). Scale bar: 50 µm. GSIS assay was performed by A. Pelligra (B – C), while K. Griess performed the viability assay.

To gather further evidence whether islet cell functionality inversely correlates with cell protection, relevant protein and gene targets were quantified in Figure 16. After 72 h of 10 µM DXO treatment, protein expression levels of ALDH1L2 and ATF4 significantly increased, but partially restored to basal levels after a 24 h DXO washout period (Figure 16A). Similarly, mRNA levels of ***Atf4*** and serine-linked ***mt***OCM also showed a substantial decrease after the 24 h washout, returning to normal levels (Figure 16B – H). However, gene expression levels of ***Ins1*** and ***Ins2*** were not restored after washout, while ***Slc2a2*** mRNA levels fully recovered.

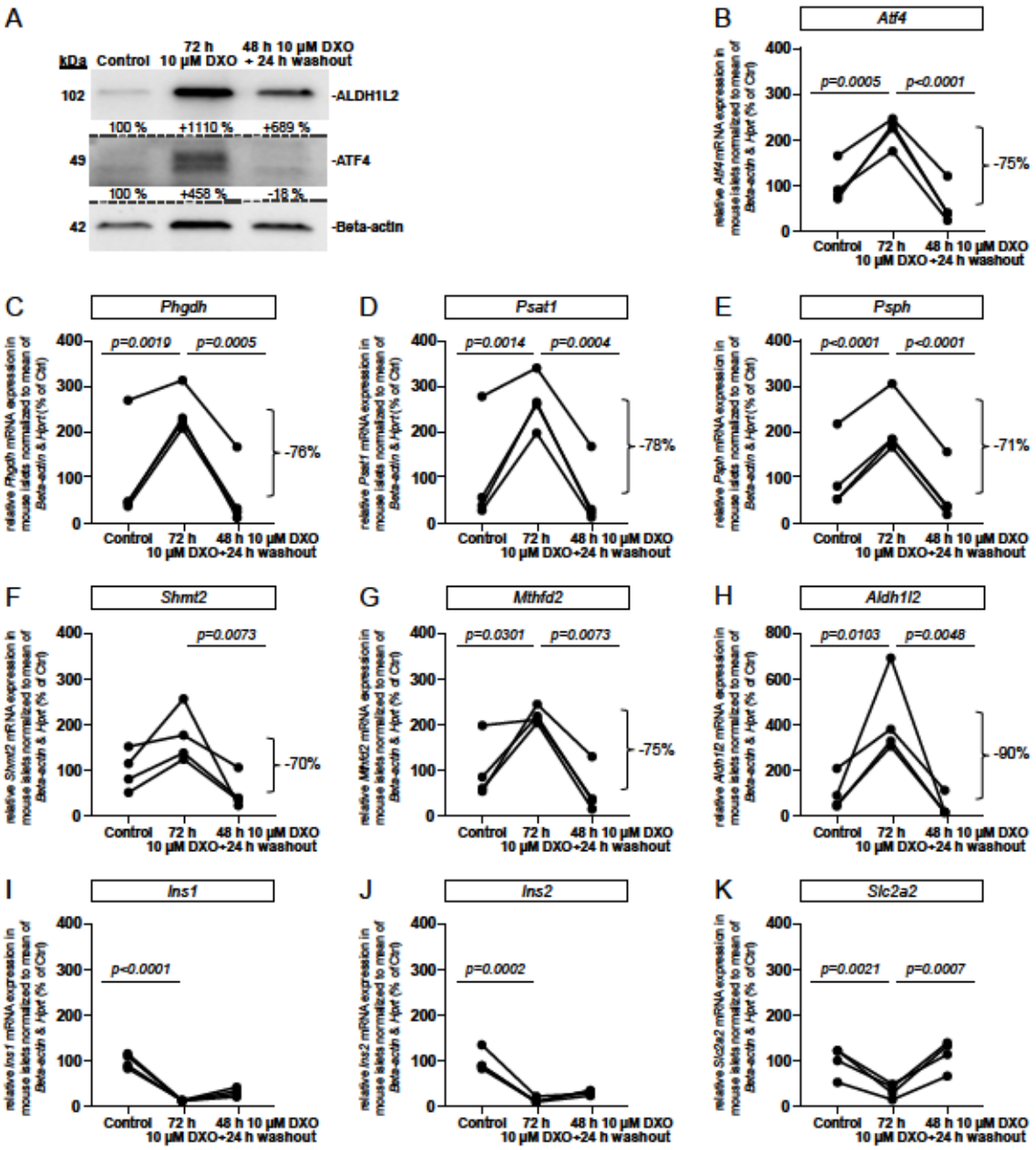

Figure 16: DXO-induced serine-linked *mt*OCM gene expression is reversible after 24 h washout.
A, Representative ALDH1L2 and ATF4 immunoblot of mouse pancreatic islets treated with 10 µM DXO for 72 h and 48 h + 24 h washout and quantification. Protein expression was normalized to Beta-actin. **B**, Gene expression levels of *Atf4*, serine-linked *mt*OCM genes **C**, *Phgdh*, **D**, *Psat1*, **E**, *Psph*, **F**, *Shmt2*, **G**, *Mthfd2*, **H**, *Aldh1l2* after 72 h, and beta cell marker genes **I**, *Ins1*, **J**, *Ins2*, and **K**, *Slc2a2* after 48 h DXO + 24 h washout (n = 4 independent experiments). Data are calculated as percentage of control with corresponding p-values. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparison test. Western blot was performed by J. Mrugala (A), while expression data were collected by A. Pelligra (B – K).

5.5 Molecular manipulation of the serine-linked *mt*OCM by GoF and LoF experiments

As demonstrated in previous figures, robust evidence was collected, indicating that ***Atf4*** and serine-linked *mt*OCM genes were highly upregulated after chronic high-dose DXO treatment in pancreatic islets. To investigate their roles in mediating the observed effects of DXO, GoF and LoF experiments of ***Atf4*** were conducted in dispersed pancreatic islet cells, which were subsequently reassembled to form pseudo-islets. Moreover, pancreatic islet cells were also transfected with si-RNA targeting serine-linked *mt*OCM genes ***Phgdh***, ***Shmt2***, and ***Mthfd2*** genes singularly, as well as simultaneously resulting in the generation of pseudo-islets, carrying an individual or triple gene-KD.

5.5.1 ATF4 induces serine-linked *mt*OCM genes and islet exhaustion

ER stress marker ATF4 was overexpressed in pseudo-islets by infecting islet cell suspension with a recombinant adenovirus, carrying the human ***ATF4*** gene, along with ***GFP*** as reporter (Ad-***ATF4***). In contrast, respective control pseudo-islets were generated by single islet cell infection with adenovirus, carrying ***GFP*** alone (Ad-***GFP***) (Figure 17A). Protein and mRNA levels of ***ATF4*** revealed a successful and robust overexpression efficiency in pseudo-islets (Figure 17B + C). Furthermore, expression levels of serine synthesis and *mt*OCM genes were all found to be significantly upregulated in Ad-***ATF4*** pseudo-islets (see Figure 17D – I).

Similar to chronic high-dose DXO treatment, the insulin secretion assay conducted with Ad-***GFP*** and Ad-***ATF4*** infected pseudo-islets showed a decreased GSIS (Figure 17J). However, ***ATF4*** overexpressing pseudo-islets exhibited enhanced insulin content compared to control pseudo-islets (Figure 17K). To investigate whether ATF4 mediates islet cell protection against STZ cell death, cell viability was quantified using flow cytometry (Figure 17L + M). Brightfield images of untreated and STZ-treated Ad-***GFP*** and Ad-***ATF4*** pseudo-islets (Figure 17L) indicate no major changes in viability of untreated pseudo-islets. However, high doses of STZ induced a stronger cell death in Ad-***ATF4*** infected pseudo-islets (94.72%) than in Ad-***GFP*** infected control islets (80.85%) (Figure 17M).

These results indicate that ATF4 expression mimics the DXO-induced upregulation of serine-linked *mt*OCM genes and the DXO-induced islet secretory dysfunction. However, it does not replicate the islet cell protective effect observed with DXO treatment.

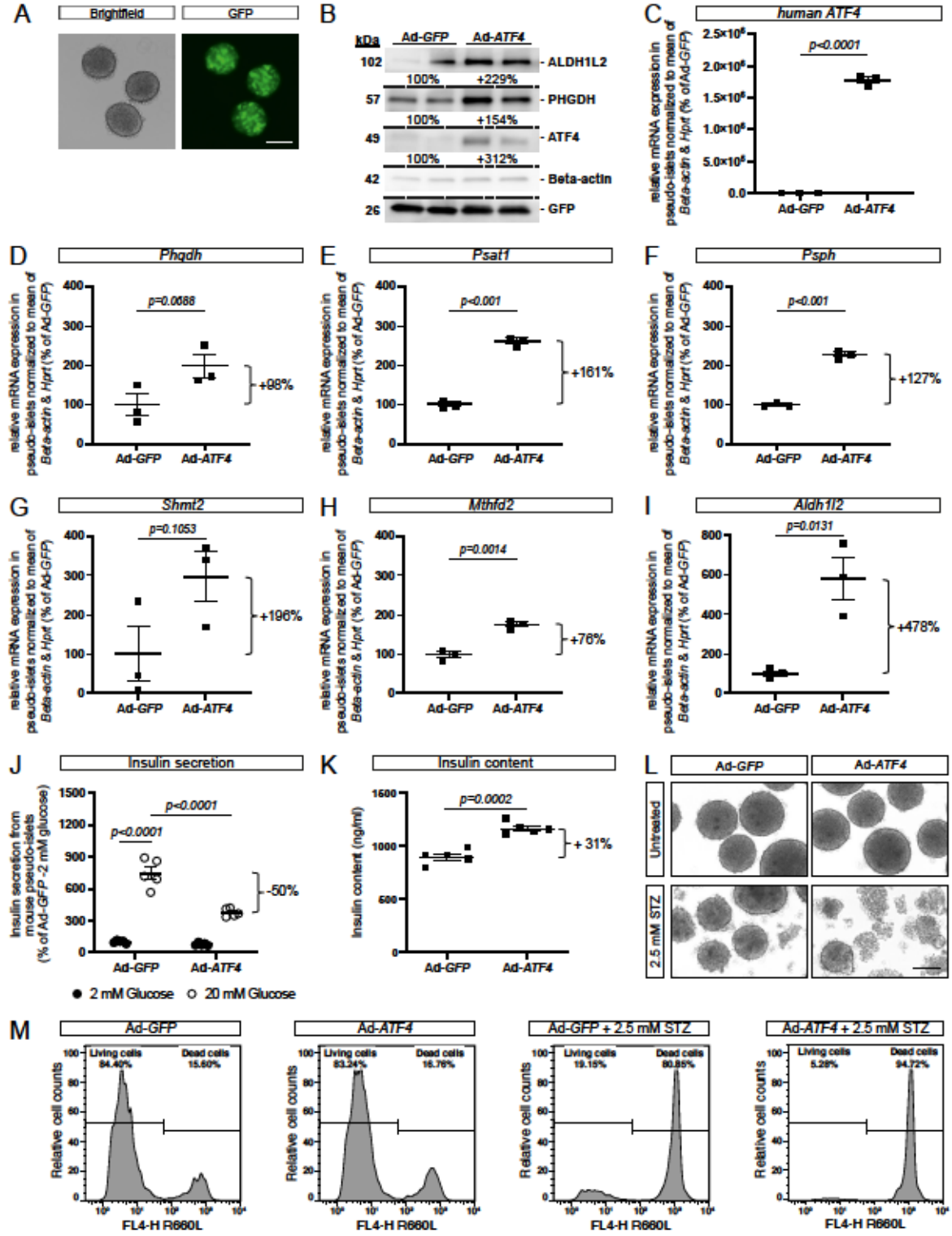

Figure 17: Overexpression of *ATF4* enhances expression of serine-linked *mt*OCM genes and induces islet dysfunction. **A**, Representative images of Ad-GFP transfected mouse pseudo-islets. Green fluorescence: GFP expressing cells. **B**, protein expression of ALDH1L2, PHGDH and ATF4 in Ad-*GFP* and Ad-*ATF4* transfected pseudo-islets and quantification. Protein expression was normalized to Beta-actin. **C**, Gene expression levels of human *ATF4* and serine-linked *mt*OCM genes **D**, *Phgdh*, **E**, *Psat1*, **F**, *Psph*, **G**, *Shmt2*, **H**, *Mthfd2*, and **I**, *Aldh1l2* in Ad-*GFP* and Ad-*ATF4* transfected pseudo-islets (n = 3 technical replicates). **J**, GSIS and **K**, Insulin content of Ad-*GFP* and Ad-*ATF4* transfected mouse pseudo- islets. Insulin secretion was measured at 2 mM and 20 mM glucose (n = 5 wells). **L**, Representative images of untreated and 24 h 2.5 mM STZ treated Ad-*GFP* and Ad-*ATF4* transfected mouse pseudo-islets and **M**, Representative histograms. FL4-H negative depict living and FL4-H positive dead cell populations. Cell viability was analyzed using FVS660. Data are shown as mean ± SEM with corresponding p-values and calculated as percentage of control. Statistical significance was determined by two-tailed unpaired student's t-test (C – I, and K) and two-way ANOVA followed by Tukey's multiple comparison test (J). Scale bar 100 µm. All data analyses were performed by K. Griess (E).

5.5.2 ATF4 activates serine-linked *mt*OCM genes and regulates GSIS, glycolytic flux, and islet cell survival against STZ-induced cell death

To investigate whether *Atf4* silencing could attenuate the effects induced by chronic DXO treatment LoF experiments were conducted. Dispersed primary islet cells were subjected to si-RNA-mediated *Atf4*-KD and reaggregated for the generation of pseudo-islets (Figure 18A). The KD efficiency was evaluated by measuring basal protein expression levels of ATF4 and ALDH1L2, showing a pronounced reduction in lysates of pseudo-islets treated with the ER stress inducer thapsigargin (Thapsi) from +622% to -19% compared to untreated si-*Ctrl* transfected pseudo-islets (Figure 18B). On mRNA level, the *Atf4*-KD efficiency reached -84% (Figure 18C). Further, transcription levels of serine-linked *mt*OCM genes were analyzed in untreated (basal) and 48 h 10 µM DXO treated si-*Ctrl* and si-*Atf4* transfected pseudo-islets (Figure 18D – I). Notably, the upregulation of target genes nearly vanished, despite chronic insulin secretagogue treatment. The functional effects of *Atf4*-KD in pancreatic islets were assessed through GSIS analysis with and without chronic DXO pre-treatment (Figure 18J – K). Interestingly, *Atf4* suppression strongly enhanced GSIS in untreated pseudo-islets (Figure 18J), while depleting insulin content (Figure 18K). Although 48 h DXO pre-treated pseudo-islets exhibited a substantial decrease in GSIS in both si-*Ctrl* and si-*Atf4* transfected groups, the KD of *Atf4* resulted in a slight enhancement of insulin secretion by 19% in DXO pre-treated pseudo-islets (Figure 18J). Finally, the effects of si-*Atf4* transfection were also analyzed concerning DXO-induced cell protection against STZ treatment (Figure 18L – M). While the basal and STZ-induced cell death rates were comparable between si-*Ctrl* and si-*Atf4* transfected pseudo-islets, the DXO-induced islet cell survival against STZ was partially but significantly attenuated after *Atf4*-KD.

Taken together, these results demonstrate on the one hand that ATF4 is required for induction of serine-linked *mt*OCM genes in pancreatic islets and for the regulation of islet secretory function, and on the other hand that it contributes to DXO conferred islet cell protection against STZ-induced stress.

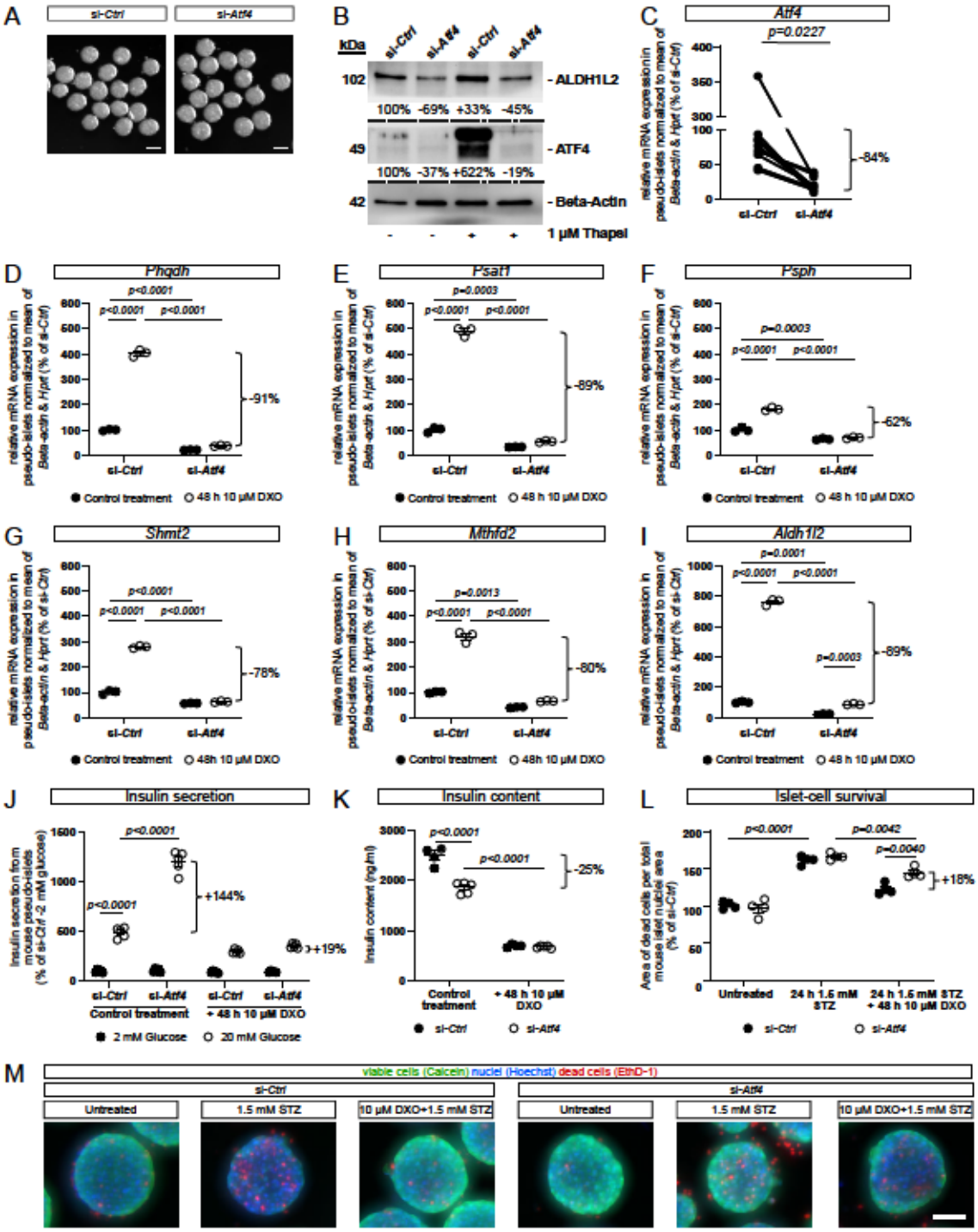

Figure 18: Knockdown of *Atf4* attenuates DXO-induced expression of serine-linked *mt*OCM genes, enhances insulin secretion and reduces DXO-induced islet cell protection. A, Representative images of si-*Ctrl* and si-*Atf4* transfected mouse pseudo-islets. **B**, protein expression of ALDH1l2 and ATF4 untreated and 24 h 1 µM thapsigargin (Thapsi) treated si-*Ctrl* and si-*Atf4* transfected mouse pseudo-islets and quantification. Protein expression was normalized to Beta-actin. **C**, Gene expression levels of *Atf4* and serine-linked *mt*OCM genes **D**, *Phgdh*, **E**, *Psat1*, **F**, *Psph*, **G**, *Shmt2*, **H**, *Mthfd2*, and **I**, *Aldh1l2* in untreated and 48 h 10 µM DXO treated si-*Ctrl* and si-*Atf4* transfected mouse pseudo-islets (n = 3 technical replicates). **J**, GSIS and **K**, Insulin content of untreated and 48 h 10 µM DXO treated si-*Ctrl* and si-*Atf4* transfected mouse pseudo-islets. Insulin secretion was measured at 2 mM and 20 mM glucose (n = 5 wells). **L**, Quantification of cell death in untreated and 24 h 1.5 mM STZ treated pancreatic mouse islets in absence or presence of 10 µM DXO for 48 h (n = 4 stainings) and **M**, Representative microscopy images. Data are shown as mean ± SEM with corresponding p-values and calculated as percentage of control. Statistical significance was determined by two-tailed paired student's t-test (C), two-way ANOVA followed by Tukey's multiple comparison test (D – K), and one-way ANOVA followed by Dunnett's multiple comparison test (D + H). Scale bar: 100 µm (A) and 50 µm (M). Representative images of A, gene expression data of C and insulin secretion assay of J + K were collected by A. Pelligra. Western blot, gene expression analysis and cell survival data were performed by K. Griess (B – I, L+M). O. Nortmann contributed to gene expression data shown in C.

