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ARTICLE

Metallothionein 1 negatively regulates glucose-stimulated insulin secretion and is differentially expressed in conditions of beta cell compensation and failure in mice and humans

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Abstract

Aims/hypothesis The mechanisms responsible for beta cell compensation in obesity and for beta cell failure in type 2 diabetes are poorly defined. The mRNA levels of several metallothionein (MT) genes are upregulated in islets from individuals with type 2 diabetes, but their role in beta cells is not clear. Here we examined: (1) the temporal changes of islet *Mt1* and *Mt2* gene expression in mouse models of beta cell compensation and failure; and (2) the role of *Mt1* and *Mt2* in beta cell function and glucose homeostasis in mice.

Methods *Mt1* and *Mt2* expression was assessed in islets from: (1) control lean (chow diet-fed) and diet-induced obese (high-fat diet-fed for 6 weeks) mice; (2) mouse models of diabetes (*db/db* mice) at 6 weeks old (prediabetes) and 16 weeks old (after diabetes onset) and age-matched *db/+* (control) mice; and (3) obese non-diabetic *ob/ob* mice (16-week-old) and age-matched *ob/+* (control) mice. *MT1E*, *MT1X* and *MT2A* expression was assessed in islets from humans with and without type 2 diabetes. *Mt1-Mt2* double-knockout (KO) mice, transgenic mice overexpressing *Mt1* under the control of its natural promoter (Tg-*Mt1*) and corresponding control mice were also studied. In MIN6 cells, MT1 and MT2 were inhibited by small interfering RNAs. mRNA levels were assessed by real-time RT-PCR, plasma insulin and islet MT levels by ELISA, glucose tolerance by i.p. glucose tolerance tests and overnight fasting-1 h refeeding tests, insulin tolerance by i.p. insulin tolerance tests, insulin secretion by RIA, cytosolic free Ca^{2+} concentration with Fura-2 leakage resistant (Fura-2 LR), cytosolic free Zn^{2+} concentration with Fluozin-3, and NAD(P)H by autofluorescence.

Results *Mt1* and *Mt2* mRNA levels were reduced in islets of murine models of beta cell compensation, whereas they were increased in diabetic *db/db* mice. In humans, *MT1X* mRNA levels were significantly upregulated in islets from individuals with type 2 diabetes in comparison with non-diabetic donors, while *MT1E* and *MT2A* mRNA levels were unchanged. Ex vivo, islet *Mt1* and *Mt2* mRNA and MT1 and MT2 protein levels were downregulated after culture with glucose at 10–30 mmol/l vs 2–5 mmol/l, in association with increased insulin secretion. In human islets, mRNA levels of *MT1E*, *MT1X* and *MT2A* were downregulated by stimulation with physiological and supraphysiological levels of glucose. In comparison with wild-type (WT) mice, *Mt1-Mt2* double-KO mice displayed improved glucose tolerance in association with increased insulin levels and enhanced insulin release from isolated islets. In contrast, isolated islets from Tg-*Mt1* mice displayed impaired glucose-stimulated insulin secretion (GSIS). In both *Mt1-Mt2* double-KO and Tg-*Mt1* models, the changes in GSIS occurred despite similar islet

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Research in context

What is already known about this subject?

- The mechanisms responsible for beta cell compensation in obesity and beta cell failure in type 2 diabetes are poorly defined
- Several metallothionein genes are upregulated in islets from individuals with type 2 diabetes but their role in beta cells is not clear

What is the key question?

- Do changes in *Mt1* and/or *Mt2* gene expression play a role in the modulation of beta cell function?

What are the new findings?

- *Mt1* and *Mt2* mRNA levels are downregulated in the islets of obese mice that can compensate for insulin resistance by increasing insulin secretion, whilst they are upregulated in the islets of mouse models of diabetes that fail to compensate
- *Mt1* inhibition enhances glucose-stimulated insulin secretion and improves glucose tolerance in mice, whereas its overexpression attenuates the secretory response
- *Mt1* modulation of beta cell function does not involve classical pathways, such as changes in glucose metabolism, intracellular Ca^{2+}, intracellular Zn^{2+} and insulin content

How might this impact on clinical practice in the foreseeable future?

- Our findings suggest that *Mt1* is a key negative regulator of beta cell function. The inhibition of *Mt1* may represent an attractive therapeutic target to augment insulin secretion in type 2 diabetes

insulin content, rises in cytosolic free Ca^{2+} concentration and NAD(P)H levels, or intracellular Zn^{2+} concentration vs WT mice. In MIN6 cells, knockdown of MT1 but not MT2 potentiated GSIS, suggesting that *Mt1* rather than *Mt2* affects beta cell function. **Conclusions/interpretation** These findings implicate *Mt1* as a negative regulator of insulin secretion. The downregulation of *Mt1* is associated with beta cell compensation in obesity, whereas increased *Mt1* accompanies beta cell failure and type 2 diabetes.

Keywords Beta cell compensation · Beta cell failure · Glucose-stimulated insulin secretion · Islets · Obesity · Type 2 diabetes

Abbreviations

BAT	Brown adipose tissue
EDL	Extensor digitorum longus
ER	Endoplasmic reticulum
$\lambda_{ex/em}$	Excitation/emission wavelength
FCCP	Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
Fura-2 LR	Fura-2 leakage resistant
GSIS	Glucose-stimulated insulin secretion
HFD	High-fat diet
KO	Knockout
LCM	Laser-capture microdissection
MT	Metallothionein
siRNA	Small interfering RNA
Tg-*Mt1*	Transgenic mice overexpressing *Mt1* under the control of its natural promoter
TPEN	*N*,*N*,*N*′,*N*′-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine
WAT	White adipose tissue
WT	Wild-type
$[Zn^{2+}]_i$	Intracellular free Zn^{2+} levels

Introduction

Type 2 diabetes results from the complex interplay of genetic and environmental risk factors, among which obesity plays a predominant role. Interestingly, most obese individuals compensate for insulin resistance by increasing insulin secretion, thereby maintaining normoglycaemia at the price of hyperinsulinaemia. However, this compensation can be sidestepped by a phase of decompensation in which beta cells fail to uphold an adequate secretory response [1–3]. This leads to hyperglycaemia and subsequent glucotoxic alterations of beta cell mass and function [4, 5]. Identifying genes involved in beta cell compensation in obesity, and in beta cell failure in

type 2 diabetes may provide new insights into beta cell pathophysiology and reveal novel therapeutic targets to preserve beta cell function in individuals with (pre) type 2 diabetes.

Metallothioneins (MTs) are a family of low molecular mass, cysteine-rich, metal-binding proteins, the (patho)physiological functions of which have not been fully characterised. Their predominant roles are heavy metal detoxification, metal ion (including zinc) homeostasis, and the regulation of cellular redox status and antioxidant defences. Among the four different murine genes encoding MTs, *Mt1* and *Mt2* are expressed ubiquitously, *Mt3* is mainly expressed in neurons and *Mt4* is expressed in squamous epithelium cells. In humans, in addition to *MT2* (also known as *MT2A*), *MT3* and *MT4*, there are eight *MT1* isoforms for a total of 11 functional MT genes [6, 7]. Interestingly, polymorphisms in *MT1A* and *MT2A* have been associated with increased risk for type 2 diabetes and diabetic complications [8–10]. Moreover, *MT1E*, *MT1M*, *MT1X* and *MT2A* mRNA levels were significantly upregulated in islets obtained by laser-capture microdissection (LCM) of pancreatic sections from type 2 diabetes donors [11]. However, the role of MTs in the pathophysiology of type 2 diabetes remains unclear. We, therefore, verified whether changes in *Mt1* and/or *Mt2* gene expression plays a role in the modulation of beta cell function.