Previously shown data within this study demonstrate that ATF4 is sufficient to induce the upregulation of serine-linked *mt*OCM genes, thereby mediating their expression, and regulating both, islet secretory function and survival upon cell stress, in response to DXO treatment. These data indicate that ATF4 plays a fundamental role not only in DXO-mediated effects, but also in basal islet cell physiology. To investigate the crucial role of ATF4 in determining islet cell fate, its influence in regulating glucose flux towards the serine-linked pathway was analyzed by performing isotope tracing experiments, using untreated and 48 h 10 µM DXO-treated si-*Ctrl* and si-*Atf4* transfected pseudo-islets (Figure 19A + B). The quantified [U-^{13}C]-units incorporation into citrate revealed no difference between si-*Ctrl* and si-*Atf4* transfected pseudo-islets under untreated condition (Figure 19A). However, 48 h of DXO treatment, enhanced incorporation of [U-^{13}C]-units into higher citrate isotopologues (M+3 – M+5) in si-*Atf4* transfected pseudo-islets, accompanied by decreased levels of unlabeled citrate (M+0) (Figure 19A). Conversely, si-*Atf4* transfected pseudo-islets exhibited significantly increased levels of unlabeled serine in untreated conditions compared to si-*Ctrl* transfected pseudo-islets (Figure 19B). This effect was even more pronounced after 48 h of DXO treatment, which led to significantly less incorporation of [U-^{13}C]-units into higher serine isotopologues (M+1 – M+2). Considering that ATF4 was previously shown to mediate DXO-induced gene expression of serine-linked *mt*OCM enzymes (Figure 18D – I), expression levels of beta cell marker genes such as *Slc2a2*, *Ins 1*, and *Ins2*, were as well analyzed after 48 h of DXO treatment (Figure 19C – H). In contrast to the serine-linked *mt*OCM genes, the downregulation of beta cell marker genes induced by DXO was not attenuated in si-*Atf4* transfected pseudo-islets.

In summary, ATF4 mediates the shift in glycolytic flux towards serine synthesis induced by chronic high-dose DXO treatment, but does not mediate the DXO-induced beta cell dedifferentiation.

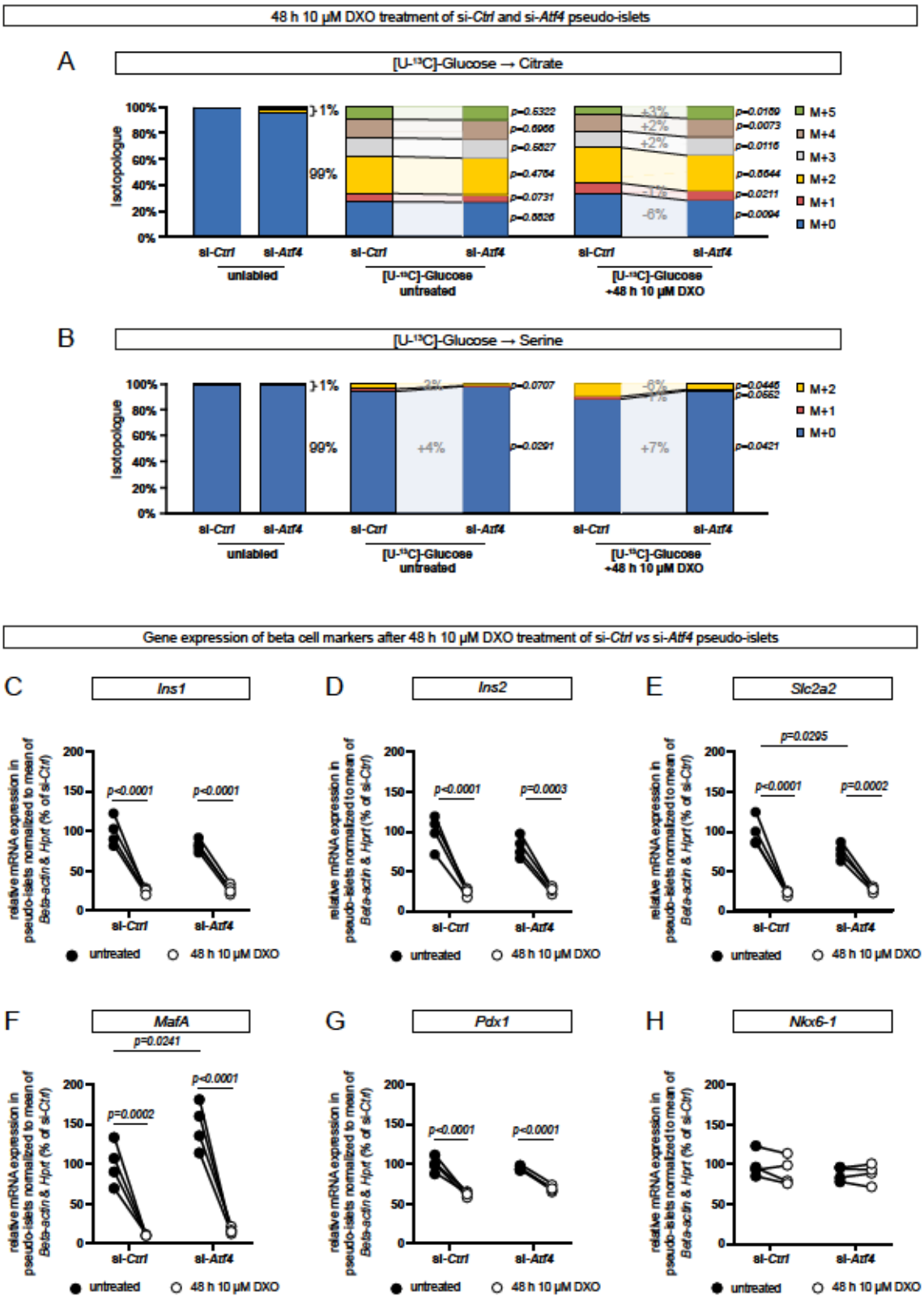

Figure 19: Knockdown of *Atf4* attenuates DXO-induced glucose flux into serine but not its dedifferentiating effects.
A, Metabolic tracing and incorporation into the TCA cycle intermediate citrate and **B**, the *de novo* serine synthesis product serine of untreated and 48 h 10 µM DXO treated si-*Ctrl* and si-*Atf4* transfected mouse pseudo-islets (n = 3 independent experiments). **C**, Gene expression levels of beta cell marker genes *Ins1* **D**, *Ins2*, **E**, *Slc2a2*, **F**, *MafA*, **G**, *Pdx1*, and **H**, *Nkx6-1*, in untreated and 48 h 10 µM DXO treated si-*Ctrl* and si-*Atf4* transfected mouse pseudo-islets (n = 4 independent experiments). All quantifications are shown as percent of control and with corresponding p-values. Significance was determined by two-tailed unpaired student's t-test (A + B) and two-way ANOVA followed by Tukey's multiple comparison test (C – H). Treatments and sample collection for flux analyses were performed by A. Pelligra (A + B), while flux analyses were performed and evaluated by P. Westhoff (AG Weber) (A + B). Gene expression analyses were performed by K. Griess (C – H).

5.5.3 KD of *Phgdh*, *Shmt2*, and *Mthfd2* enhance GSIS, while their simultaneous KD reduces islet cell survival

Besides ATF4, genes encoding for serine-linked *mt*OCM enzymes, were as well analyzed for potential involvement in islet functionality and DXO-mediated cell protection. In specific, *Phgdh*, *Shmt2*, and *Mthfd2* were individually targeted by si-RNA transfection, resulting in robust KD efficiencies of -75 to -84% at protein expression levels (Figure 20A, D, G). Although, GSIS levels were respectively enhanced by +44%, +25%, and +54% in si-*Phgdh*, si-*Shmt2*, and si-*Mthfd2* transfected pseudo-islets (Figure 20B, E, and H), neither the DXO-induced islet dysfunction nor the insulin content (Suppl. Fig. 11A – B) were rescued by the performed KDs. Additionally, none of the KDs affected the viability or the DXO-induced cell protection of pseudo-islets against STZ-induced cell death (Figure 20C, F, I).

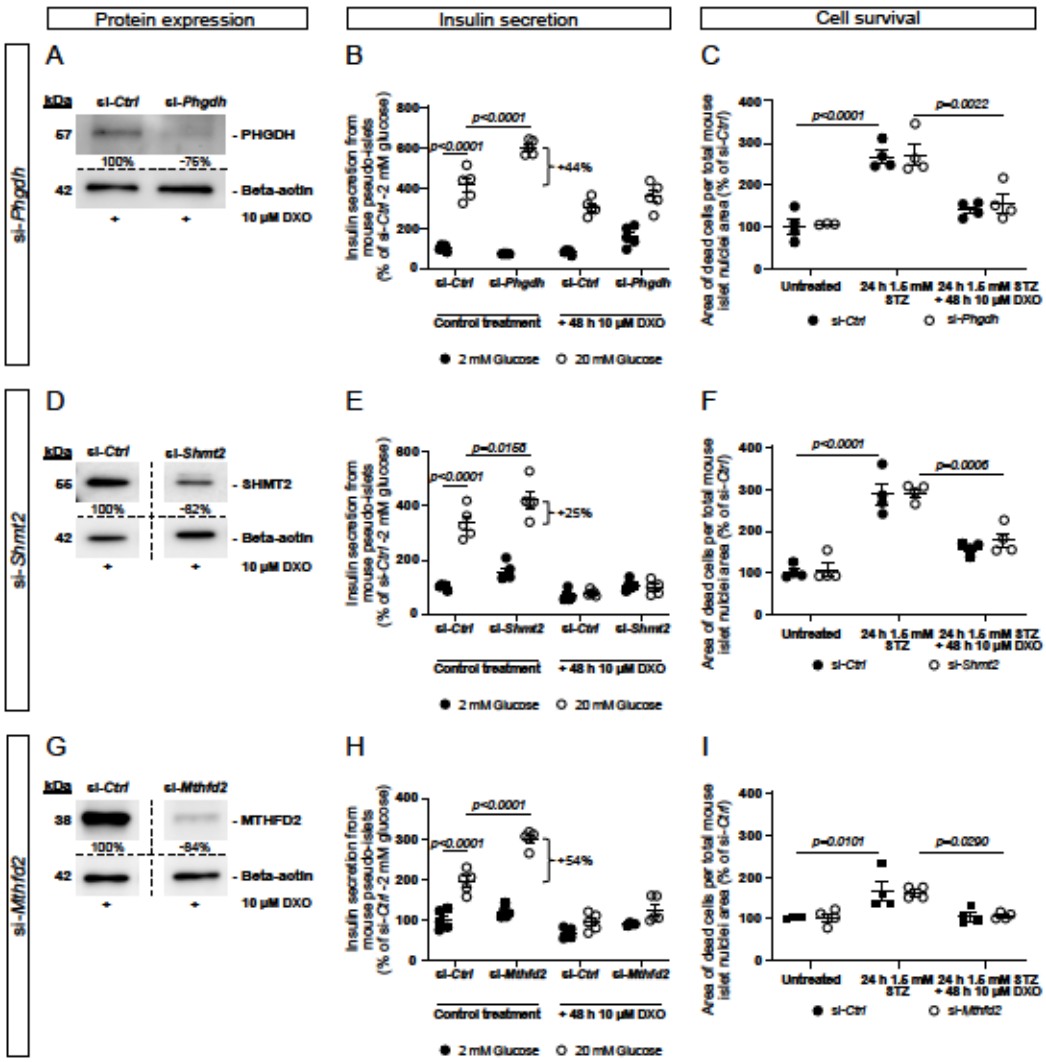

Figure 20: Single KD of *Phgdh*, *Shmt2* and *Mthfd2* were able enhance islet insulin secretion.
A, Protein expression, **B**, GSIS, and **C**, Quantification of cell death in untreated and 24 h 1.5 mM STZ treated si-*Ctrl* and si-*Phgdh* transfected mouse pseudo-islets in absence or presence of 10 µM DXO for 48 h (n = 4 stainings). **D**, Protein expression, **E**, GSIS, and **F**, Quantification of cell death in untreated and 24 h 1.5 mM STZ treated si-*Ctrl* and si-*Shmt2* transfected mouse

pseudo-islets in absence or presence of 10 µM DXO for 48 h (n = 4 stainings). **G**, Protein expression, **H**, GSIS, and **I**, Quantification of cell death in untreated and 24 h 1.5 mM STZ treated si-Ctrl and si-Mthfd2 transfected mouse pseudo-islets in absence or presence of 10 µM DXO for 48 h (n = 4 stainings). Protein expression was normalized to Beta-actin. Insulin secretions were measured at 2 mM and 20 mM glucose (n = 5 wells). Data are shown as mean ± SEM with corresponding p-values and calculated as percentage of control. Statistical significance was determined by two-way ANOVA followed by Tukey's multiple comparison test. Knockdown experiments of *Phgdh* and *Shmt2* were performed by A. Pelligra (A – F), while K. Griess performed all analyses of the *Mthfd2*-KD (G – I).

In comparison to single si-RNA treatments, simultaneous triple si-RNA treatment of pseudo-islets resulted in less strong KD efficiencies of -59% to -76% at protein expression levels (Figure 21A). Nevertheless, the simultaneous triple-KD induced a much stronger GSIS (+80%) compared to the single gene KD (Figure 20A, D, G) at high glucose concentrations. However, the DXO-induced islets dysfunction and the depletion of insulin content (Suppl. Fig. 11D) were not attenuated by the simultaneous KD of *Phgdh*, *Shmt2*, and *Mthfd2* (Figure 21B). Notably, triple-KD pseudo-islets exhibit increased susceptibility to STZ-induced cell death, even though DXO-induced cell protection was only tendentially attenuated in these pseudo-islets (Figure 21C).

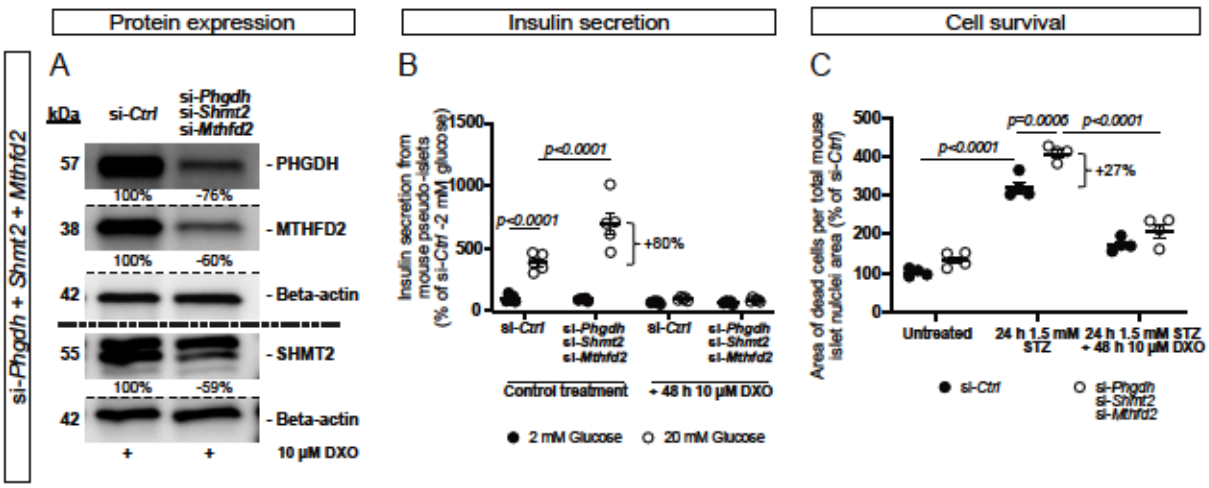

Figure 21: Simultaneous KD of *Phgdh*, *Shmt2* and *Mthfd2* enhances susceptibility against STZ-induced cell death.
A, Protein expression, **B**, GSIS, and **C**, Quantification of cell death in untreated and 24 h 1.5 mM STZ treated *si-Ctrl* and si-*Phgdh* + *Shmt2* + *Mthfd2* transfected mouse pseudo-islets in absence or presence of 10 µM DXO for 48 h (n = 4 stainings). Protein expression was normalized to Beta-actin. Insulin secretions were measured at 2 mM and 20 mM glucose (n = 5 wells). Data are shown as mean ± SEM with corresponding p-values and calculated as percentage of control. Statistical significance was determined by two-way ANOVA followed by Tukey's multiple comparison test. All si-RNA transfections, protein expression, and cell survival analyses were performed by A. Pelligra (A + C). The insulin secretion assay was conducted by A. Pelligra (B), while K. Griess performed the respective insulin ELISA.

5.6 Induction of islet dysfunction, upregulation of the serine-linked *mt*OCM, and enhanced survival are not DXO-specific effects

5.6.1 NMDAR antagonist MK-801 and glucolipotoxicity enhance serine-linked *mt*OCM in pancreatic islets

DXO is a NMDAR antagonist which induces gene expression levels of serine-linked *mt*OCM (see Figure 12 and Figure 13). To investigate whether the inhibition of the NMDAR mediates the DXO-induced effects, pancreatic islets were treated with 10 µM of the NMDAR antagonist MK-801 for 24 h, and used for RNA-seq analysis (Figure 22). The transcriptome analysis revealed an upregulation of the ATF4-associated ER stress branch (Figure 22A), genes of the *de novo* serine synthesis genes (Figure 22B), the *mt*OCM enzymes and *Cth* (Figure 22C) similar, but to a lesser extent compared to 24 h 10 µM DXO treatment (shown in Figure 12). Additionally, MK-801 also induced a slight downregulation of *Slc2a2* and *MafA* (Figure 22D).

Overall, the inhibition of NMDAR induces similar, but less pronounced effects to DXO, suggesting the potential involvement of additional mechanism in the DXO-induced effects.

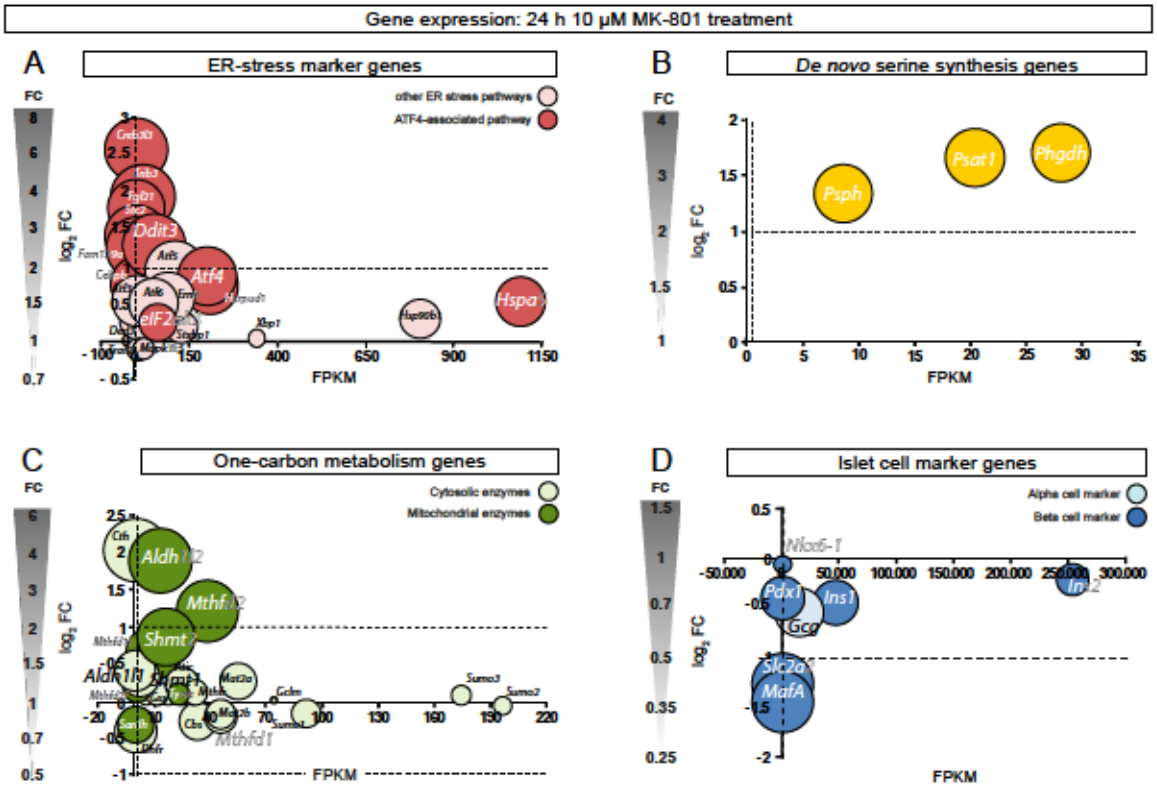

Figure 22: Chronic (48 h) treatment with NMDAR antagonist MK-801 induces the expression of serine-linked OCM genes. **A**, Transcriptome analysis of ER stress marker genes, **B**, *De novo* serine synthesis genes, **C**, OCM genes, and **D**, Islet cell marker genes, after 48 h 10 µM MK-801 treatment of pancreatic mouse islets. Graphs show log_2 FC, FPKM and p-values of genes based on RNA-seq data. Horizontal axes show FPKM values (Fragments Per Kilobase Million), vertical axes show log_2 FC. Dashed lines: cut-off of the FPKM and the log_2 FC. Gradients on the left indicate the FC. Genes of the ATF4-associated branch and the folate cycle are highlighted by larger font size. RNA-seq data were collected by J. Mrugala.