Methods

Reagents Fura-2 leakage resistant (Fura-2 LR), diazoxide (Dz), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) and $ZnCl_2$ were from Sigma (St Louis, MI, USA). *N*,*N*,*N*′,*N*′-tetrakis(2-pyridinylmethyl)-1,2-ethanediamine (TPEN) was from Abcam (Cambridge, UK). Fluozin-3, control non-targeting small interfering RNA (siRNA), ON-TARGETplus SMARTpool siRNA and DharmaFECT3 (transfection reagent) were from Thermo Fisher Scientific (Lafayette, CO, USA).

Mice Six- and 16-week-old male and female C57BL/KsJ *db/db* mice and age-matched lean control mice (C57BL/KsJ), 16-week-old male and female C57BL/6J *ob/ob* mice and age-matched lean control mice, and the 16-week-old wild-type (WT) male mice (C57BL/6JAusb) used in diet-induced obesity experiments were from the Garvan Institute breeding colonies (Australian BioResources, Moss Vale, NSW, Australia). Male *Mt1-Mt2* double-knockout (KO) mice at 4–5 months of age (129S7/SvEvBrd-*Mt1*^{tm1Bri}*Mt2*^{tm1Bri}/J; herein referred to as KO mice) and their sex- and age-matched controls (129S1/SvImJ) originated from the Jackson Laboratory (Bar Harbor, MA, USA). Male transgenic mice overexpressing *Mt1* under the control of its natural promoter at 3 and 9 months of age (B6.Cg-Tg(*Mt1*)174Bri/J; referred to as Tg-*Mt1*) and their sex- and age-matched controls (C57BL/6 J) were also from the Jackson Laboratory. All animals were bred in the local animal facility of the health sciences sector at UCLouvain. Mice were housed under a controlled temperature of 22°C and a 12 h light cycle, with ad libitum access to water and chow (8% energy from fat, 21% energy from protein and 71% energy from carbohydrate [wt/wt]; 10.9 kJ/g; Gordon's Specialty Stock Feeds, Yanderra, NSW, Australia) or a high-fat diet (HFD; 23% energy from fat, 19.4% energy from protein and 57.6% energy from carbohydrate and fibres [wt/wt for all]; 20.1 kJ/g; catalogue no. SF03-020; Specialty Feeds, Glen Forest, WA, Australia). Mice were randomly assigned to experimental groups using an odd/even number method by unblinded experimenters. All experiments were approved by the Institutional Committee on Animal Experimentation of the Health Sciences Sector at UCLouvain (Project 2013/UCL/MD/016) and the Garvan Institute/St Vincent's Hospital Animal Experimentation Ethics Committee.

Human islets Human islets were obtained from 24 non-diabetic and 12 diabetic individuals at the Tom Mandel Islet Transplant Program, Melbourne [12]. Human islets were isolated from heart-beating, brain-dead donors and approved for use under the ethics reference HREC011/04 (St Vincent's Hospital Human Research Ethics Committee). Characteristics of donors and islet preparations are listed in the electronic supplementary material (ESM) Table 1. To evaluate the effects of glucose on MT gene expression, islets were obtained from three non-diabetic donors through the JDRF award 31-2008-416 (European Consortium for Islet Transplantation [ECIT] Islet for Basic Research program) and approved for use under the ethics reference B403/2017/05JUL/355 (Comité d'éthique hospitalo-facultaire Saint-Luc, UCLouvain). Characteristics of these donors are indicated in ESM Table 2. Written informed consent was obtained from next of kin and all experiments were performed in accordance with relevant guidelines and regulations.

Glucose and insulin tolerance tests Intraperitoneal (i.p.) glucose tolerance tests (i.p.GTTs) and fasting-refeeding tests were performed on overnight-fasted mice and i.p. insulin tolerance tests (i.p.ITTs) on 4 h-fasted mice. For fasting-refeeding tests, blood glucose levels were measured in fasted mice and 1 h after refeeding. For i.p.GTTs and i.p.ITTs, mice were injected i.p. with glucose (2 g/kg of body weight) or insulin (0.75 U/kg of body weight; Actrapid, Novo Nordisk, Bagsværd, Denmark) and blood glucose levels were measured at the indicated time points using a FreeStyle Precision Neo glucometer (Abbott, Wavre, Belgium).

Tissue collection and histological analysis Mice were killed by cervical dislocation and trunk blood collected and centrifuged for measurement of plasma insulin levels by ultra-sensitive ELISA (Crystal Chem, Downers Grove, IL, USA). The pancreas, liver, white adipose tissue (WAT) fat pads (epididymal, retroperitoneal and inguinal), interscapular brown adipose

tissue (BAT), skeletal muscles of the right leg (tibialis anterior, extensor digitorum longus [EDL], gastrocnemius and soleus) and heart of each animal were dissected, weighed, and frozen or fixed in 4% (wt/vol.) paraformaldehyde for 24 h at 4°C and embedded in paraffin for further histological analysis. Liver and epididymal fat pad sections were counter stained with haematoxylin and eosin (H&E) to assess morphology. Adipocyte size was analysed using Visiopharm software (Author module, Version 6.4.1; Hørsholm, Denmark). Insulin and glucagon immunostaining of pancreatic sections was performed as previously described [13, 14]. Beta cell mass was computed based on the relative cross-sectional beta cell area and pancreas weight. Three pancreatic sections at three different levels were analysed for each mouse. Pancreatic sections immunoprobed for insulin, glucagon and a nuclear stain (DAPI) were scanned using the panoramic 250 Flash III digital slide scanner (3DHISTECH, Budapest, Hungary) and analysed with Visiopharm.

Islet isolation and culture Islets were isolated by collagenase digestion, separated by density gradient centrifugation and handpicked under a stereomicroscope. Islets were cultured in standard RPMI medium (Invitrogen, Carlsbad, CA, USA) supplemented with 2 mmol/l glutamine, 5 g/l BSA, 100 U/ml penicillin and 100 μg/ml streptomycin.

Cell culture and treatment MIN6 cells (passage 26–43; mycoplasma negative), originally provided by J. I. Miyazaki (Osaka University Medical School, Osaka, Japan) [15], were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 25 mmol/l glucose, 10 mmol/l HEPES, 10% FCS (vol./vol.), 50 U/ml penicillin and 50 μg/ml streptomycin. Cells were transfected with 100 nmol/l control, *Mt1* or *Mt2* siRNA using DharmaFECT3 transfection reagent 24 h before experiments.

Glucose-stimulated insulin secretion (GSIS) tests Isolated islets in batches of five, or MIN6 cells seeded in 24-well plates at 2×10^5 cell per well, were preincubated for 1 h in KRB buffer (120 mmol/l NaCl, 4.8 mmol/l KCl, 2.5 mmol/l $CaCl_2$, 1.2 mmol/l $MgCl_2$, 24 mmol/l $NaHCO_3$ and 1 g/l BSA) containing 3 mmol/l glucose and then incubated for 1 h in KRB buffer containing 3 mmol/l, 15 mmol/l or 30 mmol/l glucose. At the end of the incubation, the buffer was collected for measurement of insulin by RIA, and islets were collected and disrupted by sonication in 10 mmol/l Tris, 0.2 mol/l NaCl and 10 mmol/l EDTA for measurement of their DNA and insulin content. All secretion experiments were carried out in duplicate.