To determine, whether *Atf4* and serine-linked *mt*OCM genes are also upregulated in response to diabetogenic stressors, their gene expression levels were examined in pancreatic islets following treatment with high-palmitate (500 µM) + high-glucose (HG, 25 mM) to induce glucolipotoxicity. This treatment induced a robust upregulation of *Atf4* and, with exception of *Psph*, of all other analyzed serine-linked *mt*OCM genes (Figure 23).

These findings suggest that the induction of ER stress and the upregulation of serine-linked *mt*OCM genes in pancreatic islet cells is a general response to induced cellular stress.

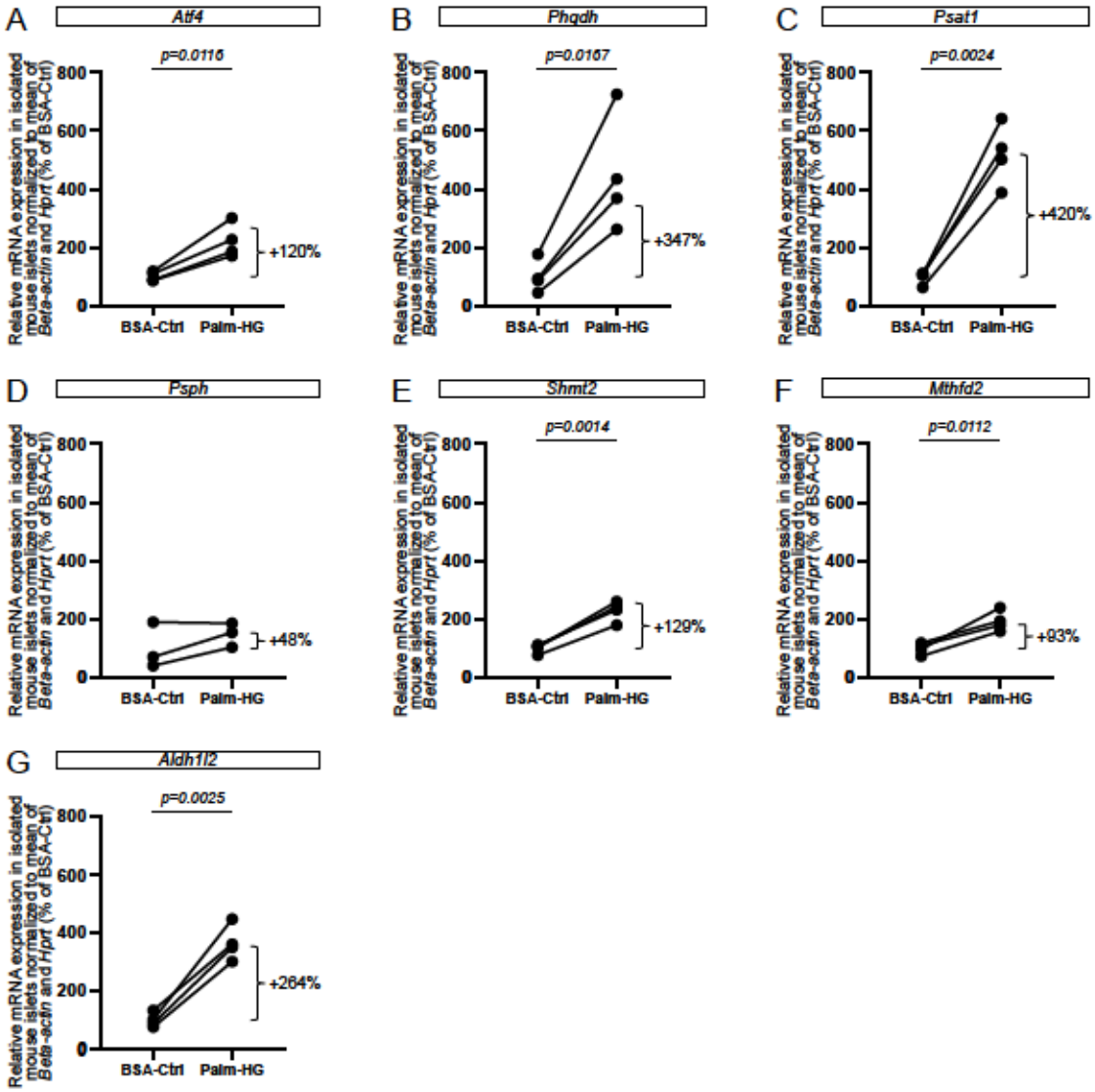

Figure 23: High-palmitate + high glucose treatment induces *Atf4* and serine-linked *mt*OCM gene expression.
Gene expression levels of **A**, *Atf4* and serine-linked *mt*OCM genes **B**, *Phgdh*, **C**, *Psat1*, **D**, *Psph*, **E**, *Shmt2*, **F**, *Mthfd2*, and **G**, *Aldh1l2* after 48 h treatment with 500 mM palmitate – 25 mM glucose (n = 3 – 4 independent experiments). Data are calculated as percentage of control with corresponding p-values. Statistical significance was determined by two-tailed paired student's t-test. Gene expression data were collected by K. Griess.

5.6.2 Islet dysfunction and protection are induced by the K_{ATP}-dependent GSIS mechanism

To investigate whether the effects induced by chronic high-dose DXO treatment were specific to NMDAR antagonization, GSIS, islet cell viability and expression levels of serine-linked *mt*OCM genes were analyzed upon treatment with different anti-diabetic drugs. For this purpose, pancreatic islets were treated for 48 h either with the sulfonylurea Glib or the GLP-1R-agonist Ex-4. Notably, 1 µM Glib treatment induced GSIS reduction (Figure 24A), to a similar extent as 48 h 10 µM DXO (see Figure 10E). The insulin content was as well decreased, but to a lesser extent, compared to DXO treatment (Figure 24B). In contrast, Ex-4 treatment did not induce islet dysfunction, but rather enhanced GSIS (Figure 24C), with no effects on the insulin content (Figure 24D).

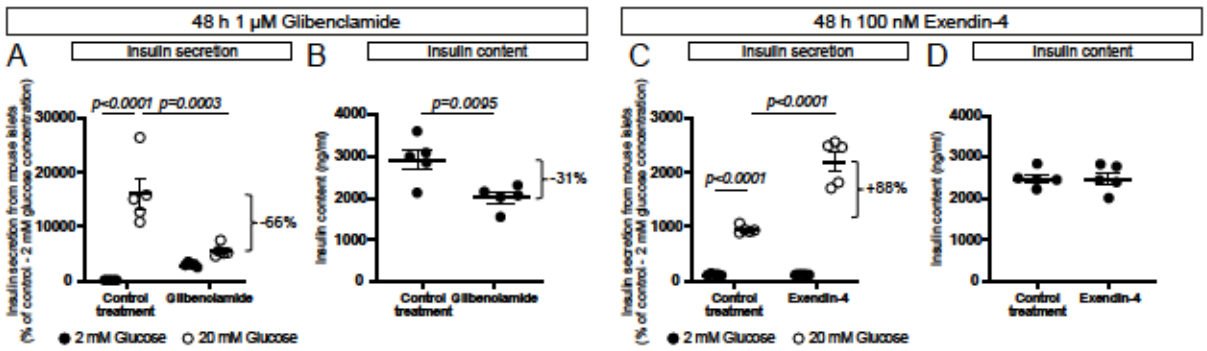

Figure 24: Chronic (48 h) glibenclamide treatment induces islet dysfunction, whereas chronic exendin-4 enhances GSIS. **A**, GSIS and **B**, insulin content of untreated and 48 h 1 µM glibenclamide (Glib) pre-treated pancreatic mouse islets. **C**, GSIS and **D**, insulin content of untreated and 48 h 100 nM exendin-4 (Ex-4) pre-treated pancreatic mouse islets (n = 5 wells). Statistical significance was determined by two-way ANOVA followed by Tukey's multiple comparison test (A + C) and two-tailed unpaired student's t-test (B + D). Data are calculated as percentage of control with corresponding p-values. Insulin secretion assays were performed by A. Pelligra.

Furthermore, the survival enhancing features of both insulin secretagogues were analyzed in flow cytometry-based cell survival assays against high-dose STZ-induced cell death. The viability analyses show that chronic Glib treatment exerted a cell-protective effect against high-doses STZ, as evidenced by a decreased cell death percentage from 46.80% upon 2.5 mM STZ to 8.27% upon 2.5 mM STZ + 1 µM Glib treatment (Figure 25A). In contrast, Ex-4 treatment did not show survival enhancing effects against STZ-induced cell death (Figure 25B). Based on the result that Glib and DXO exert comparable effects on GSIS and islet cell protection, both insulin secretagogues might potentially share similar and general MOA. In contrast, 48 h 100 nM Ex-4 emerges to have distinct effects on islet cell.

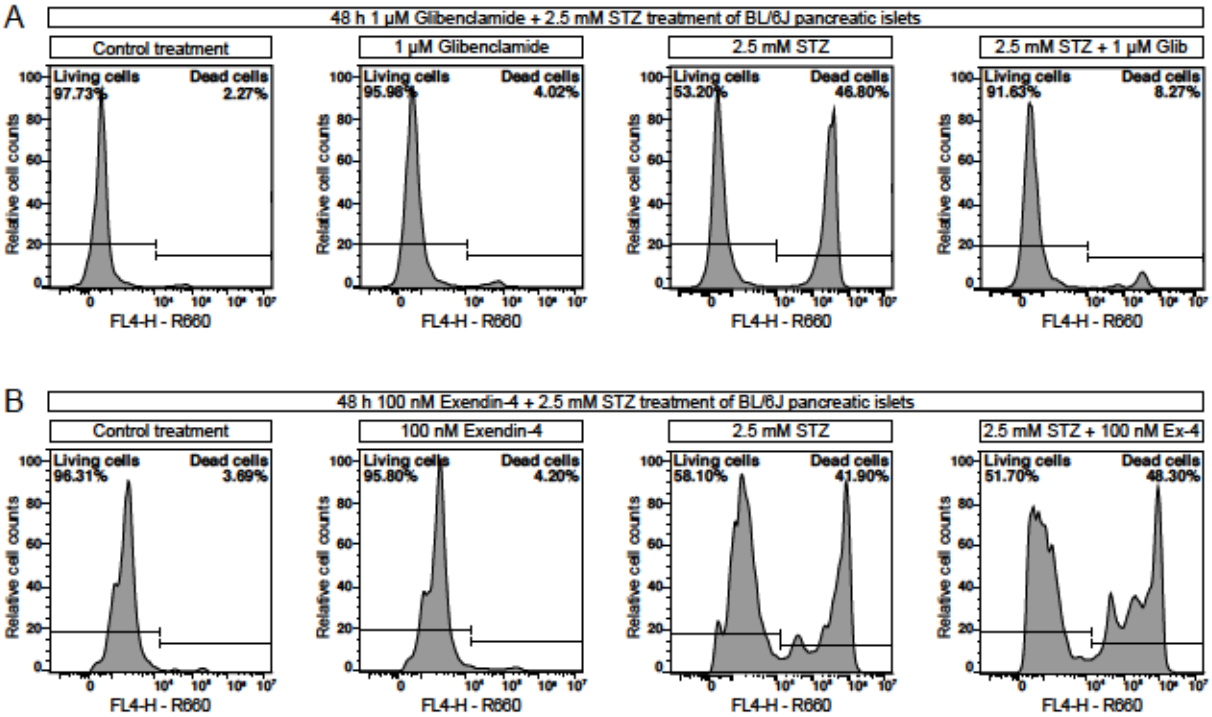

Figure 25: Chronic (48 h) glibenclamide treatment protects against STZ-induced cell death, in contrast to exendin-4.
A, Representative histograms of untreated and 24 h 2.5 mM STZ treated pancreatic mouse islets, in absence or presence of 1 µM Glib for 48 h or **B**, 100 nM Ex-4 for 48 h. FL4-H negative populations depict living cells and FL4-H positive populations depict dead cell. Cell viability was analyzed using FVS660. Flow cytometric analyses were performed by K. Griess (E) and P. Kirschner (F).

Moreover, gene expression levels were analyzed, to investigate whether chronic Glib or Ex-4 treatments enhance *Atf4* and serine-linked *mt*OCM genes in pancreatic islets, in a similar way as chronic high-dose DXO (Figure 26). According to the results shown in Figure 26, Glib induces a robust and highly significant upregulation of all analyzed genes, although to a lesser extent compared to the fold changes induced by DXO (see Figure 12 and Figure 13). In comparison, Ex-4 only induced a slight, but significant upregulation of *Phgdh*, *Mthfd2*, and *Aldh1l2* gene expression (Figure 26B, F, G).

Overall, these findings suggest that different chronic treatment with sulfonylureas and DXO might activate the same or similar MOA, for the induction of islet dysfunction and enhancing islet cell survival and expression levels of *Atf4* and serine-linked *mt*OCM genes. Ex-4 treatment exerted none of the chronic high-dose DXO effects, despite the upregulation of few genes.

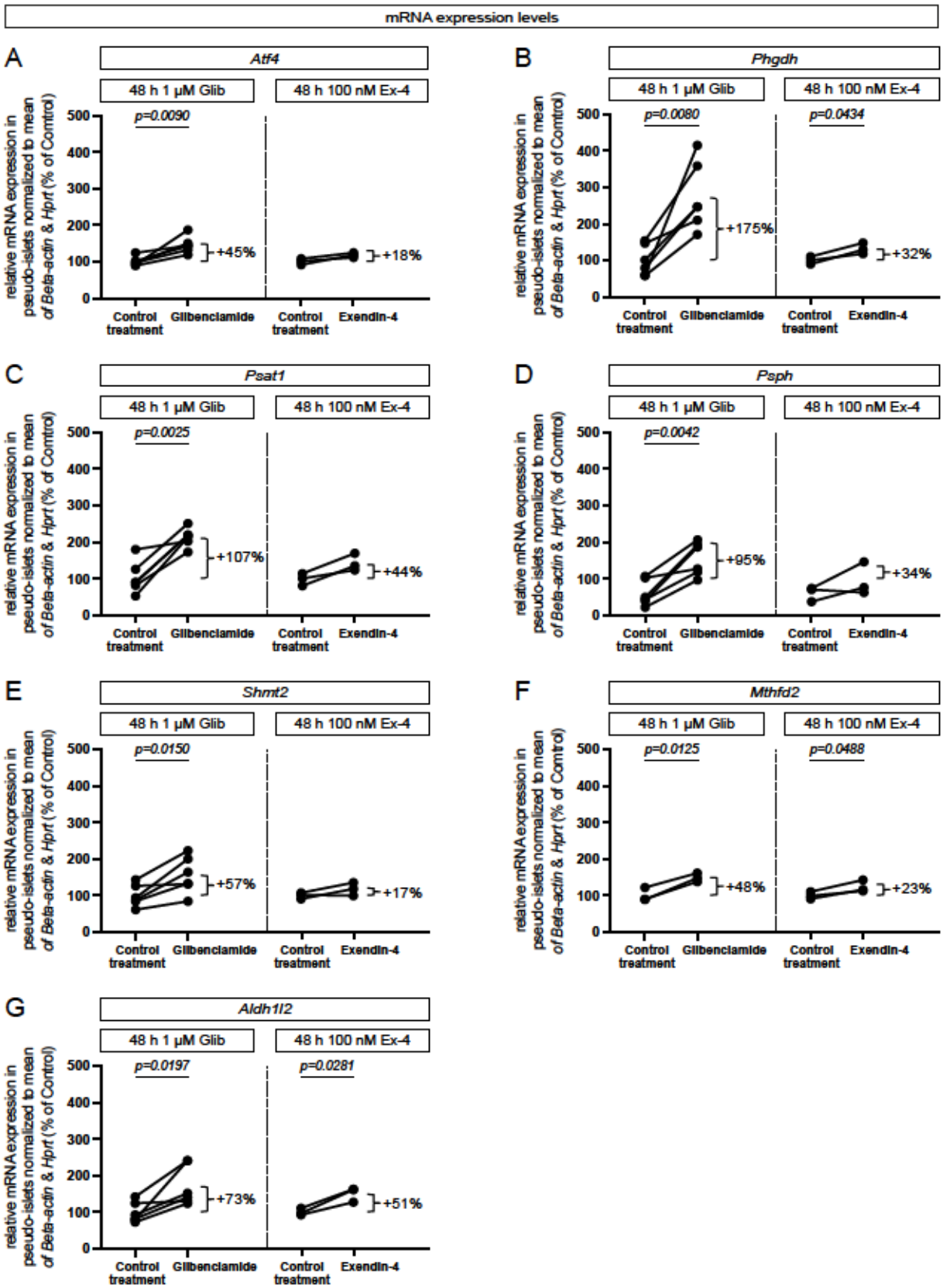

Figure 26: Chronic (48 h) glibenclamide treatment induces serine-linked *m*tOCM gene expression in pancreatic islets in contrast to exendin-4 treatment. Gene expression levels of **A**, *Atf4* and serine-linked *mt*OCM genes **B**, *Phgdh*, **C**, *Psat1*, **D**, *Psph*, **E**, *Shmt2*, **F**, *Mthfd2*, and **G**, *Aldh1l2* after 48 h treatment with 1 µM glibenclamide (Glib) and 100 nM exendin-4 (Ex-4) (n = 3 – 6 independent experiments). Data are shown as mean ± SEM with corresponding p-values and calculated as percentage of control. Statistical significance was determined by two-tailed paired student's t-test (E – M). Glib expression data were collected by K. Griess (A – E, G) and A. Pelligra (A – G), while all Ex-4 expression data were collected by K. Griess (E – M).

6 Discussion

The major aim of this study was to elucidate the underlying molecular mechanisms for enhanced islet cell survival in the presence of induced cell death, triggered by chronic high-dose DXO treatment. For this purpose, various aspects concerning the duration and concentration of DXO treatment were analyzed.

Some of the most intriguing characteristics of DXO consist of the beneficial effects on GSIS and cell survival in both, pancreatic mouse and human islets, upon diabetogenic conditions.[33,182,184,197] Studies have demonstrated that DXO treatment delays the onset of T2DM in ***db/db*** mice and reduces ***post prandial*** BG excursions in individuals with diabetes after acute single-dose DXM administration, making it a promising insulin secretagogue.[183,184,186] However, continuous (48 h) high-dose (10 µM) DXO treatment was found to induce islet dysfunction, characterized by a strong and robust decline in GSIS and intracellular insulin content.[186] Notably, this treatment coincides with enhanced islet cell survival against cytokine-induced cell death.[184,186]

Based on an RNA-seq analysis of chronic high-dose DXO pre-treated pancreatic mouse islets, J. Mrugala identified the upregulation of ***Phgdh***, ***Psat1***, ***Psph***, ***Shmt2***, ***Mthfd2***, and ***Aldh1l2***, encoding enzymes of the serine-linked ***mt***OCM.[186] Additionally, the transcription factor ATF4, which is not only associated with ER stress but also known to induce and regulate the serine-linked ***mt***OCM gene expression, was found to be upregulated by continuous DXO treatment.[114,206-208] According to literature, there is robust evidence that these enzymes enhance the cellular redox state and overall cell survival.[199-205] Hence, the RNA-seq analysis identified valuable potential candidate genes and revealed a metabolic pathway, which might be responsible for enhanced islet cell survival and impaired GSIS, induced by chronic high-dose DXO treatment.[186] However, the hypothesized metabolic model and the specific roles of the involved enzymes remained to be validated and analyzed in primary pancreatic islets.

Overall, this study provides robust comprehensive insights about the regulation of transcriptional and metabolic adaptations of islet cell physiology in response to induced cell death. The uncovered metabolic interlinkages and pathways serve for the development of novel preventive or therapeutic treatments in the field of T2DM.

6.1 Chronic insulin hypersecretion induces the serine-linked *mt*OCM to mitigate cellular damage

Understanding the interplay between the DXO treatments duration and concentration with their induced effects might be crucial for the development of potential therapeutic drugs and even for understanding general islet cell physiology. This study explores the effects of acute and continuous high doses of DXO on isolated pancreatic islets, focusing on GSIS and islet cell survival against induced cell stress. Studies, in particular those conducted by J. Mrugala, suggest a critical dependency on the duration of the DXO treatment behind its induced beneficial effects.[184,186] Further, the induction of the hypothesized metabolic pathway by DXO and the potential cause-and-effect relationships between the upregulated genes, as well as the observed DXO-induced effects, were investigated by different approaches. First, GSIS and islet cell survival were specifically investigated after chronic (48 h) and acute (1 h) DXO treatment (Figure 10). Second, the respective RNA-seq data with high (10 µM) or low (1 µM) DXO doses were reanalyzed for specific genes, including those encoding for ER stress markers, serine synthesis, and OCM enzymes (Figure 12 and Figure 13). Finally, glycolytic flux analyses were conducted on both, acute and chronic high-dose DXO treated pancreatic islets (Figure 14).

6.1.1 DXO enhances beta cell survival against ROS and oxidative stress

Among all outcomes, this study reveals that high doses of DXO enhance the GSIS of isolated pancreatic islets after only 1 h of pre-incubation (Figure 10). This result is in line with previous observations from a clinical trial, where a single acute administration of DXM enhanced *post prandial* insulin secretion of T2DM patients.[183] However, acute high-dose DXO treatment did not exert positive effects on islet cell survival. In contrast, chronic high-dose DXO pre-treatment enhances the resilience and survival of islet cells against low and high doses of STZ. STZ is a glucose analog and oxidative stress inducer, specifically targeting beta cells via the GLUT-2 transporter.[223] Besides STZ, DXO-derivatives were additionally found to enhance the cell survival of islets which were exposed to H_2O_2 and menadione.[189] These results suggest that DXO exert cell protective effects against different types of ROS and oxidative stress-induced cell death in general, although this hypothesis remains to be proven by the conduction of respective experiments. Nevertheless, this beneficial effect coincides

with the induction of islet cell dysfunction, characterized by a robust decrease in GSIS and intracellular insulin content. Considering that strongly depleted intracellular insulin levels are usually associated with a decline in beta cell mass, it was crucial to prove that continuous DXO treatment protects the beta cells of pancreatic islets against high doses of their specific toxin, STZ (Figure 11).[145] In addition to the anti-diabetic potential of DXO, the effect of preserving beta cell viability represents a feature of particular relevance in regard of novel drug proposals. Notably, the RNA-seq analysis revealed that 24 h pre-treatment with high-dose DXO additionally induces a severe downregulation of beta cell key genes *Ins1*, *Ins2*, *MafA*, and *Slc2a2* (Figure 12). These findings not only provide an intriguing correlation between islet dysfunction and potential de- or trans-differentiation into other endocrine cell types, but might also be an indicator of a reduced beta cell population or an attenuated efficiency of the STZ-induced cell death. In fact, dedifferentiation resulting for example from chronic hyperglycemia and prolonged insulin hypersecretion, is considered a striking hallmark of T2DM and has been observed to cause rapid endocytosis and degradation of the GLUT-2 channel.[224-226] Despite the severe *Slc2a2* downregulation, this study clearly evidences that the stability or protein expression of GLUT-2 encoding mRNA remains unaffected by continuous DXO treatment, at the analyzed time point. This finding is crucial and suggests that the DXO-conferred islet cell protection against STZ-induced cell death relies on metabolic adaptations, rather than GLUT-2 internalization.

Impaired islet functionality is a characteristic feature of the early-onset of both T2DM and T1DM in response to stress conditions such as increased ROS levels, serving as a mechanism to evade cell death.[99,125,227-229] Based on this hypothesis and due to the observation of unaltered GLUT-2 protein expression levels, these results potentially indicate that chronic high-dose DXO treatment might induce insulin hypersecretion and possibly only a modest or initial form of islet dysfunction and dedifferentiation. Notably, insulin secretagogues in general have previously been associated with the induction of dedifferentiation, which is thought to be initiated by the depletion of the insulin content.[230,231]

In summary, these results reveal that high doses of DXO enhance the GSIS of pancreatic islets after acute pre-treatment, and improve the overall beta cell survival against STZ and potentially against oxidative cell stress in general, after chronic pre-treatment. However, the beta cell protective effect is accompanied by the induction of dysfunction and a moderate or initial beta cell dedifferentiation, which does not significantly affect GLUT-2

biosynthesis and thus might be comparable to dedifferentiation observed during pre- or early diabetes. Despite, or potentially even due to the downregulation of beta cell markers, the DXO enhanced islet cell survival is likely to underly specific cellular adaptations, rather than dedifferentiation.

6.1.2 The glycolytic flux regulates islet cell functionality and survival in islets

In recent years, the increasingly recognized involvement of the serine-linked *mt*OCM in the development of diseases, in particular in cancer, has attracted substantial interest, becoming subject of numerous studies, aiming at elucidating its roles in both, normal physiological functions and disease-related contexts.[155,156,232-234] Studies concerning the OCM have usually been conducted in immortalized beta cell lines, therefore, not much is yet known about its functions in healthy or dysfunctional islets.[131,202,205,235] Thus, this study provides first comprehensive insights about the role of the serine-linked *mt*OCM in primary pancreatic islets.

Herein, robust evidence has been collected indicating an inverse regulation of islet cell survival and functionality upon persisting insulin secretion induced by chronic high-dose DXO. Previous studies provide intriguing aspects, which directly connect impaired cell functionality to the upregulation of the OCM, by the deviation of 3-PG from glycolytic flux into the serine synthesis pathway.[131,207] The TCA cycle is a critical metabolic pathway, generating key analytes required for cellular respiration.[236] Therefore, its blockade or bypassing limits downstream processes, such as ATP synthesis, which are considered indispensable in the K_{ATP}-dependent GSIS mechanism of beta cells.[21,237] According to this study and as previously suggested by J. Mrugala, the herein observed deviation of glucose intermediates into the serine synthesis pathway induced by continuous DXO treatment, is likely to be the main cause of the observed decrease in GSIS.[186] Hence, the here conducted glycolytic flux analyses revealed complex interlinkages, clearly indicating that persisting cellular stress, such as insulin hypersecretion, does not necessarily lead to cell death, but triggers an alternative pathway instead, causing the deviation of glucose resources into a pro-survival rather than functional pathway (Figure 14). Similar was also reported by DeNicola *et al.* and Ye *et al.*, who observed enhanced cell survival and proliferation upon glycolytic flux deviation toward the serine synthesis and the *mt*OCM as well.[207,238] Although the OCM is of fundamental importance for the synthesis of nucleotides and cell proliferation in general, RNA-seq data of chronic high-dose DXO treated

islets shown in Pelligra *et al.*, did not reveal the upregulation of common proliferation marker genes such as *proliferating-cell-nuclear-antigen* (*Pcna*), *mitogen-activated protein kinase 1* (*Mapk1*), *signal transducer and activator of transcription* 3 (*Stat3*), and various kinases, but rather indicate their downregulation.[209] This result suggests that the upregulation of the *mt*OCM by DXO is likely not serving for proliferating reasons. Otherwise, enhanced proliferation could as well explain the reduced number of apoptotic cells upon STZ-induced cell death in chronic high-dose DXO treated islets.

Further evidence suggesting that diabetic stress might not directly decrease the viability of dysfunctional beta cells, but instead trigger metabolic adaptations to hyperglycemia, is provided by various scientific investigations. Studies revealed that beta cell mass and volume were not significantly or only slightly reduced in patients with impaired glucose tolerance and pre-diabetes, compared to those of non-T2DM patients, suggesting that a reduced functional beta cell mass acts as major risk factor for T2DM.[14,229,239,240] The hypothesis that beta cells prioritize a viable but less functional state when exposed to continuous stress, might find additional support in the existence of various beta cell sub-populations, differing in functionality and resilience.[35-37] Interestingly, it has also been reported that ATF4 impairs the TCA cycle, which in turn promotes serine synthesis, *mt*OCM activity, and enhances GSH synthesis.[241,242] Consistent with this study, thapsigargin-induced ER stress led to INS-1E beta cell dysfunction, depletion of ATP levels, and the accumulation of *mt*ROS levels, rather than apoptosis.[131] These findings suggest that ATF4 activation might also play a crucial role in deviating the glycolytic flux and maintaining cell viability (see chapter 6.2.1).

It is important to note that the *de novo* synthesized serine might undergo rapid processing within an enhanced *mt*OCM. Therefore, the levels of generated serine and incorporated [U-^{13}C] units quantified in this study, might only represent a small fraction of the actual total generated serine. However, it is likely that continuous DXO treatment induces a strong glycolytic flux deviation, in line with the enhanced fold changes shown in protein expression levels of ATF4, PHGDH, and ALDH1L2, the severity of induced islet dysfunction, and the robustness of DXO-conferred cell protection against high dose STZ-induced cell death. The induction of effects with such a substantial impact is likely to be critically dependent from the treatment‘s duration, concentration, and the cellular metabolic adaptations. In fact, glycolytic flux analyses conducted with acute high-dose DXO-treated islets reveal no substantial

alteration in [U-^{13}C]-unit incorporation in citrate and serine, indicating that full TCA cycle activity and its downstream processes are required for proper beta cell functionality. Thus, the glycolytic flux deviation reflects a metabolic adaptation, which is required to mitigate intracellular stress against persistent and excessive insulin secretion induced by chronic high-dose DXO treatment, resulting in enhanced beta cell survival even in the presence of STZ.

Although this aspect is not directly assessable in this study, quantifying absolute serine and citrate levels in untreated and chronic high-dose DXO-treated islets could provide valuable insights into the degree of glucose flux deviation that is required or sufficient to potentially induce both, enhanced GSIS and survival against induced cell death. Interestingly, chronic treatment with low dose (1 µM) DXO tendentially, and also partially significantly, induces the upregulation of ***Atf4*** and serine-linked ***mt***OCM genes (Figure 13). The same low-dose treatment was previously reported to also enhance the survival of pancreatic islets upon cytokine-induced cell death.[186] Further, ***db/db*** mice treated for two weeks with 3 mg/ml oral DXM administration and displaying DXO blood plasma levels of 1 µM, showed enhanced GSIS and reduced BG levels.[184,186] Taken together, these results point to the possibility that a slight and controlled upregulation of the serine-linked ***mt***OCM might induce a glucose flux deviation at a degree which enhances both, islet cell survival in the presence of cell stress and GSIS. However, the glycolytic flux of chronic low-dose DXO treated islets remains to be analyzed.

In summary, this study confirms that chronic high-dose DXO treatment deviates the glycolytic flux away from K_{ATP}^-dependent GSIS mechanism, favoring the serine synthesis over the TCA cycle, as suggested in previous studies.[186] Consequently, the deviation of the glycolytic flux and the concomitant upregulation of the serine-linked ***mt***OCM pathway are likely to play a critical role in impaired islet cell functionality and their enhanced resilience. Hence, dysfunctional beta cells might not necessarily be doomed to cell death but rather undergo dysfunction by prioritizing pro-survival pathways. Therefore, targeting the glycolytic flux from the glycolysis into the serine-linked OCM of beta cells might represent a promising therapeutic approach to restore beta cell functionality.

6.1.3 Chronic high-dose DXO enhances the serine-linked *mt*OCM and improves the cellular redox homeostasis upon hyperglycemia

Besides *Atf4*, the RNA-seq data of pancreatic islets treated for 24 h with 10 µM DXO shown within this study, also revealed the upregulation of *Ddit3*, *Fgf21, Hspa5*, and *Trib3*, which are related to the activation of the ATF4-associated UPR branch of the ER (Figure 12).[222,243-245] ATF4 is a versatile transcription factor, regulating expression levels of numerous pathways. One of its prominent targets is the pro-apoptotic transcription factor CHOP (encoded by *Ddit3*), for which ATF4 is commonly associated with cell death and the induction of apoptosis.[103,109,246] Conversely, robust evidence has been collected, demonstrating also protective features of ATF4, even in pancreatic islet cells of hyperglycemic mice.[112,247,248] Although the mechanism by which ATF4 induces cell protection is not fully understood, the upregulation of its downstream target genes, encoding serine-linked *mt*OCM enzymes is thought to play a pivotal role in these beneficial effects.[111,112,249] In line with these notions, this study provides robust evidence that chronic high-dose DXO treatment enhances the serine-linked *mt*OCM also on protein expression levels, as previously suggested, but only partially demonstrated by J. Mrugala (Figure 12).[186] Notably, the ATF4-associated UPR branch and the serine-linked *mt*OCM were strongly enhanced only after chronic, but not acute DXO treatment (Figure 12 and Figure 13). As islet cell protection was only observed in chronic high-dose DXO treated islets (Figure 10), this result points to the possibility that ATF4 and the upregulation of the serine-linked *mt*OCM might be responsible for this beneficial effect. Due to its general functions, the serine-linked *mt*OCM was found to enhance survival of various cell types including neurons, hepatocytes, endothelia, and several cancer cells.[149,156,199,250-252] Therefore, the dual role of ATF4 in determining cell fate might not only rely on the severity and duration of cell stress or the intensity of its activation, but also on the prompt activation of the *mt*OCM and the metabolic adaptation to manage cellular stress.[114,242,253]

Since serine levels were found to be decreased in diabetic patients, recent studies identified its deficiency as novel risk factor for DM.[254,255] Intracellular, serine is mainly used as substrate for the synthesis of sphingolipids and as donor of C1-units in the OCM.[153,256] The OCM substantially contributes to a healthy cellular homeostasis and is therefore found in numerous mammalian cell types.[150-152] Notably, the OCM includes a cytosolic and a mitochondrial folate cycle, comprising isoenzymes which catalyze homologous reactions, although encoded by distinct genes.[151,159,160] It is not fully elucidated whether the duplication

of this pathway exhibit compartment-dependent advantages, but both are prominently involved in amino acid synthesis and maintaining the cellular redox balance.[151,159,160] Krupenko *et al.* suggested that one major role of *mt*OCM enzyme ALDH1L2 is to provide NADPH, serving as co-factor for the glutathione reductase (GSR) in recycling oxidized glutathione (GSSG) back to its active reduced form (GSH).[257-259] In fact, NADPH does not only act as reducing agent for the direct neutralization of harmful ROS, but is also essential in maintaining a robust GSH/GSSG ratio, which is considered crucial in the cellular antioxidant system.[258-260] J. Mrugala reported about the upregulation of the NADPH-generating enzyme ALDH1L2, which was one of the most striking candidates in the RNA-seq gene expression analysis (FC = 3.9) upon chronic high-dose DXO treatment.[153,159,186] In the context of this study, the enhanced *mt*NADPH/NADP$^+$ levels quantified by UPLC analyses, might be attributable to this reaction, and probably indicate cellular adaptations to highly elevated endogenous ROS levels, arising from DXO-induced excessive insulin secretion. Although endogenous ROS is known to be of fundamental importance in triggering the GSIS, *in vitro* conducted studies on insulinoma cells of mice, rats, and human pancreatic islets report about dramatically high ROS generation and in particular of elevated H_2O_2 levels during hyperglycemia and GSIS, directly deriving from the ETC.[122-125,134] As previous studies have demonstrated that acute high-dose DXO treatment enhance the GSIS at glucose concentrations of 10 mM, it is very likely that a continuous high-dose DXO treatment, conducted in culture media containing the same glucose concentration, induces insulin hypersecretion and excessive ROS generation in pancreatic islets.[184,186] Thus, the DXO induced upregulation of the serine-linked *mt*OCM pathway might be a metabolic adaptation to mitigate oxidative stress, which is strong enough and sufficient to enhance islet cell survival even against STZ or cytokine treatment.[184,186] Of note, elevated ROS levels do not only cause physical damage for example by inducing DNA-strand breaks, but act as well as signaling molecules, inhibiting GSIS and the synthesis of insulin in beta cells, possibly contributing or exacerbating the DXO-induced islet cell dysfunction.[125,261] Taking into account that NADPH would be constantly required to maintain the cellular redox homeostasis and support viability in DXO-treated pancreatic islets, the actual *mt*NADPH/NADP$^+$ ratio could be expected to be much higher than the herein quantified (Figure 14). In line with this result, dysfunctional beta cells of diabetic rats displayed as well enhanced NADPH/NADP$^+$ levels in concomitance to increased GSH levels, considered to serve for self-protection against ROS and oxidative damage.[262]

Within this study neither GSH/GSSG ratio nor ROS levels were analyzed. Consequently, the roles of these factors can only be hypothesized in the observed DXO-induced islet cell protection and require further analyses. Nevertheless, an additional hint pointing to a prominent involvement of GSH, derives from the RNA-seq analysis, revealing the upregulation of *cystathionine gamma-lyase* (*Cth*) (Figure 12). This gene encodes a cytosolic OCM enzyme which reduces cystine to cysteine, which in turn is one of the main substrates for GSH biosynthesis.[259]

Considering that ATF4 has recently been described as a key regulator of GSH synthesis, this study suggests that the observed protective effect induced by chronic high-dose DXO treatment might derive from an enhanced cellular redox state and a well-adapted antioxidant defense.[242] Last but not least, considering that cytokines and STZ lead to the production of ROS and other highly reactive molecules such as reactive nitrogen species (RNS) which is as well neutralised by GSH, the cellular adaptations induced by continuous high-dose DXO treatment might basically be the same, resulting in similar protective outcomes for both cell death-inducing agents.[263,264]

Taken together, this study reveals that chronic high-dose DXO treatment of pancreatic islets leads to the activation of ATF4, which mediates the upregulation of the serine-linked *mt*OCM. This upregulation is likely to be responsible for improved cellular redox state and to enhance cell survival in dysfunctional islets, even upon severe cell death induction by high STZ concentrations. However, further investigations, aiming to quantify GSH/GSSG levels could prove that islet cell survival can be enhanced by adequate metabolic adaptations, such as the upregulation of the serine-linked *mt*OCM.

6.1.4 Alleviating insulin hypersecretion might restore the functionality of dysfunctional islet cells

Current anti-diabetic medications have been ineffective in halting the progressive beta cell decline observed in DM, therefore a definitive cure from DM remains elusive.[190] Thus, DM is considered an incurable chronic disease, requiring lifelong treatment. However, studies investigating T2DM patients have reported promising signs of remission following drastic interventions, including severe caloric restriction and bariatric surgeries, independently of substantial weight loss.[71,265,266] Therefore, moderating nutrition excess, thus preventing persistent hypersecretion of insulin and alleviating endogenous stress in beta cells, emerges

to be crucial for restoring islet cell functionality and reverse T2DM in patients and ***db/db*** mice.[89,265-268] To investigate, whether removal of the insulin secretagogue DXO might be sufficient to reverse islet dysfunction, washout experiments were performed (Figure 15 and Figure 16). In line with previously reported findings, removal of the cause for persistent insulin secretion and cell stress was found to be sufficient to restore the insulin secretory capacity, thus functionality of islet cells.[89,265-268] However, these effects come along with vanishing DXO-conferred islet cell protection and decrease in ***Atf4*** and serine-linked ***mt***OCM gene expression levels. Therefore, this study provides strong evidence that i) islet cell functionality and viability are inversely regulated, ii) the uncovered molecular mechanism is tightly linked to the activation of ATF4 and the serine-linked ***mt***OCM, and iii) that its induction specifically responds to the insulin secretagogue DXO. As indicated by decreasing ***Atf4*** and serine-linked ***mt***OCM gene expression levels, these observations are in line with previous studies, claiming that reducing the demand in insulin and restoring beta cell functionality relieves the ER and reverses oxidative stress.[269-272] However, the restoration of beta cell functionality does not coincide with a complete recovery of insulin content or the expression levels of critical beta cell genes, such as ***Ins1*** and ***Ins2***, at the analyzed time point. As previously discussed, the DXO-induced dedifferentiation might be comparable to that observed in the stages of pre- or early-onset DM. Considering that beta cell dedifferentiation in ***db/db*** mice was reported to be potentially restorable by long-term caloric restriction and that effectiveness and durability of remission from T2DM were found to be more favorable in patients with early disease onset (age <40 years) and/or a diagnosed pathogenesis of less than 10 years, the complete recovery of insulin content, as well as normalized ***Ins1*** and ***Ins2*** expression levels, might require a longer period than 24 h of washout, for full recovery from chronic high-dose DXO treatment.[40,71,265,268]

Restoration of islet cell functionality appears to be intricately linked to the removal of persistent cell stress and demand in insulin secretion. According to the validated metabolic model, reversing insulin hypersecretion corresponds to removing the causes of persistent insulin secretion and cell stress, which induces a metabolic rearrangement consisting in the downregulation of the antioxidant defense system and reprioritization of functional- over pro-survival pathways (Figure 16). Hence, the serine-linked ***mt***OCM genes result in downregulation to normal levels after washout, likely to allow the glycolytic flux to be redirected towards the TCA cycle. This redirection might support the K_{ATP}-dependent GSIS

mechanism, ultimately resulting in a restored beta cell functionality. This study does not provide evidences for a redirection of the glycolytic flux from serine synthesis to TCA cycle in restored pancreatic islets. However, one of the early benefits observed in T2DM patients *post*-bypass surgery, consist of enhanced beta cell function, which points to the possibility that metabolic readaptations, such as the redirection of the glycolytic flux towards the TCA cycle, could indeed be responsible for remission.[68,70] Furthermore, the observation of remission in T2DM patients overall evidences the presence of viable but dysfunctional beta cells. These beta cells might deviate their cellular resources towards pro-survival pathways, and therefore become dysfunctional. Noteworthy, remission is observed to be not sustained in cases of severe T2DM, implying that the potential for reversibility reaches a point of no return, likely to be based on the duration of a hypersecretory state, the severity of induced islet cell stress, and the extent of islet dysfunction.[40,71] However, removing the cause of cell stress and excessive insulin secretion might potentially contribute to prevent further progression of T2DM.