Live-cell imaging NAD(P)H autofluorescence (excitation/emission wavelength [$\lambda_{ex/em}$], 360/470 nm) was measured every 5 s and expressed as the percentage of the fluorescence level measured after 15–20 min of treatment with 10 μmol/l FCCP in KRB buffer containing 30 mmol/l glucose. For measurements of intracellular Ca^{2+} concentrations, islets were loaded for 2 h with 2 μmol/l Fura-2 LR acetoxymethyl ester and the fluorescence ratio ($\lambda_{ex/em}$, 340/510 to 380/510 nm) was measured every 5 s. For measurements of intracellular free Zn^{2+} levels ($[Zn^{2+}]_i$), islets were loaded for 2 h with 2 μmol/l Fluozin-3 acetoxymethyl ester and fluorescence ($\lambda_{ex/em}$, 490/510 nm) was measured every 10 s. TPEN (a zinc chelator) was used as a negative control to lower $[Zn^{2+}]_i$ and used at 50 μmol/l. Islets from control and KO/Tg-*Mt1* mice were simultaneously perifused side by side with KRB buffer continuously gassed with 5% CO_2 air mix to maintain pH 7.4 at a flow rate of 1 ml/min at 37°C on the stage of an inverted microscope.

RNA analysis Total RNA was extracted and reverse transcribed as previously described [16, 17]. Real-time RT-PCR was performed using the SYBR Green method and a 7900HT Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) or a CFX96 optical cycler detection system (Bio-Rad, Hercules, CA, USA). Primer sequences are listed in ESM Table 3. The value obtained for a specific gene product was normalised to the control gene cyclophilin A or *TBP* and expressed as fold change of the value in the control condition. TaqMan assays were used to assess the mRNA levels of *MT1E* (Hs01938284_g1), *MT1X* (Hs00745167_sH), *MT2A* (Hs02379661_g1) and the control gene 18S rRNA (Hs03003631_g1) in human islets from control and type 2 diabetes donors (Applied Biosystems). AmpliTaq Gold reagents (Thermo Fisher Scientific) were used on a Light Cycler 480 Instrument II (Roche, Risch-Rotkreuz, Switzerland).

Protein analysis Islet MT1 and MT2 protein levels were quantified by ELISA (Frontier Institute, Ishikari, Japan). Absorbance values were normalised to total protein content measured with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

Statistical analysis Results are means ± SEM for the indicated number of experiments. Statistical significance was assessed by unpaired two-tailed Student's *t* test, one-way ANOVA with a Newman–Keuls post hoc test, or two-way ANOVA with a Bonferroni post hoc test.

Results

***Mt1* and *Mt2* mRNA levels were differentially regulated between conditions of beta cell compensation and failure** Islet *Mt1* and *Mt2* mRNA levels were markedly downregulated in mice fed for 6 weeks with a HFD vs a chow diet. This was associated with increased body-weight gain and plasma insulin levels (compensation) (Fig. 1a,b and ESM Fig. 1). Similarly, islet *Mt1* and *Mt2* mRNA levels were significantly

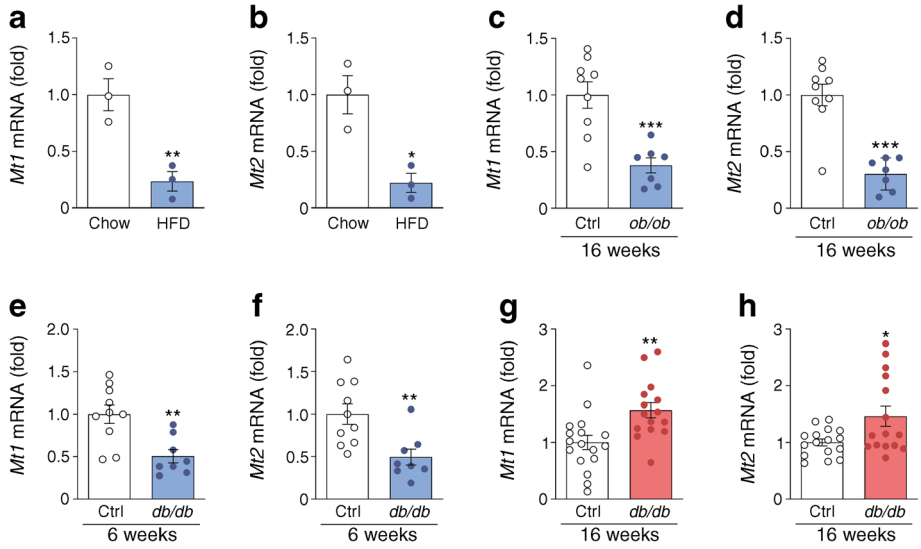

Fig. 1 *Mt1* and *Mt2* mRNA levels are downregulated in the islets of obese compensating mice and upregulated in the islets of decompensating diabetic mice. (**a**–**h**) Changes in the mRNA levels of *Mt1* and *Mt2* in the islets of (**a**, **b**) chow-fed and HFD-fed WT mice, (**c**, **d**) control and *ob/ob* mice at 16 weeks of age, (**e**, **f**) control and *db/db* mice at 6 weeks of age, and (**g**, **h**) control and *db/db* mice at 16 weeks of age. Data are means ± SEM. (**a**, **b**) $n = 3$, (**c**, **d**) $n = 7$–9, (**e**, **f**) $n = 8$–10, (**g**, **h**) $n = 15$–17 animals per group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs chow-fed or control mice, unpaired two-tailed Student's *t* test. Ctrl, control

downregulated in 16-week-old *ob/ob* mice (Fig. 1c,d), a model of successful beta cell compensation [17, 18], vs control mice. In *db/db* mice, which progress from successful beta cell compensation to beta cell failure between 6 and 16 weeks of age [17], islet *Mt1* and *Mt2* mRNA levels were downregulated in 6-week-old mice (Fig. 1e,f) and upregulated in 16-week-old mice (Fig. 1g,h). These results reveal that, in vivo, beta cell compensation is associated with *Mt1* and *Mt2* downregulation, whereas beta cell failure is associated with *Mt1* and *Mt2* upregulation.

Glucose stimulation downregulated the expression of *Mt1* and *Mt2* WT mouse islets were cultured at various glucose concentrations, ranging from low, non-stimulating concentrations (2–5 mmol/l) to the optimal concentration for culture of rodent islets (10 mmol/l) to a very high glucose concentration (30 mmol/l) [16, 19, 20]. *Mt1* and *Mt2* mRNA and MT1/2 protein levels were markedly downregulated after culture at glucose at 10 mmol/l vs 2 and 5 mmol/l, with little or no further decrease at 30 mmol/l vs 10 mmol/l glucose (Fig. 2a–c). These effects were anti-paralleled by the stimulation of insulin secretion and the upregulation of the antioxidant genes *Mt3*, *Srxn1* and *Gpx2* (Fig. 2d–g). These findings show that, compared with other antioxidant genes, *Mt1* and *Mt2* have a specific gene expression pattern in response to glucose stimulation. They also show an ex vivo association between islet *Mt1* and *Mt2* downregulation and the stimulation of insulin secretion.

***MT1X* mRNA levels were upregulated in human islets from individuals with type 2 diabetes and were affected by glucose stimulation** *MT1E*, *MT1X* and *MT2A* were the most upregulated MT genes in islets obtained by LCM from pancreases of individuals with type 2 diabetes vs islets from non-diabetic donors [11]. Among them, *MT1X* was the only isoform showing significantly upregulated mRNA levels in islets isolated from type 2 diabetes vs non-diabetic donors. Besides, *MT1X* displayed higher mRNA levels than *MT1E* and *MT2A* in islets isolated from non-diabetic donors (Fig. 3a–d). When islets from non-diabetic donors were cultured in the presence of a low, non-stimulating glucose concentration (2.2 mmol/l), the optimal glucose concentration for culture of human islets (5.5 mmol/l), a high glucose concentration (11.1 mmol/l) and a very high glucose concentration (22 mmol/l) [21, 22], *MT1E*, *MT1X* and *MT2A* mRNA levels were all downregulated between treatment with 2.2 mmol/l and 11.1 mmol/l glucose, while, with 22 mmol/l glucose, *MT1E* and *MT1X* mRNA levels returned to a similar level as with 5.5 mmol/l glucose (Fig. 3e–g). In parallel, treatment with glucose at 11.1 and 22 mmol/l significantly increased insulin secretion during culture vs treatment with 2.2 and 5.5 mmol/l glucose (Fig. 3h).

These results show that *MT1X* upregulation is associated with beta cell failure in human type 2 diabetes. They also reveal a specific gene expression pattern of human MT genes in response to glucose stimulation that partly resembles that of *Mt1* and *Mt2* in mouse islets.

Deletion of *Mt1/Mt2* improved glucose tolerance The potential role of *Mt1* and/or *Mt2* in the modulation of glucose homeostasis and beta cell function was investigated in a global *Mt1-Mt2* double-KO mouse model [23]. Compared with WT mice, KO mice displayed higher body weight and daily food intake (ESM Fig. 2a,b). They also showed increased liver weight (ESM Fig. 2c) without macroscopical or histological

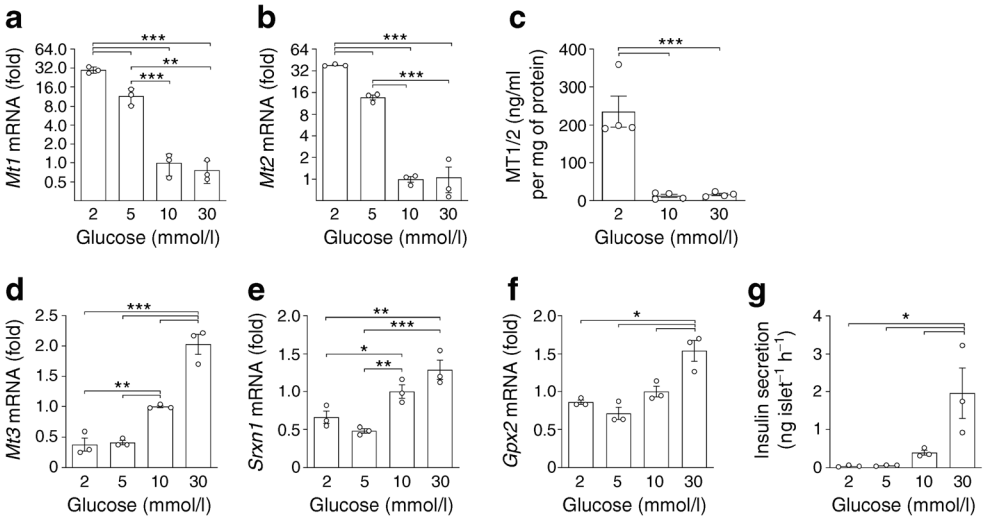

Fig. 2 *Mt1* and *Mt2* mRNA and MT1/MT2 protein levels are downregulated by glucose stimulation in a concentration-dependent manner. Isolated islets from WT mice were cultured for 24 h (for mRNA analysis) or 48 h (for protein analysis) in the presence of increasing glucose concentrations: 2 mmol/l, 5 mmol/l, 10 mmol/l and 30 mmol/l. (**a**–**c**) Changes in mRNA levels of (**a**) *Mt1* and (**b**) *Mt2*, and (**c**) MT1/MT2 protein levels. (**d**–**g**) Changes in mRNA levels of (**d**) *Mt3*, (**e**) *Srxn1* and (**f**) *Gpx2* and (**g**) in insulin secretion. Changes in mRNA levels were normalised to cyclophilin A and expressed relative to levels with treatment with 10 mmol/l glucose. Data are means ± SEM of $n = 3$–4 experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, one-way ANOVA with Newman–Keuls post hoc test

signs of steatosis (ESM Fig. 2d), and increased weight of different leg muscles, including tibialis anterior, EDL and gastrocnemius (ESM Fig. 2e–g). However, soleus muscle and heart weights were similar (ESM Fig. 2h,i). Interestingly, the weight of epididymal, inguinal and retroperitoneal fat pads and the sum of the three fat pads (WAT), thereof, were reduced in KO mice (ESM Fig. 2j–m), whereas BAT weight was similar between KO and WT mice (ESM Fig. 2n). Histological sections of epididymal fat pads also revealed reduced adipocyte surface in KO mice (ESM Fig. 2o,p).

Fed blood glucose levels were similar in KO and WT mice (Fig. 4a), overnight-fasted blood glucose levels were slightly higher in KO mice (Fig. 4b) and fed and fasted plasma insulin levels were not significantly different (Fig. 4c,d). Interestingly, glucose tolerance during i.p.GTT was markedly improved in KO mice, together with significantly increased plasma insulin

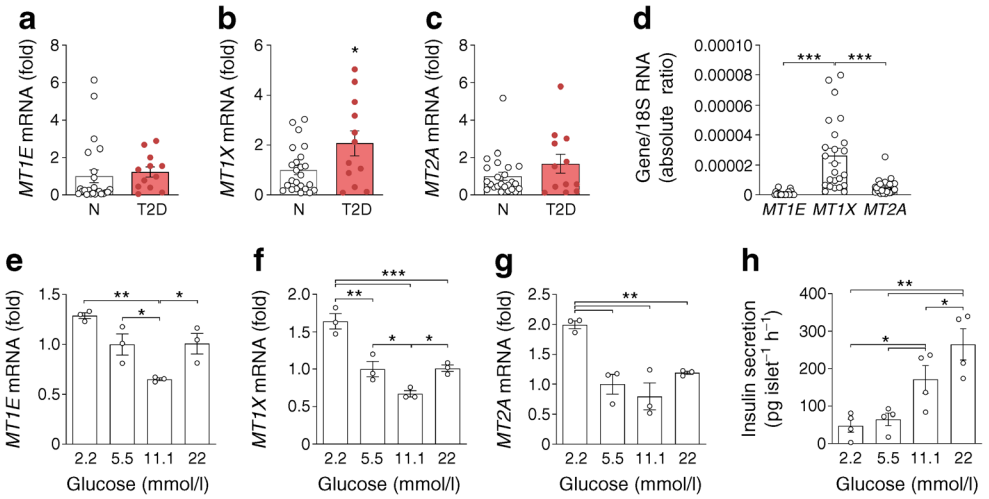

Fig. 3 *MT1X* mRNA levels are upregulated in the islets of human diabetic donors and MT gene isoforms are affected by glucose stimulation. (**a**–**c**) Changes in the mRNA levels of (**a**) *MT1E*, (**b**) *MT1X* and (**c**) *MT2A* in the islets of non-diabetic individuals (N) and type 2 diabetic donors (T2D). The mRNA levels of each gene were normalised to 18S RNA. (**d**) Comparison of the mRNA levels of *MT1E*, *MT1X* and *MT2A* in the islets of non-diabetic individuals. The mRNA levels of each gene were normalised to 18S RNA and the absolute ratios compared. (**e**–**h**) Human islets from non-diabetic donors were cultured for 24 h in the presence of increasing glucose concentrations. (**e**–**g**) Changes in the mRNA levels of (**e**) *MT1E*, (**f**) *MT1X* and (**g**) *MT2A* during culture. The mRNA levels of each gene were normalised to *TBP* and expressed relative to levels with treatment with 5.5 mmol/l glucose. (**h**) Changes in insulin secretion during culture. Data are means ± SEM; (**a**–**d**) $n = 24$ non-diabetic and $n = 12$ type 2 diabetic donors; (**e**–**h**) $n = 3$–4 experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs non-diabetic donors or as shown; in (**a**–**c**), unpaired two-tailed Student's *t* test; in (**d**–**h**), one-way ANOVA with Newman–Keuls post hoc test