Although, initial remission results are reported to be achieved regardless of substantial weight loss, long-term remission success is observed to positively correlate with higher and constant weight loss.[70,71,265,266] This observation might indicate that reducing obesity, hence reducing cellular stress might significantly contribute to restore islet cell functionality.

Overall, this study provides evidence that functional impairments of beta cells are potentially fully reversible in early stages. As restoring beta cell functionality results to be intricately linked to the removal of the cause for persistent insulin secretion, cellular detoxification from excessive nutrients and ROS, emerges as a key factor in restoring GSIS and as a potential therapeutic approach in T2DM management, in particular of the MOD cluster subtype. This study challenges the notion that DM is an irreversible metabolic disorder and underscores the importance of early intervention and treatment strategies to restore beta cell functionality and achieving successful T2DM remission.

6.2 GoF and LoF: ATF4 and the serine-linked *mt*OCM regulate the GSIS

6.2.1 ATF4 regulates the serine-linked *mt*OCM and GSIS but is not sufficient to enhance islet cell survival

ATF4 is a pivotal transcription factor with significant implications for cellular fate. Although, this study provides no data about the origin of ER stress in chronic high-dose DXO treated islets, excessive insulin secretion induced by the insulin secretagogue at glucose concentrations of 10 mM, may represent a major trigger.[184,273,274] Further, hyperglycemia and hyperinsulinemia are reported to activate mammalian target of rapamycin complex1 (mTORC1) as well.[275,276] The signaling complex mTORC1 is a prominent upstream regulator of ATF4, known on the one hand to directly contribute to islet dysfunction and on the other hand to be crucial in proper protein synthesis, glycolysis, and mitochondrial metabolism.[206,208,276-279] Although mTORC1 was not object of this study, it could have contributed in the activation of ATF4 in beta cells. In turn, ATF4 is the prominent transcription factor for the genes encoding all serine synthesis enzymes PHGDH, PSAT1, and PSPH, as well as for *mt*OCM enzymes SHMT2, MTHFD2 and ALDH1L2.[114,200,206-208,238] Additionally, ATF4 is also considered an essential mediator of neuroprotection and found to be crucial in Parkinson disease.[232]

Gene expression analyses of *ATF4*-OE and *Atf4*-KD pseudo-islets confirmed on the one hand that ATF4 activation is sufficient to enhance mRNA levels of serine-linked *mt*OCM genes, and that their DXO-induced upregulation is mediated by ATF4 on the other hand (Figure 17 and Figure 18). Although ATF4 overexpression was much higher compared to protein levels induced by chronic high-dose DXO treatment (+312% vs +187%), expression levels of serine-linked *mt*OCM genes were not as highly upregulated, compared to the fold changes induced by chronic high-dose DXO treatment (Figure 17 and Figure 12). This result potentially indicates that the upregulation of these genes does not exclusively rely on ATF4, but on a yet unknown factor as well. However, it also has to be considered that the overexpressed human *ATF4* encodes for a protein sharing 85% identity with the murine ATF4, potentially resulting in a less efficient transcription factor (Suppl. Fig. 12).

Notably the overexpression of *ATF4* strongly decreases the GSIS of pseudo-islets, indicating that its activation might also be sufficient to induce islet dysfunction. In contrast to islet dysfunction induced by chronic high doses of DXO, the overexpression of *ATF4* does not deplete the insulin content, but significantly increases it instead. In line with unpublished data,

indicating a tendential upregulation of *Ins1* and *Ins2* gene expression levels, this finding might indicate that ATF4 does not negatively affect insulin synthesis itself, but rather affects the exocytosis of insulin, as previously observed by Liew *et al.*[115] In fact, the opposite effect, namely a strongly enhanced GSIS was found after *Atf4* silencing, which additionally decreased the insulin content. These results are in line with previous reports, revealing that ATF4 regulates the GSIS in beta cells by attenuation.[280-282]

Investigations concerning the mediative role of ATF4 in the DXO-induced islet effects, revealed that the transcription factor indeed partially mediates the induction of islet cell dysfunction, as the GSIS is only partially rescued in si-*Atf4* transfected and DXO pre-treated pseudo-islets. However, GSIS and insulin contents of both, si-*Ctrl* and si-*Atf4* transfected pseudo-islets were severely decreased by DXO compared to untreated pseudo-islets, indicating that DXO does not affect the exocytosis of insulin, but rather induces continuous and excessive insulin secretion, in contrast to *Atf4*-OE. Even though ATF4 was found to mediate the transcription of serine-linked *mt*OCM, the downregulation of beta cell marker genes caused by chronic high-dose DXO treatment, could not be rescued by *Atf4*-KD. Therefore, the observed downregulation of beta cell marker genes (Figure 12) are rather a consequence of continuous treatment with the insulin secretagogue DXO and the induction of islet dysfunction, as previously observed.[224,225] However, silencing *Atf4* decreases *Slc2a2* and enhances *MafA* gene expression levels, independently from chronic high-dose DXO treatment, likely as responses to strongly enhanced GSIS, observed in *Atf4*-KD pseudo-islets (Figure 19).[226]

Within this study, GoF and LoF experiments served as well to investigate the dual role of ATF4 in determining beta cell fate. According to literature, robust evidence has been collected, claiming a protective role of ATF4 in beta cells, even in hyperglycemic mice.[112,248] In contrast to these studies, *ATF4*-OE did not protect pseudo-islets from STZ-induced cell death within this study, but rather made them possibly more prone for apoptosis as indicated by the images and the FACS analysis, displaying disrupted structures of the pseudo-islets. Notably, Ad-*ATF4* transduced, untreated pseudo-islets did not display morphological differences or enhanced cell death compared to their respective controls. Despite the role of ATF4 as a transcriptional activator of the pro-apoptotic factor CHOP, this observation suggests that the substantial upregulation of *Atf4* alone does not significantly affect the overall viability of islet cells. Since strong ATF4 protein levels did not enhance, but rather decrease islet cell survival

in the presence of high doses of STZ, these results might point on the one hand to the possibility that Ad-*ATF4* transduced pseudo-islets were more resilient to low doses (1.5 mM) of STZ, or on the other hand that the serine-linked *mt*OCM and the antioxidant defense system were not sufficiently upregulated to enhance cell survival. Consequently, the overexpression of *ATF4* was not sufficient to mimic the cell survival enhancing effects of DXO, but the induction of oxidative stress by STZ treatment might rather overstrain the cellular stress management.

In contrast to GoF experiments, the suppression of *Atf4* partially attenuated the cell survival enhancing effect induced by chronic DXO treatment in pseudo-islets. These data provide further evidence that ATF4 is at least partially essential for mediating the DXO-induced effects on islet cell protection, or that the residual activity of ATF4 (-84% on mRNA level), which was not targeted by si-*Atf4* transfection is still sufficient to promote cell survival. Notably, the KD of *Atf4* impaired the deviation of the glycolytic flux towards serine synthesis in chronic high-dose DXO treated pseudo-islets (Figure 19). This finding is consistent with studies conducted by DeNicola *et al.*, who observed reduced serine and glycine incorporation in GSH and cystathionine synthesis in cancer cells following *Atf4*-KD.[207] However, these cells remained viable despite the decreased glycolytic flux directed towards serine synthesis, which is considered crucial in cancer cells.[207]

In summary, GoF and LoF experiments of ATF4 conducted in this study highlight its critical role in islet cell physiology, acting as an upstream regulator for serine-linked *mt*OCM genes, impairing GSIS and modulating the glycolytic flux in primary pancreatic islets. Moreover, this study provides evidence that ATF4 partially mediates the DXO-induced islet cell protection, although the overexpression of *ATF4* was shown to be not sufficient to enhance islet cell survival against high doses of STZ. Hence, the potential key regulator responsible for enhancing beta cell survival remains to be identified. However, islet cell dysfunction and dedifferentiation observed upon chronic high-dose DXO treatment could not be rescued by *Atf4* silencing and are likely to be consequences of persistent hyperinsulinemia and indicator for islet cell exhaustion. These findings contribute to a deeper understanding of the intricate regulatory mechanisms behind beta cell functionality.

6.2.2 The serine-linked *mt*OCM regulates GSIS and viability of pancreatic islet cells

Folate is an essential C1-units carrier, which cannot be biosynthesized in mammalian eukaryotic cells.[153] In order to provide sufficient C1-units for numerous cellular key metabolic processes, such as nucleotide synthesis, redox control, recycling of amino acids, and methylation reactions, dietary folate is gradually metabolized within the cytosolic and mitochondrial branches of the OCM.[150,153,161] This process involves *mt*OCM enzymes like SHMT2, MTHFD2, and ALDH1L2.[150,153,161] Therefore, THF is initially generated in presence of the non-essential amino acid (NEAA) serine, which can either derive from dietary uptake or from the ***de novo*** serine synthesis pathway.[153,255,256] THF is further metabolized to 5,10-methyl-THF by SHMT2, to 10-formyl-THF by MTHFD2 and into formate by MTHFD1L or alternatively be converted back into THF by ALDH1L2.[153] These reactions additionally generate glycine, serving for GSH synthesis, and recycle NADPH from $NADP^+$, contributing to proper cell functionality and a healthy redox homeostasis.[153,283] Due to the generation of products and analytes with antioxidant features, both, the entire OCM pathway and the single enzymes involved are considered as crucial in healthy and cancer cells.[161,250,284,285]

To investigate the role of the serine-linked *mt*OCM in pancreatic islets and determine whether it or its metabolites are responsible for the DXO-induced beta cell protection, the whole pathway was targeted by performing single and simultaneous KD of the genes encoding PHGDH, SHMT2, and MTHFD2 (Figure 20 and Figure 21). PHGDH has been reported to regulate cell survival in hepatocytes, neurons, and endothelial cells.[149,199,250-252] Conversely, the KD of ***Shmt2*** is known to impair mitochondrial respiration, and its deficiency is lethal during the embryonic state.[286,287] In turn, Mthfd2 is associated with the regulation of DNA repairing mechanisms.[288-290] Thus, all three enzymes are reported to be critical for cell survival. Contrary to the findings of the mentioned studies, the KD of each individual gene neither enhanced the basal, nor the STZ-induced cell death in pseudo-islets within this study. Considering that the KD of each gene was expected to lead to severe cell death, it is likely that the deletions of these enzymes are circumvented or compensated by related mechanisms to prevent cell death. The complex network of reactions within the OCM includes numerous reversible reactions, homologous isoenzymes, and intercompartmental exchanges, potentially alleviating consequences of single deficient or dysfunctional enzyme.[153,255,256] The existence of intercompartmental homologous isoenzymes catalyzing the same reactions is not yet fully understood in terms of conferring specific advantages, but pathway duplication might be a

plausible and useful tool to compensate dysfunctions in fundamental and critical metabolic pathways such as the folate cycle.[152,160] However, the KD of each individual gene enhances the GSIS of pseudo-islets, aligning with findings from J. Mrugala and S. Krupenko (unpublished data). Both, observed enhanced GSIS and increased plasma insulin levels in ***Aldh1l2***-KO mice after glucose injections.[186] These findings were additionally validated by ***ex vivo*** experiments, using pancreatic islets of these mice.[186] Similar to experiments conducted on ***Aldh1l2***-KO islets, the single KD of ***Phgdh***, ***Shmt2***, and ***Mthfd2*** did neither enhance the susceptibility of pseudo-islets against induced cell-death.[186] As none of the gene KDs was even able to rescue the DXO-induced islet cell dysfunction or to attenuate the protective effect of DXO, these findings suggest that the serine-linked OCM might still deprive glucose intermediates from the TCA cycle after DXO treatment. Residual gene expression activity of the targeted enzymes might be sufficient to provide a robust antioxidant defense to alleviate the STZ-induced oxidative cell stress, as well as compensatory or circumventing processes. On the other hand, these experiments indicate that the pharmacologic action of DXO is not addressable by the KD of single serine-linked *mt*OCM genes.

To induce greater KD effects, ***Phgdh***, ***Shmt2***, and ***Mthfd2*** were knocked-down simultaneously (Figure 21). In comparison to the single KDs, untreated pseudo-islets which carry the triple-KD, displayed a potentiated beneficial effect on GSIS, suggesting that the deficiency of these three *mt*OCM genes might be difficult to compensate or circumvent. Indeed, these si-***Phgdh*** + ***Shmt2*** + ***Mthfd2*** transfected pseudo-islets also displayed decreased viability upon STZ treatment. These findings confirm that the serine-linked *mt*OCM plays a major physiological role in islet cell viability, and that the OCMs functions require a severe impairment, such as a simultaneous KD of three fundamental genes, to be affected. In addition to these experiments, it would have been interesting to investigate whether the opposite effect, namely enhanced viability, could be induced in pseudo-islets which simultaneously overexpress ***Phgdh***, ***Shmt2***, and ***Mthfd2***. An overexpression was also carried out by J. Mrugala, who examined the viability of pseudo-islets overexpressing the human ***ALDH1L2*** gene. However, ***ALDH1L2***-OE pseudo-islets did not exhibit enhanced viability, suggesting that the overexpression or upregulation of one single enzyme involved in such a complex pathway might not be sufficient to observe pro-survival effects.[186]

Notably, simultaneous ***Phgdh***, ***Shmt2***, and ***Mthfd2***-KD was neither sufficient to block the induction of islet cell dysfunction, nor to significantly attenuate the protective effects of

DXO against STZ-induced cell death. These observations apparently suggest that the serine-linked *mt*OCM does not mediate the islet cell protective effects and that other, yet unknown factors might be involved in these effects. However, the residual enzyme activity of the genes might still be sufficient to exert their functions upon chronic high-dose DXO treatment. Additionally, it has been suggested that the cytosolic folate cycle could possibly compensate for the loss of the mitochondrial branch. Hence, the enzymes of the cytosolic pathway might take over the roles of the downregulated *mt*OCM enzymes.[152] Therefore, analyzing gene expression levels of ***Shmt1*** and ***Mthfd1*** in triple-KD pseudo-islets, encoding cytosolic isoenzymes, could potentially provide further insights into the role of the serine-linked *mt*OCM in DXO-mediated cell protection, and might reveal compensatory adaptations within the OCM upon gene-KD.

Taken together, these findings provide valuable insights into the complex regulatory mechanisms behind islet cell functionality and survival. In fact, this study unveiled the role of the serine-linked *mt*OCM in islet cell physiology and survival, possibly also revealing that islets with enhanced GSIS are more vulnerable against cell stress. Moreover, these results suggest that the serine-linked *mt*OCM is not or not solely mediator of DXO's pharmacological action, and that the KD of this metabolic branch is not sufficient to disrupt the machinery underlying the cellular antioxidant defense system, requiring extended investigations into the cytosolic branches of the OCM. In addition, a simultaneous overexpression of multiple *mt*OCM genes, might be a promising approach to further investigate the role of this pathway concerning enhanced islet cell viability.

6.3 The upregulation of serine-linked *mt*OCM genes is a general cellular response to persisting stress induction

6.3.1 The serine-linked *mt*OCM is also inducible by MK-801 and glucolipotoxicity

To evaluate the specificity, reliability, and physiological relevance of the effects induced by chronic treatment with high doses of DXO on pancreatic islets, the upregulation of specific genes was investigated upon an additional NMDAR antagonist and glucolipotoxicity as diabetogenic stressor. For this purpose, RNA-seq or qPCR data, obtained from respective pancreatic islet treatments with MK-801 and high-palmitate + high-glucose were analyzed (Figure 22 and Figure 23).

NMDARs are membrane-bound ionotropic glutamate receptors prevalently located on the surface of neurons, playing a critical role in synaptic transmission and plasticity in the CNS.[291,292] Whereas the role of NMDARs is extensively characterized for neurons, not much is known about their function on other tissue cell types, including beta cells. Notably, studies suggest a controversial and much more complex role of NMDARs on pancreatic islets, which is not yet fully understood.[16] In neurons and synapsis, NMDAR hypofunction is associated with ER- and oxidative stress induction by increased ROS production and GSH depletion.[293-295] In contrast, sustained NMDAR activation is reported to mediate a vast number of negative effects in beta cells functionality and survival, reaching from the induction of the ATF4-associated UPR branch, of beta cell dedifferentiation and dysfunction, finally leading to apoptosis.[109,195,296] However, treatment of diabetic mice and dysfunctional beta cells using NMDAR antagonists memantine or MK-801, are reported to enhance pancreatic islet cell survival and reverse islet dysfunction, subsequently to prolonged high-glucose treatment.[109] Therefore, NMDAR antagonists have been proposed as potential anti-diabetic drugs.[182,184]

MK-801 is a selective and specific inhibitor of NMDAR, which was demonstrated to enhance GSIS via K_{ATP} channels in pancreatic islets, after acute (1 h) treatment.[184,297] The RNA-seq analysis revealed that the inhibition of NMDAR by chronic (48 h) treatment with 10 µM MK-801 induces similar, but less pronounced upregulation of genes encoding for members of the ATF4-associated UPR branch and those of the serine-linked *mt*OCM, compared to chronic treatment with 10 µM DXO (Figure 22). Since DXO is a less selective NMDAR antagonist, these results suggest that other mechanisms might also contribute to the induction of islet dysfunction, the strong upregulation of serine-linked *mt*OCM genes, and the enhancement of cell survival. In fact, blocking the NMDAR is likely to be not sufficient to achieve these effects, as pancreatic islets of mice lacking the *Grin1* gene (GluN1-KO), which encodes the essential NMDAR subunit GluN1, did not exhibit protection against cytokines.[186] However, viability assays with pancreatic islets of GluN1-KO mice demonstrated that functional NMDARs are indispensable for the DXO-mediated islet cell protection against cytokine induced cell death.[186] Even though cell protective features of MK-801 were not scrutinized in this study, previous notion and here presented data suggest that NMDAR inhibition by DXO is not uniquely responsible for enhanced cell survival. In this context, the persisting cell stress situations, such as those induced by chronic high-dose DXO treatment and the resulting hyperinsulinemia, might underly the strong cell protection conferred by DXO.

Chronically elevated concentrations of glucose and fatty acids have deleterious effects on beta cells, resulting in strongly impaired GSIS and beta cell death.[298-300] In combination, both factors develop a synergistic effect, which unfolds a more severe toxicity, termed glucolipotoxicity.[96,301,302] As glucolipotoxicity induces endogenous cell stress leading to beta cell failure, it is considered the actual key factor and driver of T2DM.[32,59] To scrutinize whether chronic high-dose DXO treatment revealed new physiological insights of beta cells or induces non-physiological artifacts, it was crucial to validate the transcriptomic changes induced by DXO in a typical diabetogenic milieu, through high-palmitate + high-glucose treatment (Figure 23). As expected and previously reported, high-palmitate + high-glucose treatment induced a robust upregulation of almost all analyzed genes. In fact, chronic lipotoxicity was already reported to induce ER stress via the ATF4-associated UPR branch, and to enhance oxidative stress.[303,304] The upregulation of ***Shmt2***, ***Mthfd2*** and ***Aldh1l2*** is also in line with the previous notion that glucolipotoxicity induces serine-linked *mt*OCM genes.[100,305,306] Even though this study did neither analyze islet cell functionality nor survival upon high-palmitate + high-glucose treatment, the strong upregulation of ***Atf4*** and the serine-linked *mt*OCM genes might indicate that islet cells undergo a metabolic adaptation to alleviate the induced cell stress, potentially reducing the secretory function. However, literature does not provide useful insights in regard of possible expectations. Although numerous studies have previously been conducted, investigating the effects of glucolipotoxicity on pancreatic islets, the overall emerging conclusion is that numerous factors, including the used lipid species, the treatments duration, the free fatty acids concentrations, the type of glucose used within the glucolipotoxic treatment, as well as the susceptibility of the isolated pancreatic islets are likely to induce different outcomes.[96,307] Therefore, the herein missing analyses on GSIS and islet cell survival upon glucolipotoxic treatment have to be performed according to the herein used methodology, in order to interlink the upregulation of ***Atf4*** and the serine-linked *mt*OCM genes with the achieved effects and for further data interpretation.

Overall, the inhibition of NMDAR and chronic exposure of pancreatic islets to diabetogenic stress contributed to the same transcriptomic changes as observed upon chronic high-dose DXO treatment. These results provide preliminary evidence that the upregulation of the serine-linked *mt*OCM and the unveiled pathway might play a universal and pivotal role in beta cell functionality and physiology upon different GSIS-inducing and cellular stress conditions.