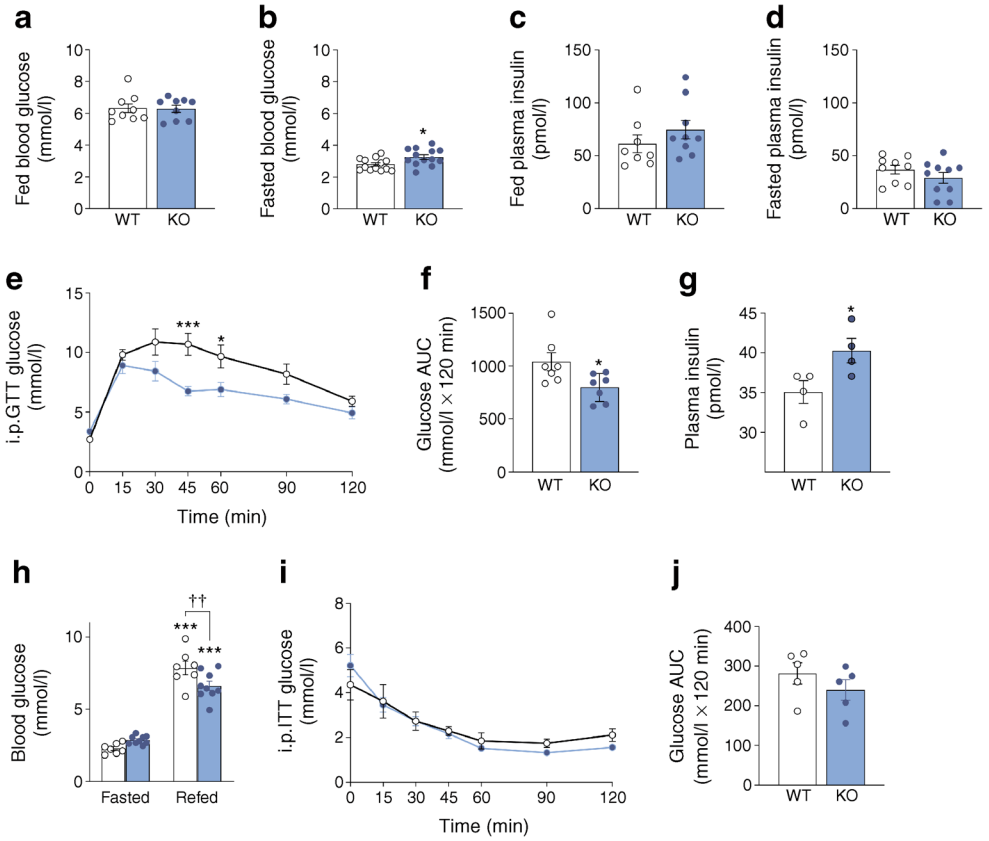

Fig. 4 *Mt1-Mt2* deletion improved glucose tolerance in vivo. (**a**–**d**) Changes in (**a**) fed and (**b**) fasted blood glucose levels, and (**c**) fed and (**d**) fasted plasma insulin levels in WT and KO mice. (**e**) Changes in blood glucose levels and (**f**) AUC during an i.p.GTT in overnight-fasted WT and KO mice. (**g**) Plasma insulin levels 30 min following i.p.GTT. (**h**) Changes in blood glucose levels in WT and KO mice at the fasted state and 1 h after refeeding. (**i**) Changes in blood glucose levels and (**j**) AUC during an i.p.ITT in 4 h-fasted WT and KO mice. WT, white bars/circles; KO mice, blue bars/circles. Mice were used between 4 and 5 months of age. Data are means ± SEM; (**a**) $n = 9$, (**b**) $n = 13$, (**c**) $n = 8$–9, (**d**) $n = 9$–10, (**e**, **f**) $n = 7$, (**g**) $n = 4$, (**h**) $n = 7$–9, (**i**, **j**) $n = 5$ animals per group. $^{*}p < 0.05$, $^{***}p < 0.001$ vs WT. In (**h**) $^{\dagger\dagger}p < 0.01$ as shown; $^{***}p < 0.001$ for the effect of refeeding. In (**b**), (**f**) and (**g**), unpaired two-tailed Student's *t* test; in (**e**) and (**h**), two-way ANOVA with Bonferroni post hoc test

levels (30 min following i.p.GTT) (Fig. 4e–g). Similarly, during an overnight fasting/1 h refeeding test, blood glucose levels after refeeding were lower in KO mice (Fig. 4h). On the other hand, insulin sensitivity during i.p.ITT was similar in KO and WT mice (Fig. 4i,j). Together, these findings suggest that *Mt1-Mt2* deletion leads to improved glucose tolerance due to increased insulin secretion rather than changes in insulin action.

MT deletion potentiated GSIS The mechanism underlying improved glucose tolerance in KO mice was further investigated in isolated islets. In WT islets, MT1/2 protein levels were expressed under control culture conditions and upregulated by treatment with $ZnCl_2$ (a potent inducer of MT expression). In contrast, MT1/2 proteins were not detected in KO islets, even after treatment with $ZnCl_2$, confirming the lack of *Mt1-Mt2* expression (Fig. 5a). Interestingly, GSIS was potentiated in KO vs WT islets after acute stimulation with 15 mmol/l glucose, and to a stronger extent after stimulation with 30 mmol/l glucose (Fig. 5b), while islet insulin content was unchanged in KO vs WT mice (Fig. 5c). In agreement, MT1 but not MT2 knockdown in MIN6 cells potentiated GSIS (Fig. 5d–f), highlighting the role of *Mt1*, rather than *Mt2*, in the negative regulation of insulin secretion.

We also measured the effects of acute stepwise increases in glucose concentration on intracellular NAD(P)H and Ca^{2+} levels and found no differences between WT and KO islets (Fig. 5g,h). These findings indicate that the potentiation of GSIS in KO islets results from an effect downstream of the stimulation of glucose metabolism and Ca^{2+} influx.

MTs are known for their metal-binding properties and proposed to play a role in metal ion, including zinc, homeostasis. As zinc plays a key role in beta cell biology, we used Fluozin-3 to compare the dynamic changes in $[Zn^{2+}]_i$ in response to glucose and zinc supplementation/chelation in islets from WT and KO mice (ESM Fig. 3a). In islets from both mouse types, $[Zn^{2+}]_i$ was increased upon supplementation of 3 mmol/l glucose-KRB buffer with 10 μmol/l $ZnCl_2$, slightly decreased upon subsequent stimulation with 30 mmol/l in the continued presence of 10 μmol/l $ZnCl_2$, rapidly decreased upon ensuing zinc chelation using TPEN, and markedly increased upon final addition of 1 mmol/l $ZnCl_2$ to the medium. These effects were almost identical in WT and KO islets. In agreement, the mRNA

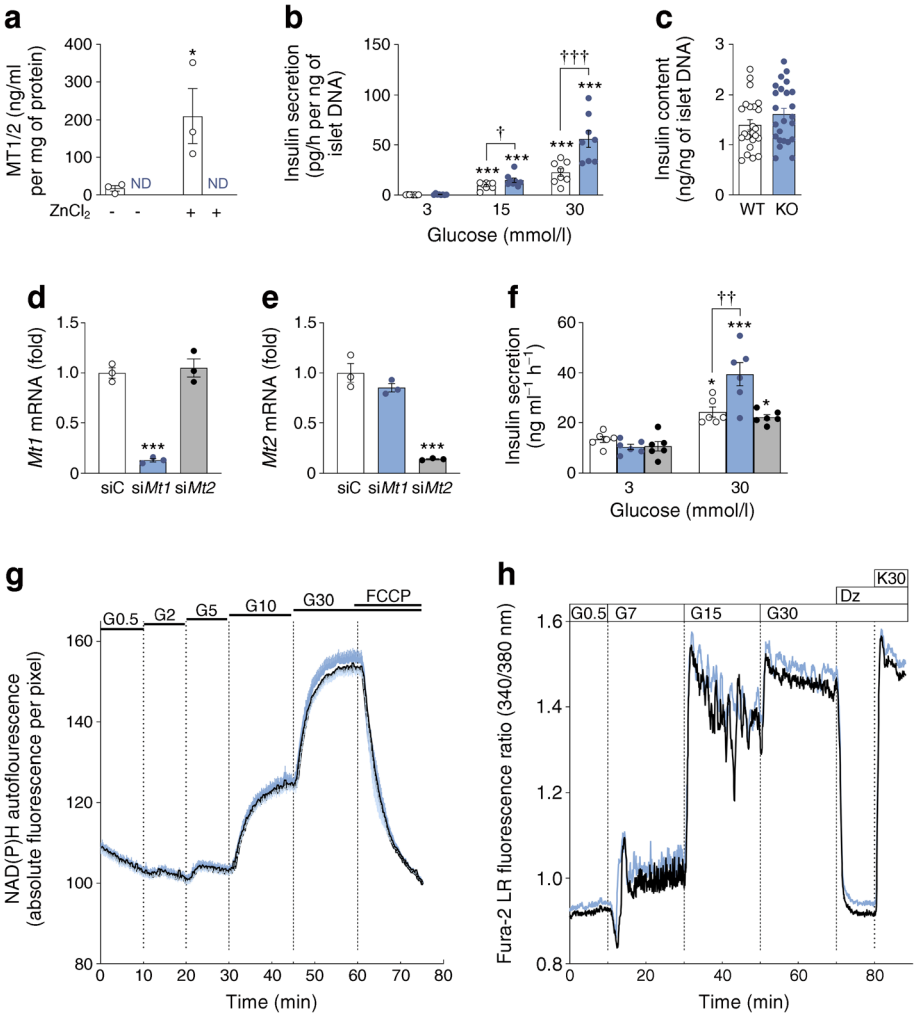

Fig. 5 *Mt1* deletion potentiated GSIS. (**a**) Changes in MT1/MT2 protein levels after 24 h culture of WT and *Mt1-Mt2* double-KO islets in the absence or presence of 100 μmol/l $ZnCl_2$. (**b**) GSIS in WT (white bars) and KO (blue bars) islets and (**c**) islet insulin content. (**d**, **e**) Changes in the mRNA levels of *Mt1* and *Mt2* and (**f**) GSIS in MIN6 cells transfected with either control siRNA (siC; white bars), *Mt1* siRNA (si*Mt1*; blue bars) or *Mt2* siRNA (si*Mt2*; grey bars). (**g**) Changes in NAD(P)H autofluorescence normalised for each experiment to the fluorescence level after 15 min treatment with FCCP. (**h**) Changes in Fura-2 LR fluorescence ratio after 2 h loading with 2 μmol/l of the Ca^{2+} probe. WT and KO islets were perifused simultaneously in the same chamber. WT, black traces; KO, blue traces. Data are means ± SEM. (**a**) $n = 3$ experiments, (**b**) $n = 4$ experiments (in duplicate), (**c**) $n = 24$ islets, (**d**–**f**) $n = 3$ experiments (**f** in duplicate), (**g**) $n = 4$ experiments, (**h**) $n = 5$ experiments. $^{*}p < 0.05$, $^{***}p < 0.001$ for the effect of $ZnCl_2$ or glucose. $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ for the effect of genotype or siRNA. In (**d**, **e**), one-way ANOVA with Newman–Keuls post hoc test; in (**a**), (**b**) and (**f**), two-way ANOVA with Bonferroni post hoc test. Dz, diazoxide; K30, 30 mmol/l KCl; G*n*, *n* mmol/l glucose

levels of the key beta cell zinc transporters *Zip6* (also known as *Slc39a6*), *Zip7* (*Slc39a7*), *Znt1* (*Slc30a1*) and *Znt8* (*Slc30a8*) were similar between KO and WT islets (ESM Fig. 3b–e). These results rule out a potential role of changes in $[Zn^{2+}]_i$ levels in the potentiation of GSIS in KO islets.

Assessment of the pancreas morphology revealed no difference in islet architecture between islets from WT and KO mice (ESM Fig. 4a). There were no significant changes in pancreatic weight, beta and alpha cell masses, or the percentage of alpha/beta cells per islet area between KO and WT mice (ESM Fig. 4b–e). Moreover, islets from WT and KO mice displayed no difference in the mRNA levels of the beta cell-enriched genes preproinsulin, *Pdx1*, *Glut2* (also known as *Slc2a2*), *Pcx* and *Gpd2* (ESM Fig. 4f–j). Similarly, there were no changes in the mRNA levels of the endoplasmic reticulum (ER) stress-response genes *Hspa5* and *Ddit3* (ESM Fig. 4k,l). Importantly, the deletion of *Mt1* and *Mt2* was not compensated for by upregulation of other MT genes. Thus, *Mt3* mRNA levels were unchanged in islets from KO vs WT mice (ESM Fig. 4m), while *Mt4* mRNA levels were undetected after 40 cycles of PCR amplification in both islet types.

Altogether, these findings strongly support a role for *Mt1* as a negative modulator of GSIS.

***Mt1* overexpression attenuated GSIS** To further assess the implication of *Mt1* in the negative regulation of GSIS, we examined islets isolated from global transgenic mice overexpressing mouse *Mt1* under the control of its natural promoter (Tg-*Mt1*)

[24, 25]. In comparison with WT mice, Tg-*Mt1* mice exhibited similar body and liver weights (ESM Fig. 5a–c). They displayed no significant difference in the weight of epididymal ($p = 0.0519$), inguinal and retroperitoneal fat pads or the sum of WAT, thereof (ESM Fig. 5d–g). There was also no difference in BAT weight (ESM Fig. 5h). Tg-*Mt1* mice also displayed similar fed blood glucose levels (Fig. 6a) and plasma insulin levels (Fig. 6b) to WT mice, and a fasting-refeeding test revealed no significant differences between the two groups (Fig. 6c). Similarly, i.p.GTT tests were similar in 3-month-old and 9-month-old animals (Fig. 6d,e). In contrast, i.p.ITT tests revealed a notable difference between WT and Tg-*Mt1* mice, with Tg-*Mt1* mice displaying lower blood glucose levels during the test (Fig. 6f,g). This effect may stem from an impact of *Mt1* overexpression on peripheral tissues.

In isolated islets, MT protein levels were markedly upregulated in Tg-*Mt1* islets, confirming the overexpression of *Mt1* (Fig. 7a). Interestingly, in contrast with its potentiation in KO vs WT islets, GSIS was significantly attenuated in Tg-*Mt1* vs WT islets after acute stimulation with 30 mmol/l glucose (Fig. 7b), while islet insulin content was similar between the two islet types (Fig. 7c). This attenuation in GSIS occurred despite similar rises in intracellular NAD(P)H and Ca^{2+} levels in islets from WT and Tg-*Mt1* mice in response to stepwise increases in glucose (Fig. 7d,e), indicating that the alteration of GSIS in Tg-*Mt1* mice vs WT mice lies at a step downstream of the stimulation of glucose metabolism and Ca^{2+} influx.

Similarly, changes in $[Zn^{2+}]_i$ in response to glucose and zinc supplementation/chelation were not different between islets from WT and Tg-*Mt1* mice (ESM Fig. 6a). In agreement, the mRNA levels of zinc transporters *Zip6*, *Zip7*, *Znt1* and *Znt8* were unchanged in islets from Tg-*Mt1* mice vs WT mice (ESM Fig. 6b–e).