6.3.2 Persistent hyperinsulinemia induced by K_{ATP}-dependent insulin secretagogues enhances islet cell survival at the expense of their functionality

As previously discussed in chapter 6.3.1, the serine-linked *mt*OCM is also enhanced independently from DXO treatment and NMDAR inhibition. To evaluate whether insulin hypersecretion is a general trigger of *Atf4* upregulation, islet dysfunction, and enhanced islet cell survival, the investigations on pancreatic mouse islets were extended to chronic treatment with insulin secretagogues Glib and Ex-4 (Figure 24, Figure 25, and Figure 26).

Although acute administration of insulin secretagogues, including DXO, Glib and Ex-4, has been shown to effectively restore impaired GSIS, prolonged and persistent use of insulin secretagogues often proves to be ineffective in the long-term, leading to a decline in islet cell functionality.[39,175,178] Ultimately, patients with severe T2DM require insulin injections to compensate for the loss of islet secretory function. Therefore, it was interesting to investigate whether these effects may underly the same metabolic adaptations as observed upon chronic high-dose DXO treatment. Notably, continuous treatment with Glib induces a comparable islet dysfunction, decreased insulin content, and the upregulation of gene expression levels as chronic high doses of DXO, whereas Ex-4 has still GSIS enhancing properties without affecting the insulin content (Figure 24). Interestingly, Remedi and Nichols also reported about Glib-induced islet dysfunction after prolonged treatment.[176] Contrary to their expectations, this dysfunction did not lead to islet cell apoptosis but was instead found to be rapidly reversible.[176] In addition to their observations, this study reveals that chronic treatment with Glib also protects pancreatic islet cells from STZ-induced cell death, similar like DXO. As both secretagogues induce insulin secretion via K_{ATP} channels, the enhanced islet cell survival might potentially be induced by a common MOA, via the upregulation of the serine-linked *mt*OCM (Figure 25).

In contrast to this study, other studies report that Ex-4 improves beta cell survival during ER stress, in primary human-, *db/db* and rat's islets.[113,308] Since continuous Ex-4 treatment also shows only slight and few significant upregulation of some serine-linked *mt*OCM genes, it is likely that this upregulation is not sufficient to induce survival enhancing effects, even less against high-dose STZ-induced cell death (Figure 26).[113] Even though gene expression levels of *Phgdh*, *Mthfd2*, and *Aldh1l2* were significantly upregulated by Ex-4, the fold changes are very low compared to those induced by Glib treatment. It would have been worth to use higher Ex-4 concentrations, to possibly induce both beneficial effects which are

associated with Ex-4 and GLP-1R agonists in general, namely enhanced GSIS and islet cell survival.[113,309] However, it was reported that human islets which were transplanted into the anterior chamber of the eyes of mice, developed signs of dysfunction when treated daily with a GLP-1R agonist for 250 days.[177] The slight upregulation in gene expression levels potentially indicate a developing islet dysfunction, which might progress and onset after a longer incubation.

Taken together, the chronic treatment with insulin secretagogues Glib and Ex-4 revealed that those which act via the K_{ATP}-dependent MOA, such as Glib and DXO, strongly induce ***Atf4*** and serine-linked *mt*OCM gene expression levels, and enhance islet cell survival against STZ-induced cell stress. Conversely, these insulin secretagogues also induce islet cell dysfunction in a long term. These results once more suggest that the unveiled metabolic pathway is part of a general physiological mechanism of pancreatic islet cells which prioritize survival over functionality. However, the activation of this pathway is probably strictly reliant on the duration and concentration of the insulin secretagogues treatment. Therefore, it would be interesting to investigate, whether longer exposure or higher concentrations of Ex-4 would as well induce this pathway, to unveil whether enhancing islet cell dysfunction at the expense of secretory function is only limited to insulin secretagogues which trigger the K_{ATP}-dependent GSIS. Nevertheless, to obtain final proof for prolonged hyperinsulinemia's role in the upregulation of serine-linked *mt*OCM genes and enhancing islet cell survival, it would also be interesting to analyze whether blocking the secretion of insulin would impede the induction of islet dysfunction.

Overall, these findings might contribute to a better understanding of the complex mechanisms governing islet cell functionality and dysfunction in diabetes, potentially helping to improve diabetes management or find novel therapeutic approaches.

6.4 The role of the NRF2-mediated antioxidant response in islet cell functionality and survival

This study provides numerous data which collectively prove the existence of a universal metabolic pathway which intricately links the functionality and survival of pancreatic islet cells through the glycolytic flux. This pathway enhances islet cell survival at the expense of secretory function in response to hyperinsulinemia and probably to the resulting excessive ROS generation. Even though ATF4 and enzymes of the serine-linked *mt*OCM were identified as key players of this metabolic pathway, GoF and LoF experiments did not point to a specific primary mediator of the induced effects. Instead, these experiments rather suggest the involvement of an additional, yet unknown factor in this intricate interplay.

Recent scientific literature provide numerous publications pointing to the pivotal role of the nuclear factor erythroid-2-related factor 2 (NRF2) in various disease contexts, including neurodegenerative disorders, cardiovascular diseases, cancer, and diabetes mellitus.[310-314] Notably, NRF2 is directly associated with ATF4 and the upregulation of genes encoding enzymes of the serine-linked *mt*OCM.[111,207] This transcription factor is crucial for the induction of cellular defense mechanisms against oxidative stress and inflammation. In fact, it is strictly related to the ***de novo*** generation and recycling processes of glutathione.[207,313,315] NRF2 is a transcription factor, which undergoes nuclear translocation after activation by the UPR, the induction of ATF4, or by elevated intracellular ROS levels.[111,207,316] Due to its multifaceted roles, NRF2 has emerged as a particular interesting factor within the specific field of research of DM. In fact, NRF2 was found to be a pivotal regulator of the antioxidant response, survival, and proliferation of beta cells.[310,317] Therefore, the transcription factor was even proposed to be considered a potential biomarker for the identification of individuals with compromised cellular stress responses and an elevated susceptibility to DM.[311] Moreover, due to its additional beneficial effects on peripheral tissues, such as improving insulin sensitivity and induce body weight reduction, the pharmacologic activation of NRF2 was suggested as a possible approach for novel therapeutic intervention, underlining its potential in the treatment of DM.[311]

Although NRF2 is commonly praised to promote beta cell functionality and survival, and to potentially predict or even reverse DM, its prolonged activation leads to adverse side effects. In fact, prolonged NRF2 activation and in general the chronic demand for antioxidants is thought to disrupt critical and functional ROS signaling pathways, ultimately resulting in

reduced GSIS and dysfunction.[261,318-320] Most notably, NRF2 has also been found to mediate deviations of the glycolytic flux from the TCA cycle into the serine-linked OCM in cancer cells, through the upregulation of some of the genes reported within this study.[207]

Starting from the premise that neither NRF2 nor the redox homeostasis have been analyzed in this study, and considering the numerous parallels between the NRF2 action and the effects induced by treatments with acute, chronic, low-, and high doses of DXO and other GSIS-enhancing agents, this transcription factor could indeed represent the yet unknown factor which directly interlinks islet cell functionality and survival. Therefore, its prolonged activation could potentially explain the presence of a reduced functional beta cell mass, which was observed in several T2DM patients.[14,229,239,240] Furthermore, a strongly enhanced antioxidant system could potentially underly certain forms of T2DM, thus removing the cause of oxidative stress, by drastic caloric restrictions might result in a successful remission. Last but not least, NRF2 is also associated to the beneficial effects of GLP-1, in a dose dependent manner.[321]

Taken together, NRF2 and its induced adaptive antioxidant response emerge to be intricately involved in the molecular pathway, which interlinks islet cell functionality and survival. Even though NRF2 was not analyzed within this study, the activation of NRF2, which is likely but not proven to be achievable by chronic high-dose DXO treatment, potentially acts as a mediating factor in the upregulation of the serine-linked *mt*OCM pathway and its concomitant effects. Given that the upregulation of the NRF2-mediated antioxidant response seems to be a general cellular adaption to persistent and strong GSIS induction, the induction of islet dysfunction by the herein presented metabolic pathway could be considered a universal defense mechanism of islet cells against elevated ROS levels. Hence, the induction of this mechanism emerges to be triggered by various agents which excessively enhance the GSIS, beyond chronic high-dose DXO treatment. Furthermore, the uncovered pathway seems to be reversible by removing the underlying cause of cell stress, as demonstrated within this study. Thus, reducing the cause for the enhanced antioxidant response, which might be achieved by a tight glycemic control or reducing the caloric uptake, thereby alleviating hyperinsulinemia and oxidative stress, emerges to be the key to maintain or potentially restore islet cell functionality. However, further experiments on NRF2-mediated antioxidant response need to be conducted, to causally link NRF2 to DXO's MOA.

6.5 Conclusion

In conclusion, this comprehensive study provides novel insights into the physiology of islet cells and the critical role of the serine-linked *mt*OCM pathway in the regulation of islet cell functionality and survival. This study provides robust evidence that chronic high-dose DXO treatment induces the ATF4-associated UPR branch, resulting in a strong upregulation of the serine-linked *mt*OCM. The conducted experiments confirmed a previously hypothesized metabolic model that links the upregulation of ATF4 and serine-linked *mt*OCM enzymes to the induction of reversible islet dysfunction and enhanced survival against STZ-induced cell death. In this context, the analyses of the glycolytic flux indicates a deviation of glucose intermediates from the TCA cycle towards the serine-linked OCM, at the basis of induced islet cell dysfunction and enhanced cell survival. However, pathway manipulations assessed by GoF and LoF experiments targeting ***Atf4***, ***Phgdh***, ***Shmt2***, or ***Mthfd2***, did not reveal mediative roles of any of the analyzed genes in the DXO conferred islet cell protection.

Investigations of gene expression levels, GSIS, and islet cell survival upon STZ revealed the upregulation of the validated pathway in DXO independent treatments. Notably, chronic treatment with the sulfonylurea glibenclamide induced a strong upregulation of serine-linked *mt*OCM genes and enhanced islet cell survival at the expense of GSIS, comparable to chronic high-dose DXO treatment. These results suggest that the induction of the analyzed metabolic pathway is a general mechanism in islet cells responding to various treatments that induce persistent and excessive insulin secretion. Although no analyses were performed regarding cellular redox homeostasis or the quantification of ROS, several indications point to these metabolic adaptations being triggered by excessive ROS and the resulting antioxidant response, potentially mediated by NRF2.

Overall, this study provides deep insights into the metabolic regulation of islet cell functionality and survival, highlighting that impaired GSIS in early-onset T2DM might potentially depend on a reduced functional beta cell mass rather than apoptosis. The reversibility of this mechanism suggests that reducing caloric intake and maintaining tight glycemic control, thus alleviating hyperinsulinemia, could reverse early-onset T2DM or prevent further progression of late-onset T2DM. Therefore, this pathway might hold crucial implications for translational opportunities and treatments aiming at preserving islet cell functionality and survival. Elucidating these molecular interlinkages might pave the way for the development of preventive interventions for T2DM.

6.6 Outlook

In the intricate puzzle of islet cell physiology, this study contributes a crucial piece by revealing a novel metabolic pathway that links functionality and survival in various contexts, while also identifying key regulators. However, there are still missing elements waiting to be discovered, and intriguing aspects that are worthful to be explored.

To ultimately prove that the unveiled mechanism is a general metabolic pathway triggered by persistent and excessive GSIS induction, the analyses on chronic Ex-4-treated islets should be repeated at higher concentrations, inducing a serine-linked *mt*OCM gene upregulation at comparable levels as Glib. If secretory dysfunction cannot be induced, the metabolic pathway would exclusively underlie the induction of K_{ATP} dependent GSIS-inducing agents.

Although the serine-linked *mt*OCM was identified to regulate the viability and survival of pancreatic islet cells against cell death induction, the mediating key factor responsible for DXO-induced cell protection against STZ-induced cell death remains to be identified. Due to its strong anti-oxidant features, enhanced GSH levels, and its role in enhanced islet cell survival against oxidative stress, would be interesting to be investigated. Therefore, LoF experiments targeting the GSH-GSSG recycling enzyme glutathione reductase (GSR) or the cysteine generating cystathionine gamma-lyase (CTH), serving for GSH generation in combination to a sensitive mass-spectrometric, thiol quantifying read-out analysis and viability assays could unveil the crucial role of GSH in the regulation of the islet cell redox state and enhanced survival, induced by DXO. Further, its upstream regulator NRF2 requires to be investigated by a methodology which additionally comprise the analysis of the glycolytic flux, to determine whether it mediates the DXO-induced effects to a similar or even greater extent to ATF4.

Furthermore, investigating the compensatory actions within the cytosolic and mitochondrial folate cycle of the OCM could reveal interesting insights. Although challenging due to the OCM's complexity, analyzing expression levels of the cytosolic isoenzymes, SHMT1 and MTHFD1, in untreated and chronic high-dose DXO treated si-*Phgdh* + *Shmt2* + *Mthfd2* transfected pseudo-islets could be considered feasible to reveal intriguing adaptations. This analysis may reveal intriguing adaptations aimed at maintaining the viability of severely dysfunctional islets and potentially reveal new targets for DM therapeutic strategies.

Within this thesis, a prolonged and excessive insulin secretion was hypothesized as the main trigger for islet cell dysfunction, the upregulation of the serine-linked *mt*OCM pathway,

and the resulting cell protection against STZ-induced cell death. To analyze whether these effects underly the chronic high-dose DXO treatment, it would be interesting to perform the DXO-treatment on islets with pharmacologic inhibited insulin secretion. Therefore, DXO treated islets could be pre- or co-treated for example with diazoxide or nifedipine and be used for viability assays, gene expression-, and glycolytic flux analyses. In case of no cell protection, no upregulation of serine-linked *mt*OCM genes, or no glycolytic flux deviation, these experiments would causally link all effects to the DXO-induced insulin hypersecretion and ultimately reveal the cell protection against cell death induction as a consequence of the glycolytic flux deviation into the upregulated OCM.

A less feasible but theoretically research-worthy approach, is investigating the role of OCM and the adaptive antioxidant response in islets of T2DM patients. Considering that islet dysfunction is only one key characteristic of T2DM besides insulin resistance, it could be interesting to analyze the viability and serine-linked *mt*OCM gene expression levels of pancreatic islets from donors displaying secretory dysfunctions or obesity-related diabetes. These islets could additionally be used to investigate reversibility by culturing them to low glucose media (5 – 10 mM glucose), previously to a GSIS assay. However, these approaches remain elusive, due to potential artifacts generated during the islet isolation and ***ex vivo*** culturing.

7 Publications

The data shown within this dissertation were used for following publication:

Pelligra A.*, Mrugala J.*, Griess K.*, Kirschner P., Nortmann O., Bartosinska B., Köster A., Krupenko N.I., Gebel D., Westhoff P., Steckel B., Eberhard D., Herebian D., Belgardt B-F., Schrader J., Weber A.P.M., Krupenko S.A., Lammert E. **Pancreatic islet protection at the expense of secretory function involves serine-linked mitochondrial one-carbon metabolism**. Cell Reports *42*, doi.org/10.1016/j.celrep.2023.112615, 2023.
*equally contributed

Other publications:

Griess K., Rieck M., Muller N., Karsai G., Hartwig S., Pelligra A., Hardt R., Schlegel C., Kuboth J., Uhlemeyer C., Trennkamp S., Jeruschke K., Weiss J., Peifer-Weiss L., Xu W,. Cames S., Yi X., Cnop M., Beller M., Stark H., Kondadi A.K., Reichert A.S., Markgraf D., Wammers M., Lickert H., Lammert E., Roden M., Winter D., Al-Hasani H., Höglinger D., Hornemann T., Brüning J.C., Belgardt B-F. **Sphingolipid subtypes differentially control proinsulin processing and systemic glucose homeostasis**. Nature Cell Biology *25*, doi.org/10.1038/s41556-022-01027-2, 2022.

Kuhn T., Kaiser K., Lebek S., Altenhofen D., Knebel B., Herwig R., Pelligra A., Görigk., Khuong J.M-A., Vogel H., Schürmann A., Blüher M., Chadt A., Al-Hasani H. **Comparative genomic analyses of multiple back-cross mouse populations suggest SGCG as a novel potential obesity-modifier gene**. Human Molecular Genetics *31*, doi.org/10.1093/hmg/ddac150, 2022.

8 List of Abbreviations

A

AC	Adenylate cyclase
Ad	Adenovirus
ADP	Adenosine diphosphate
AGE	Advanced glycation end
ALDH1L1	Aldehyde dehydrogenase 1 family member L1
ALDH1L2	Aldehyde dehydrogenase 1 family member L2
ANOVA	Analysis of variant
Atf4	Activating transcription factor 4
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate

B

B9	Folic acid
BG	Blood glucose
BiP	Binding immunoglobulin protein
BMI	Body-mass index
bp	Base pairs
BSA	Bovine serum albumin

C

C1	One-carbon unit
Ca^{2+}	Calcium
$CaCl_2$	Calcium chloride
cAMP	Cyclic adenosine monophosphate
CC3	Cleaved Caspase 3
cDNA	Complementary DNA
CHOP	C/EBP homologous protein
CMRL	Connaught medical research laboratories medium 1066

CMV	Cytomegalovirus
CNS	Central nerve system
CO_2	Carbon dioxide
C_T	Cycle of threshold
CTH	Cystathionine gamma-lyase
Ctrl	Control

D

db/db	Mouse model for obesity and T2DM
Ddit3	*DNA damage inducible transcript 3* (CHOP)
DDZ	Deutsches Diabetes Zentrum
DIP	Diabetes mellitus in pregnancy
DM	Diabetes mellitus
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
DNSS	*De novo* serine synthesis
DPBS	Dulbecco's phosphate-buffered saline
DPP-4	Dipeptidyl peptidase 4
DXM	Dextromethorphan
DXO	Dextrorphan

E

EDTA	Ethylenediaminetetraacetic acid
eIF2α	eukaryotic initiation factor 2
ELISA	Enzyme-linked immuno-sorbent assay
ER	Endoplasmic reticulum
ETC	Electron transport chain
EthD-1	Ethidium homodimer-1
Ex-4	Exendin-4

F

FFA	Free fatty acid
f-THF	Formyl-tetrahydrofolate
FBS	Fetal bovine serum
FC	Fold change
Fgf21	*Fibroblast growth factor 21*
FP	Forward primer
FPKM	Fragments per kilobase of transcript per million mapped reads
FVS	Fixable viability stain

G

GC-MS	Gas chromatography-mass spectrometry
GDM	Gestational diabetes mellitus
GDS	German Diabetes Study
GFP	Green fluorescent protein
Glib	Glibenclamide
GLP-1	Glucagon-like peptide 1
GLP-1R	Glucagon-like peptide 1 receptor
GluN1	Subunit of the NMDAR (*Grin1*)
GluN1-KO	Knockout of *Grin1* NMDAR subunit
GLUT-2	Glucose transporter 2
GoI	Gene of interest
GoF	Gain-of-function
GPx	Glutathione peroxidase
Grin1	*Glutamate ionotropic receptor NMDA type subunit 1* (GluN1)
GRP78	78 kDa glucose-regulated protein (Hspa5, BiP)
GSH	Glutathione (reduced)
GSIS	Glucose-stimulated insulin expression
GSR	Glutathione reductase
GSSG	Glutathione (oxidized)

H

h	hours
H_2O	Dihydrogen monoxide (water)
H_2O_2	Hydrogen peroxide
Hb	hemoglobin
HbA1c	Glycated hemoglobin A1c
HBSS	Hank's Balanced Salt Solution
HCl	Hydrogen chloride
HEPES	Hydroxyethylpiperazine ethane sulfonic acid
Herpud1	*Homocysteine inducible ER protein with ubiquitin like domain 1*
HIP	Hyperglycemia in pregnancy
Hprt	*Hypoxanthine-guanine phosphoribosyl transferase*
HRP	Horseradish peroxidase
Hspa5	*Heat Shock Protein Family A (Hsp70)* Member 5 (Grp78, BiP)

I

IDDM	Insulin-dependent diabetes mellitus
IDF	International Diabetes Federation
INS-1E	Rat insulinoma beta cell line
Ins1	*Insulin 1* (Insulin)
Ins2	*Insulin 2* (Insulin)
IRE1	Inositol-requiring enzyme 1

K

K_{ATP}	ATP-sensitive potassium channels
KCl	Potassium chloride
KD	Knockdown
kDa	Kilo Dalton
KO	Knockout
KRH	Krebs Ringer

L

LANUV	Landesamt für Natur, Umwelt und Verbraucherschutz NRW
LoF	Loss-of-function
Log_2 FC	Binary logarithmic Fold Change