Importantly, in comparison with islets from WT mice, Tg-*Mt1* islets exhibited a similar expression level of the beta cell-enriched genes preproinsulin, *Pdx1*, *Glut2* and *Pcx* (Fig. 8a–d). In addition, the mRNA levels of ER stress-response genes *Hspa5* and *Ddit3* were also not different between the two mouse models (Fig. 8e,f). On the other hand, the overexpression of *Mt1* (Fig. 8g) resulted in reduced mRNA levels of *Mt2* ($p < 0.05$) and *Mt3* ($p = 0.0704$) in comparison with WT islets (Fig. 8h,i), while *Mt4* mRNA levels were not detected after 40 cycles of PCR amplification in both types of islets.

Collectively, these findings further support the implication of *Mt1* in the negative regulation of insulin secretion.

Discussion

We have unveiled a novel role of *Mt1* in beta cells as a negative regulator of insulin secretion. The key findings of the

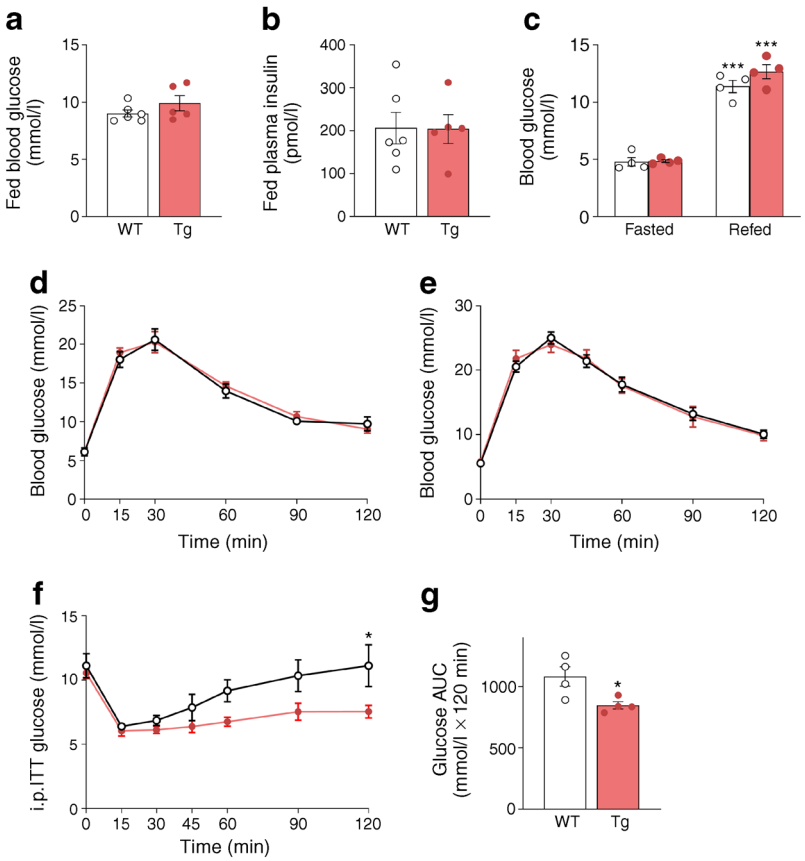

Fig. 6 *Mt1* overexpression did not affect glucose tolerance but affected insulin tolerance in vivo. (**a**) Changes in fed blood glucose levels and (**b**) fed plasma insulin levels in WT and Tg-*Mt1* mice. (**c**) Changes in blood glucose levels in WT and Tg-*Mt1* mice at the fasted state and 1 h after refeeding. (**d**, **e**) Changes in blood glucose levels during an i.p.GTT in overnight-fasted WT and Tg-*Mt1* mice at the age of (**d**) 3 months and (**e**) 9 months. (**f**) Changes in blood glucose levels during an i.p.ITT in 4 h-fasted WT and Tg-*Mt1* mice and (**g**) respective AUC. WT, white bars/circles; Tg-*Mt1*, red bars/circles. Data are means ± SEM; (**a**, **b**) $n = 5–6$, (**d**) $n = 4$, (**e**) $n = 6–8$, (**f**, **g**) $n = 4$ animals per group. *$p < 0.05$ for the effect of genotype (**f**); ***$p < 0.001$ for the effect of refeeding (**c**), two-way ANOVA with Bonferroni post hoc test. Tg, Tg-*Mt1* mice

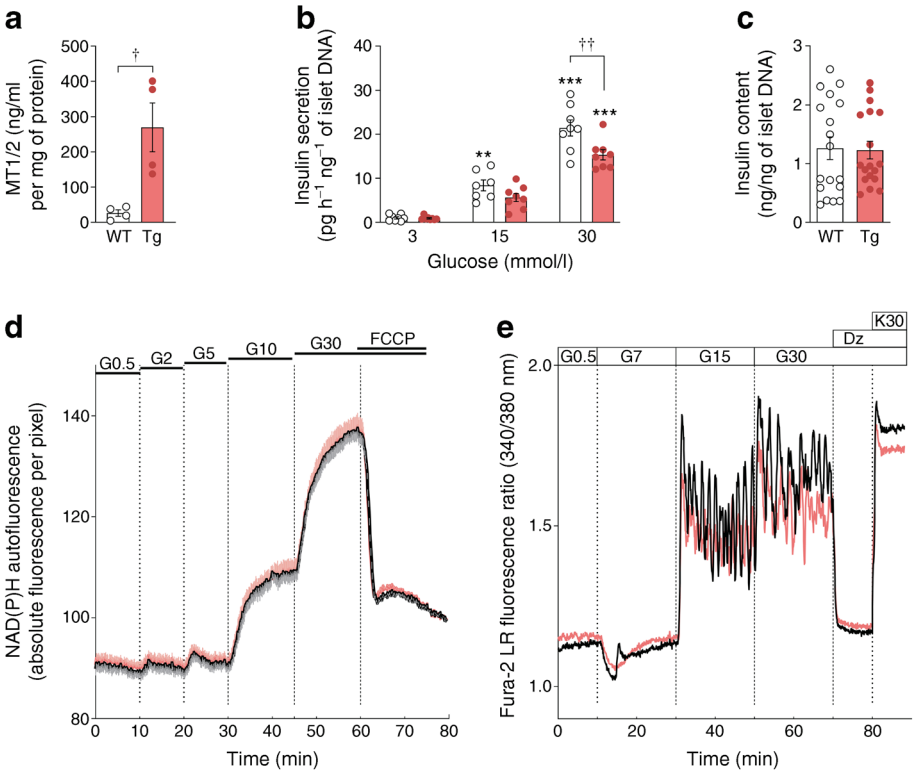

Fig. 7 *Mt1* overexpression attenuated GSIS. (**a**) Changes in MT1/MT2 protein levels in WT and Tg-*Mt1* islets. (**b**) GSIS in islets from WT (white bars) and Tg-*Mt1* (red bars) mice and (**c**) islet insulin content. (**d**) Changes in NAD(P)H autofluorescence normalised for each experiment to the fluorescence level after 15 min treatment with FCCP ($n = 4$). (**e**) Changes in Fura-2 LR fluorescence ratio after 2 h loading with 2 μmol/l of the Ca^{2+} probe ($n = 3$). WT and Tg-*Mt1* islets were perifused simultaneously in the same chamber. WT, black traces; Tg-*Mt1*, red traces. Data are means ± SEM. (**a**, **b**, **d**) $n = 4$ experiments, (**c**) $n = 18$ islets, (**e**) $n = 3$ experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ for the effect of glucose; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ for the effect of genotype. In (**a**), unpaired two-tailed Student's *t* test; in (**b**), two-way ANOVA with Bonferroni post hoc test. Dz, diazoxide; K30, 30 mmol/l KCl; G*n*, *n* mmol/l glucose; Tg, Tg-*Mt1* mice

study are: (1) *Mt1* and *Mt2* islet gene expression in obese mice was downregulated with beta cell compensation and upregulated with beta cell failure; (2) *MT1X* islet mRNA levels were upregulated in human type 2 diabetes donors; (3) physiological and supraphysiological glucose stimulation downregulated mouse and human MT islet gene expression; (4) deletion of *Mt1* and *Mt2* improved glucose tolerance in vivo and potentiated GSIS in isolated islets; (5) knockdown of MT1, but not MT2, potentiated GSIS in MIN6 cells; and (6) *Mt1* overexpression attenuated GSIS in isolated islets (Table 1). These cumulative findings strongly support the implication of *Mt1* in the negative regulation of beta cell function.