M

M	Molar
m-THF	Methyl-tetrahydrofolate
M+0 – M+5	Isotopologues
MafA	*MAF BZIP transcription factor A*
Mapk1	*Mitogen-activated protein kinase 1*
MARD	Mild age-related diabetes
min	Minute
MIN6	Mouse insulinoma cells
MK-801	Dizocilpine
MOA	Mechanism of action
MOD	Mild obesity-related diabetes
MODY	Maturity-onset of diabetes of the young
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
mt	Mitochondrial
*mt*OCM	Mitochondrial one-carbon metabolism
MTHFD1	Methyl-Tetrahydrofolate dehydrogenase 1
MTHFD1L	Methylenetetrahydrofolate Dehydrogenase ($NADP^+$) 1 Like
MTHFD2	Methyl-Tetrahydrofolate dehydrogenase 2
MTHFR	Methyl-Tetrahydrofolate reductase
MTR	Methionine synthase
mTORC1	Mammalian target of rapamycin complex 1

N

Na^+	Sodium
NaCl	Sodium chloride
NAD(H)	Nicotinamide adenine dinucleotide
NADP(H)	Nicotinamide adenine dinucleotide phosphate
NaF	Sodium fluorid
$NaHCO_3$	Sodium bicarbonate
NEAA	Non-essential amino acid
NDM	Neonatal diabetes mellitus
NFDM	Non-fat dried milk
Nkx6.1	*NK6 homeobox 1*
NMDA	N-methyl D-aspartate
NMDAR	NMDA receptor
NOD	Non-obese diabetic mouse model
NRF2	Nuclear factor erythroid-2-related factor

O

O^{2-}	superoxide anion
OCM	One-carbon metabolism
OE	Overexpression
OGTT	Oral glucose tolerance test
OH^-	hydroxyl radical

P

3-PG	3-Phospho-glycerate
PBS	Phosphate-buffered saline
P_{CMV}	Promoter of cytomegalovirus
Pcna	*Proliferating-cell-nuclear-antigen*
PCR	Polymerase chain reaction
Pdx1	*Pancreatic and duodenal homeobox 1*
PERK	Protein kinase RNA-like ER kinase

PFA	Paraformaldehyde
PHGDH	Phosphoglycerate dehydrogenase
Pi	Phosphate
PKA	Protein kinase A
PP	Pancreatic polypeptide
PSAT1	Phosphoserine aminotransferase
PSPH	Phosphoserine phosphatase
PVDF	polyvinylidene difluoride

Q

qPCR	Quantitative real-time polymerase chain reaction

R

RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RNA-seq	RNA-sequencing
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RP	Reverse primer
RT	Room temperature
RT-PCR	Reverse-transcription polymerase chain reaction

S

SAM	S-Adenosyl methionine
SEM	Standard error of the mean
SHMT1	Serine hydroxy methyltransferase 1
SHMT2	Serine hydroxy methyltransferase 2
SIDD	Severe insulin deficient diabetes
SIRD	Severe insulin-resistant diabetes
si-RNA	Small interfering ribonucleic acid
Slc2a2	*Solute carrier family 2 member 2* (GLUT-2)

SOD	Superoxide dismutase
Stat3	*Signal transducer and activator of transcription* 3
STZ	Streptozotocin

T

T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TCA	Tricarboxylic acid cycle
Td	Tandem dimer
THF	5,6,7,8-Tetrahydrofolate
Trib3	*Tribbles pseudokinase 3*

U

[U-^{13}C]	Ubiquitously labeled with Isotope 13 of Carbon atom
U	Units
UPLC	Ultra-performance liquid chromatography
UPR	Unfolded protein response

V

V	Volt
VAT	Visceral adipose tissue
VDCC	Voltage-gated Ca^{2+} channel

9 References

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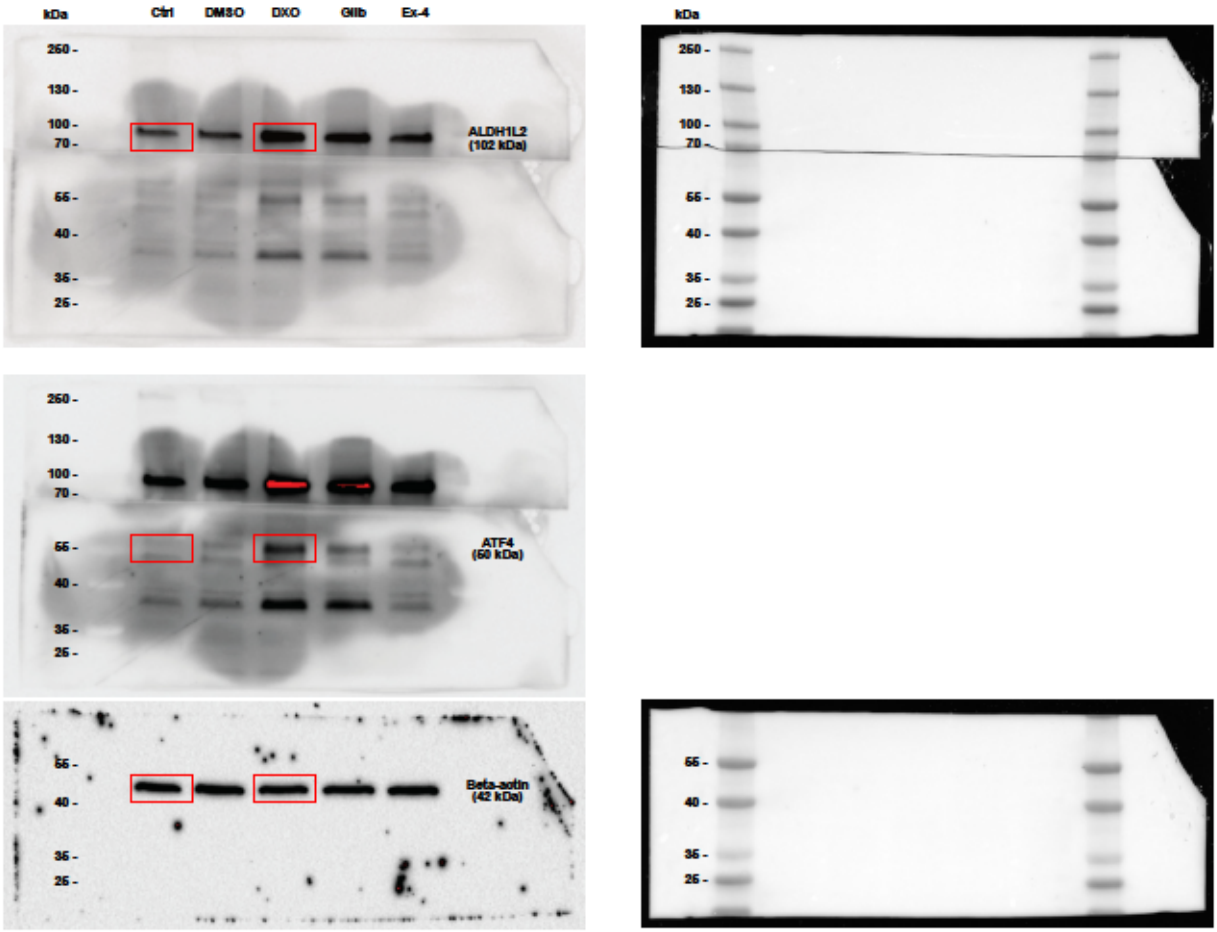
10 Supplementary Information

10.1 RNA-sequencing

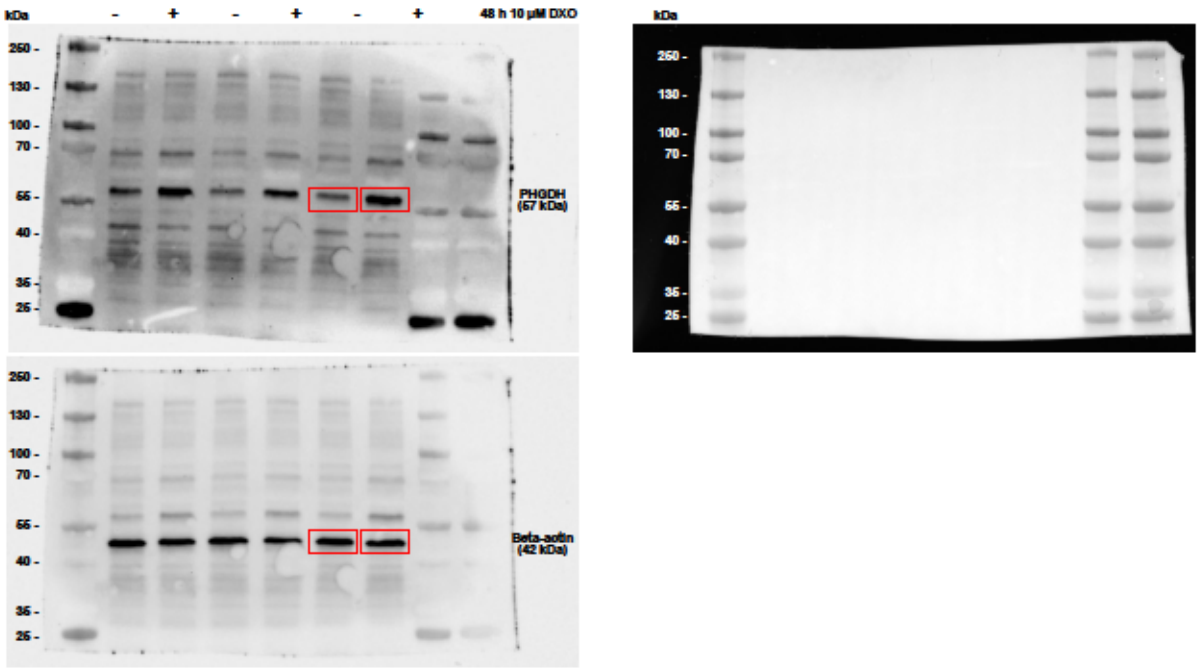
All RNA-sequencing raw and analyzed data presented in this study are published in Pelligra *et al.* and have been deposited on Mendeley Data and can be accessed through the following links[209]: https://data.mendeley.com/datasets/4rwmrc9cpr/4
https://data.mendeley.com/datasets/g4bdvw6czr/2

10.2 Western blots

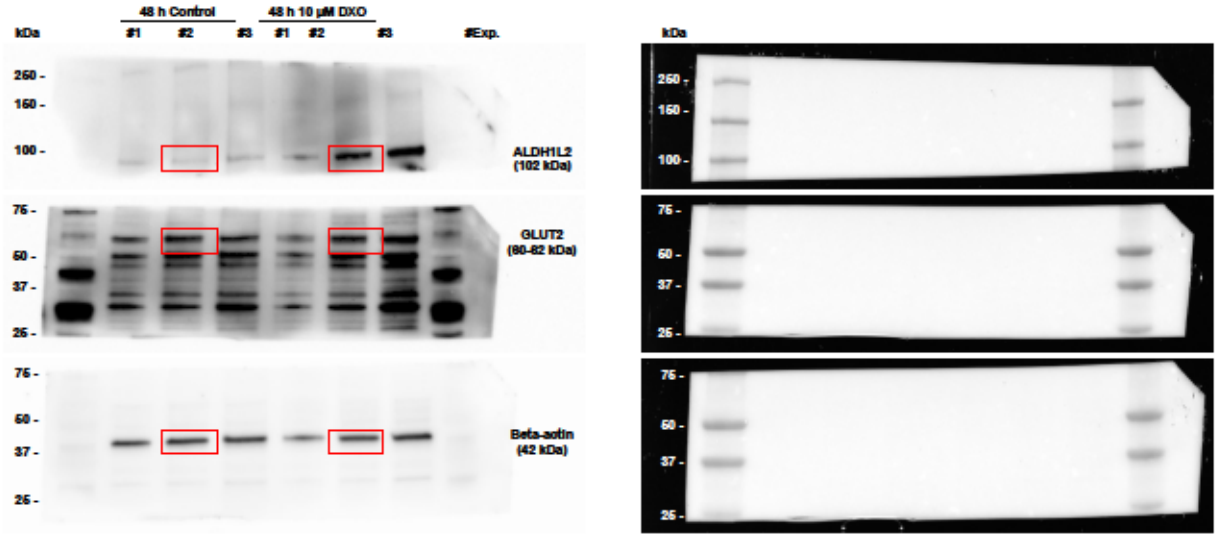
Fully uncropped western blot images shown in this study:



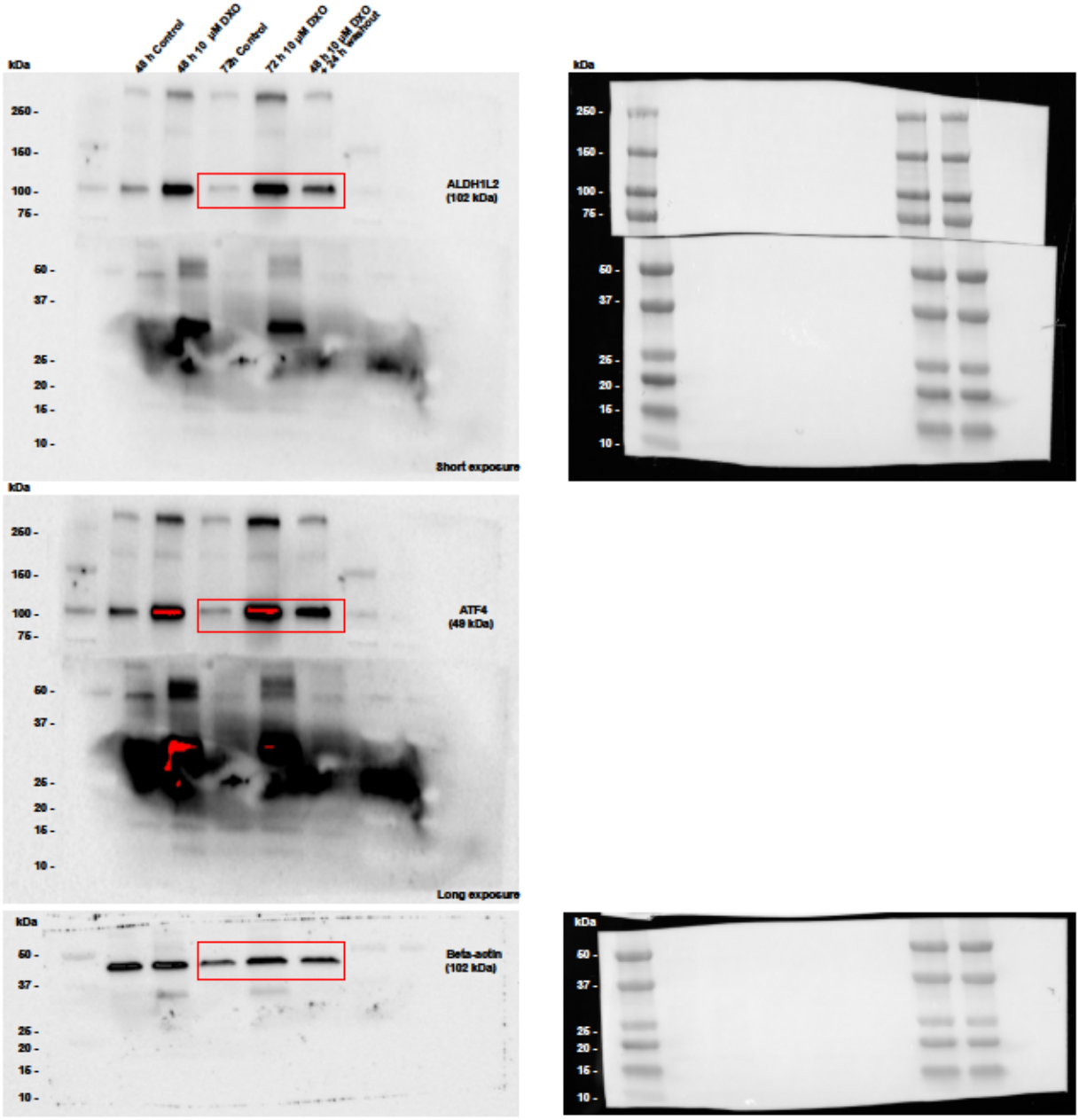
Supplementary Figure 1 to Figure 12B and H: Uncropped western blot images with molecular size marker.
Highlighted regions are depicted in the corresponding figures.



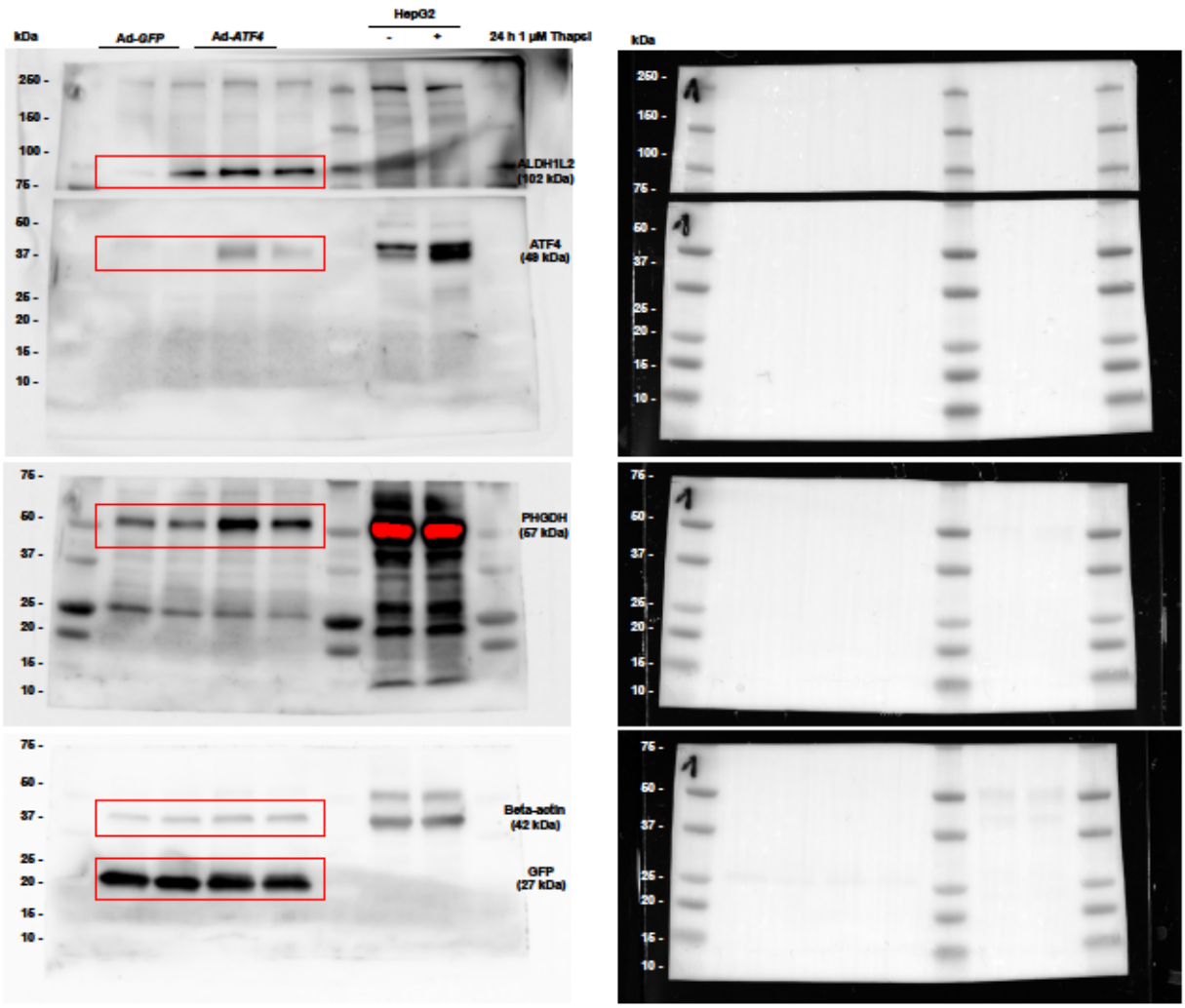
Supplementary Figure 2 to Figure 12E: Uncropped western blot images with molecular size marker.
Highlighted regions are depicted in the corresponding figures.



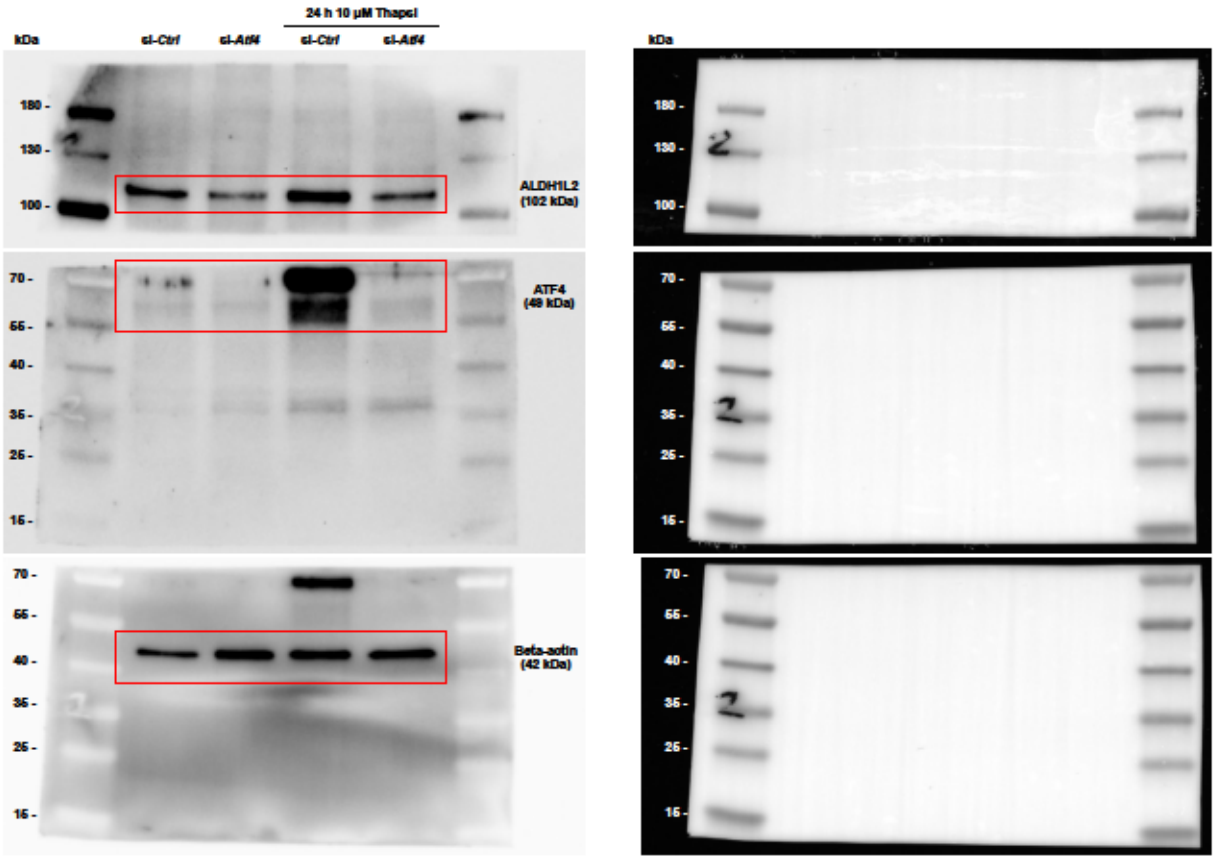
Supplementary Figure 3 to Figure 12K: Uncropped western blot images with molecular size marker.
Highlighted regions are depicted in the corresponding figures.



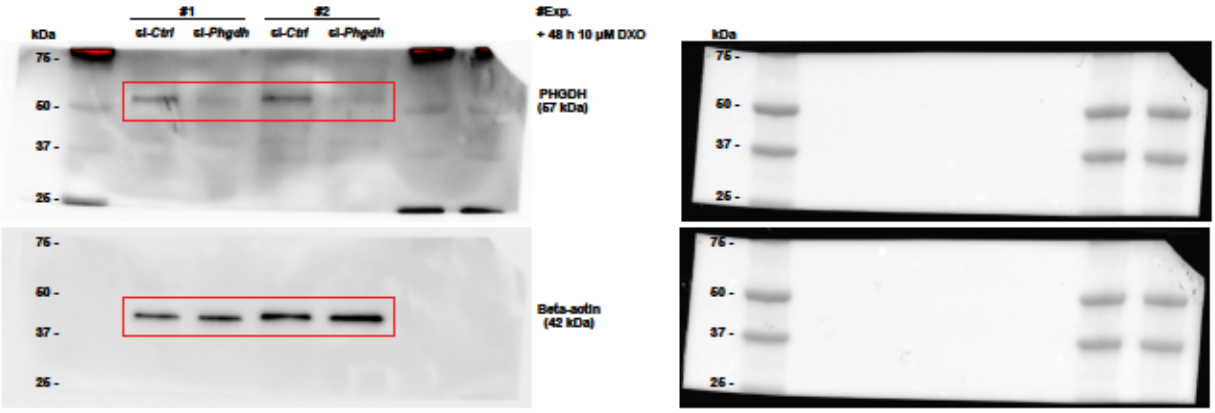
Supplementary Figure 4 to Figure 16A: Uncropped western blot images with molecular size marker.
Highlighted regions are depicted in the corresponding figures.



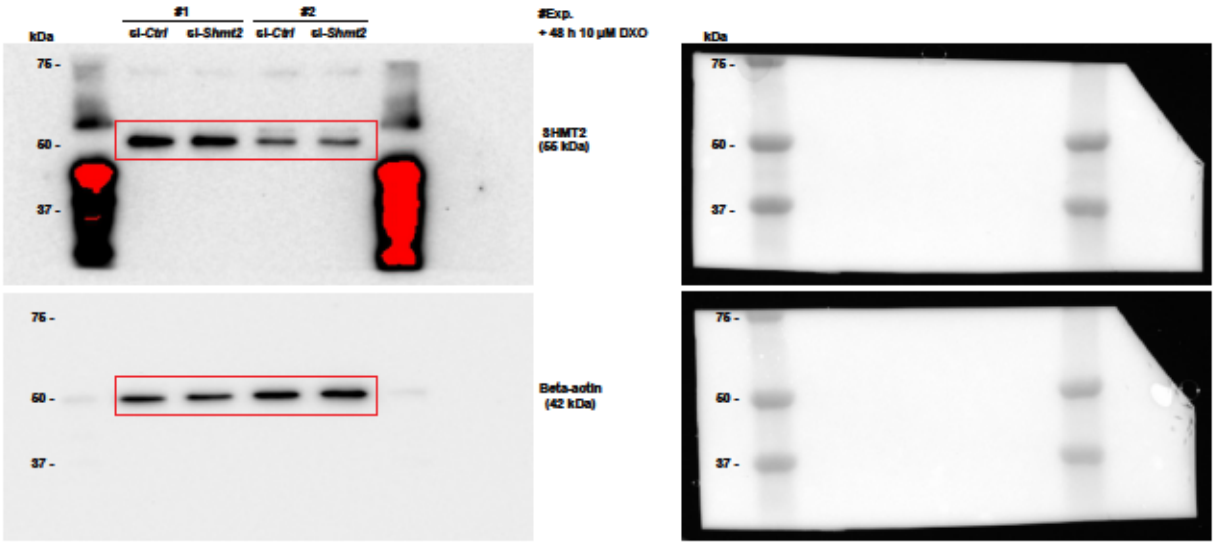
Supplementary Figure 5 to Figure 17B: Uncropped western blot images with molecular size marker.
Highlighted regions are depicted in the corresponding figures.



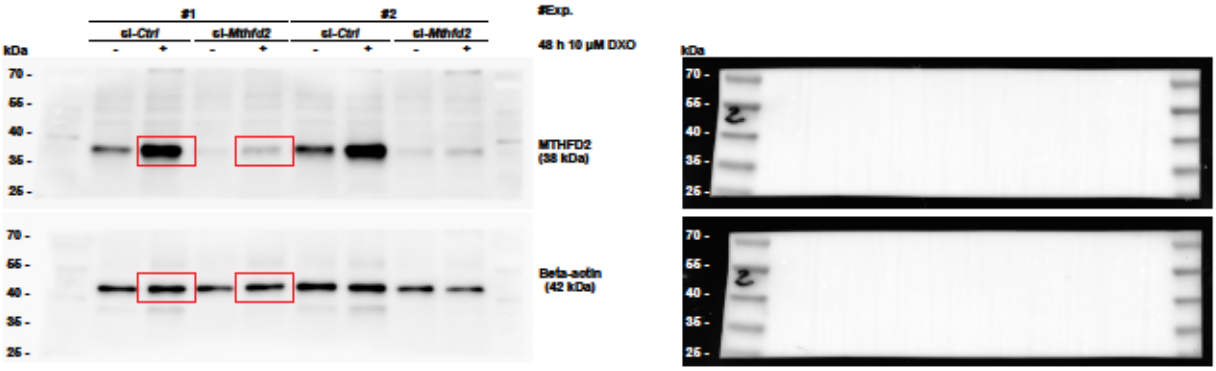
Supplementary Figure 6 to Figure 18B: Uncropped western blot images with molecular size marker.
Highlighted regions are depicted in the corresponding figures.



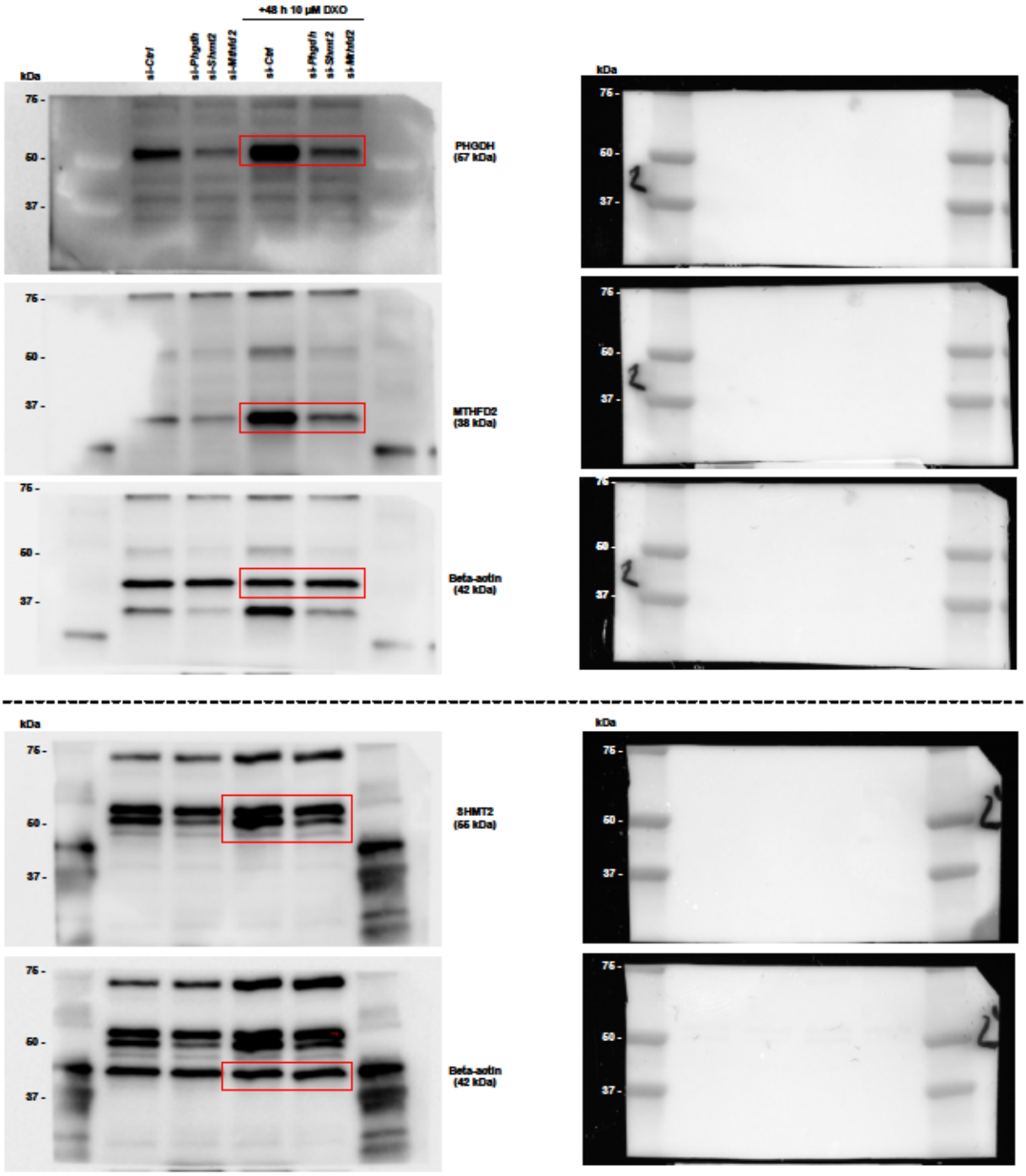
Supplementary Figure 7 to Figure 20A: Uncropped western blot images with molecular size marker.
Highlighted regions are depicted in the corresponding figures.



Supplementary Figure 8 to Figure 20D: Uncropped western blot images with molecular size marker.
Highlighted regions are depicted in the corresponding figures.



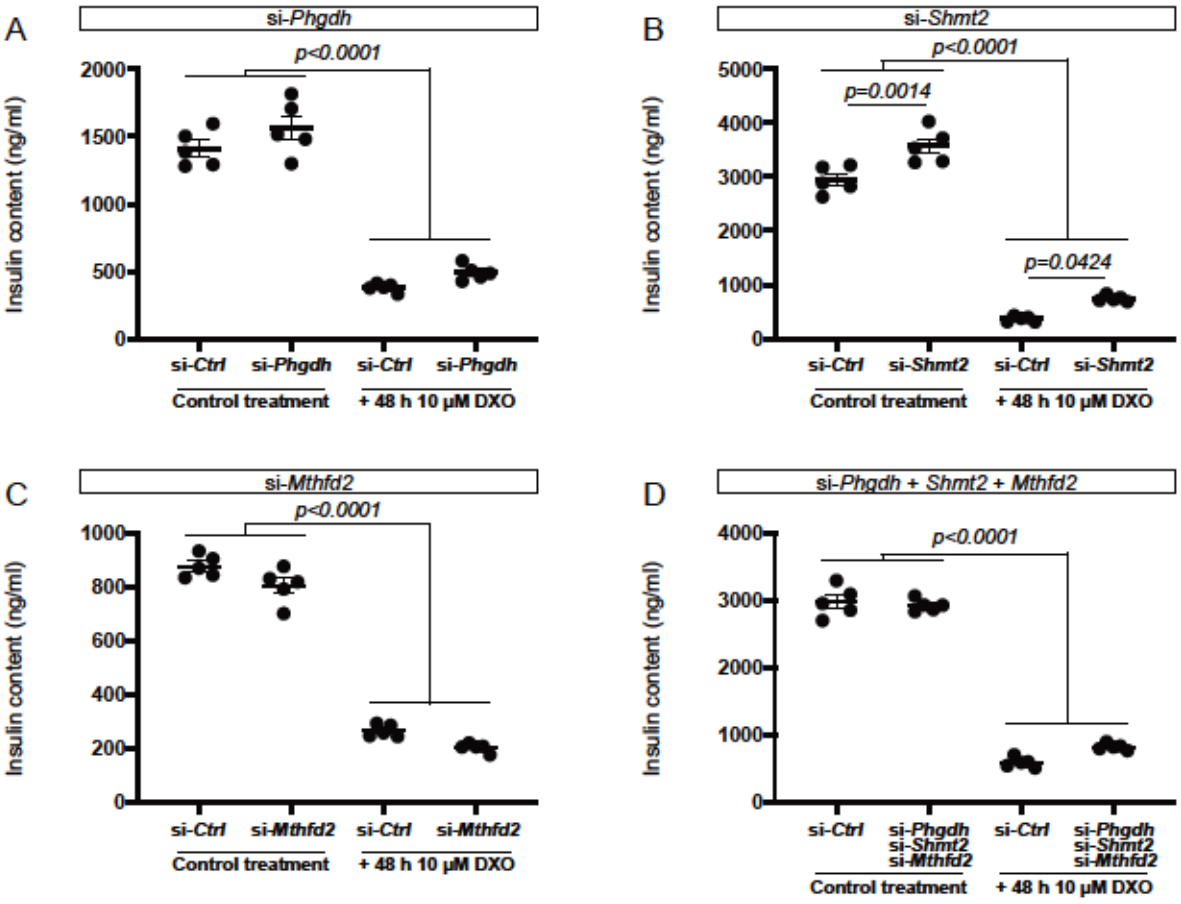
Supplementary Figure 9 to Figure 20G: Uncropped western blot images with molecular size marker.
Highlighted regions are depicted in the corresponding figures.



Supplementary Figure 10 to Figure 21A: Uncropped western blot images with molecular size marker.
Highlighted regions are depicted in the corresponding figures.

10.3 Insulin contents

Insulin contents of untreated and 48 h 10 µM DXO treated pseudo-islets which were transfected with si-*Phgdh*, si-*Shmt2*, si-*Mthfd2* or all three si-*Phgdh* + *Shmt2* + *Mthfd2* simultaneously, shown in this study:

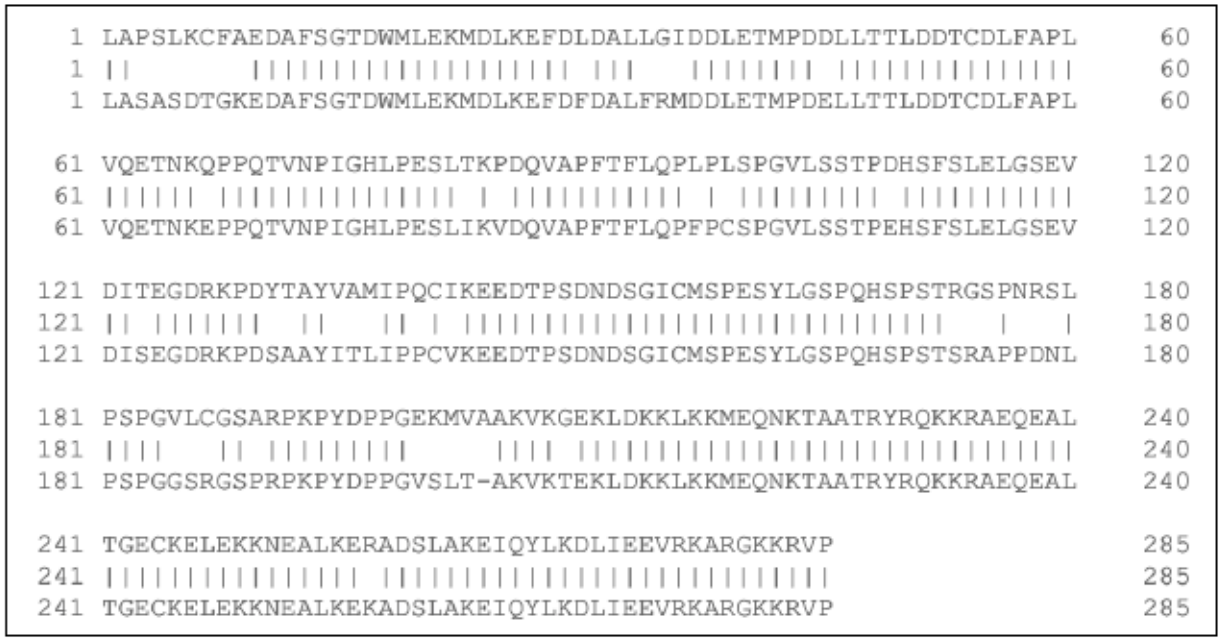


Supplementary Figure 11 to Figure 20B, E, H, and Figure 21B: Insulin content of untreated and 48 h 10 µM DXO treated pancreatic pseudo-islets after transfection with A, si-*Phgdh*, **B**, si-*Shmt2*, **C**, si-*Mthfd2*, and **D**, si-*Phgdh* + *Shmt2* + *Mthfd2* simultaneously (n = 5 wells). Data are shown as mean ± SEM with corresponding p-values. Statistical significance was determined by two-way ANOVA followed by Tukey's multiple comparison. Respective GSIS assay were performed by A. Pelligra (A ,B, D) and K. Griess (C).

10.4 Protein BLAST

DNA – Protein BLAST of ATF4, encoded by human GenID: 468, RefSeq: BC011994 and compared to C57BL/6 protein database, according to the Altschul *et al.*, using the "BLASTX 2.12.0+ BLAST" tool by Ensemble.[219,220]

Query DNA:	Human *ATF4* (RefSeq: BC011994)
Protein Database:	Mus_musculus.GRCm39.pep.all.fa
BLAST/BLAT type:	BLASTX (NCBI BLAST)
Query location:	UnnamedSeq 1187 to 2041 (+)
Database location:	ENSMUSP00000105234 66 to 349 (+)
Genomic location:	15 80140695 to 80141658 (+)
Alignment score:	366
E-value:	6.01e-121
Alignment length:	285
Percentage identity:	84.912



Supplementary Figure 12 to Chapter 4.9.1: Alignment between human and mouse ATF4 protein sequences.
Screenshot from BLAST/BLAT Alignment results.[322]

11 Eidesstaatliche Erklärung

Ich versichere an Eides Statt, dass die Dissertation „Dextrorphan uncovers a role of the serine-linked mitochondrial one-carbon metabolism in pancreatic islet cell survival at the expense of secretory function“ von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist. Ich habe die Dissertation weder in der hier vorgelegten, noch in einer ähnlichen Form, bei einer anderen Fakultät eingereicht und habe bisher keine Promotionsversuche unternommen.

Düsseldorf, den 18/12/2023

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Angela Pelligra

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