***Mt1* and *Mt2* exhibit an atypical gene expression pattern in comparison with other antioxidant genes** MTs are known for their protective antioxidant properties [26–28]. Oxidative stress plays an important role in beta cell demise and islets of humans with diabetes and animal models display upregulated expression of many antioxidant genes and markers of oxidative damage [4]. Interestingly, antioxidant genes like *Hmox1*, *Gpx1*, *Gpx2*, *Sod1* and *Nrf2* (also known as *Nfe2l2*) were also upregulated in the islets of compensating young *db/db* mice and mice fed an HFD [17, 29, 30], in sharp contrast with *Mt1* and *Mt2* downregulation (Fig. 1). Similarly, in isolated islets, treatment with glucose upregulated *Mt3*, *Srxn1* and *Gpx2*, while it downregulated *Mt1* and *Mt2* (Fig. 2 and [31, 32]). Noteworthy, our previous studies in Wistar rats revealed that islet *Mt1* expression was upregulated by fasting and downregulated upon refeeding (J-C Jonas, unpublished data). Furthermore, a recent study exploring beta cell heterogeneity by single-cell transcriptomics revealed that high activity of the insulin gene promoter was associated with low expression of *Mt1* and *Mt2* and vice versa [33]. These observations demonstrate that *Mt1* and *Mt2* behave differently from other oxidative stress-response genes. Besides, *Mt1-Mt2* deletion does not affect antioxidant/stress-response gene expression (ESM Fig. 4 and ESM Fig. 7), indicating no evident impact on islet redox status under physiological conditions. Thus, *Mt1* and/or *Mt2* may play a role in beta cell (patho)physiology that goes beyond their known antioxidant function.

***Mt1* negatively regulates GSIS** Deletion of *Mt1* and *Mt2* improved glucose tolerance and potentiated GSIS in isolated islets from KO mice. These findings contrast with a previous study using islets from *Mt1-Mt2* KO mice [34]. Although

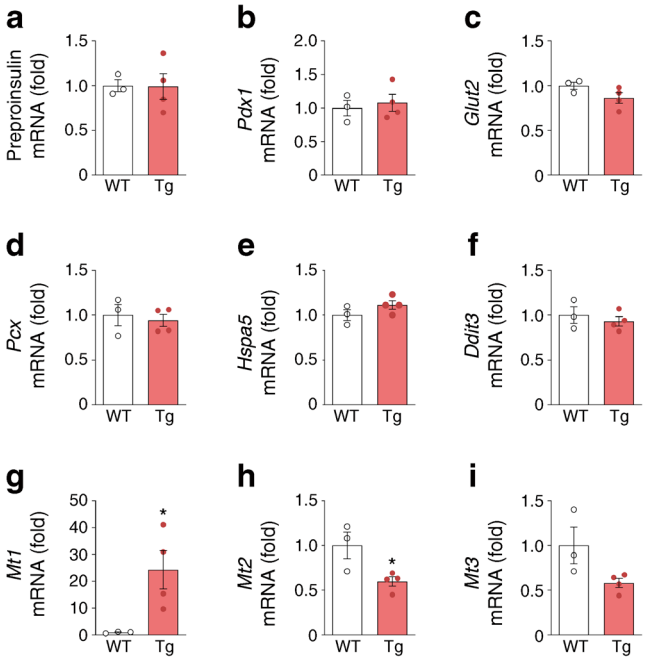

Fig. 8 *Mt1* overexpression did not affect the expression of beta cell-enriched genes and ER stress-response genes. Changes in the mRNA levels of (**a**–**d**) preproinsulin, *Pdx1*, *Glut2* and *Pcx*, and (**e**, **f**) ER stress-response genes *Hspa5* and *Ddit3* and (**g**–**i**) MT gene isoforms *Mt1*, *Mt2* and *Mt3*. Data are means ± SEM of $n = 3$–4 animals per group. $^*p < 0.05$ vs WT, unpaired two-tailed Student's *t* test. Tg, Tg-*Mt1* mice

differences in genetic backgrounds may contribute to this discrepancy, our study is more comprehensive than the previous investigation. Thus, besides our complementary in vivo and ex vivo results, the knockdown of MT1 in MIN6 cells reproduced the enhanced insulin secretory phenotype of the KO islets. Furthermore, in our study, islets overexpressing *Mt1* exhibited the reciprocal phenotype.

Our results underscore the role of *Mt1*, rather than *Mt2*, in the modulation of insulin secretion. Although these genes exhibit similar expression patterns, *Mt1* displays a higher expression level in comparison with *Mt2* and *Mt3* in primary mouse islets (ESM Fig. 8). In addition, mRNA sequence alignment analysis showed that *Mt1* and *Mt2* are only ~80% identical. Thus, the ~20% sequence difference may also underlie functional specificities. Such specificities may stem from different metal affinities and binding properties or from specific protein–protein interactions [6, 35].

How could *Mt1* impact on insulin secretion? We did not detect significant changes in classical metabolic (NAD(P)H) and ionic parameters (cytosolic free Ca^{2+} and Zn^{2+}) in response to glucose stimulation between islets from WT mice and KO or Tg-*Mt1*mice. The expression of antioxidant and other stress-response genes was not different between WT and KO islets, thereby ruling out a potential impact on redox status (ESM Fig. 4 and ESM Fig. 7). Furthermore, *Mt1-Mt2* deletion or *Mt1* overexpression had no impact on cell death in islets cultured under control conditions (10 mmol/l glucose; ESM Fig. 9). Nevertheless, insulin secretion in response to high potassium was also potentiated in islets from KO vs WT mice (ESM Fig. 10). This result strongly suggests a potential impact of *Mt1* on the beta cell exocytotic machinery. Interestingly, MT3 was previously shown to interact with Rab3A GTPase in neurons, thereby playing a role in presynaptic vesicle trafficking [36]. One may, therefore, hypothesise that MT1 interacts with a yet-to-be-identified protein of the beta cell exocytotic machinery to modulate insulin secretion.

The mechanism(s) of induction of *Mt1* in diabetes Hyperglycaemia, per se, may not be the upstream factor involved in increased expression of *Mt1* in diabetes. Indeed, although *Mt1* mRNA expression transiently increases in rat islets cultured overnight with glucose at 30 mmol/l vs 10 mmol/l [16], prolonged exposure of mouse and rat islets to high glucose (30 mmol/l) had little or no impact (vs 10 mmol/l) on *Mt1* and *Mt2* mRNA and MT1/MT2 protein levels (Fig. 2 and [32]). In human islets, culture in the presence of the already supraphysiological glucose concentration of 11.1 mmol/l vs 2.2 and 5.5 mmol/l also downregulated the mRNA levels of MT genes. Upregulation beyond this concentration may result from differences in glucose sensitivity and

Table 1 Overview of the principal findings of the study

Species	Gene	Expression during beta cell compensation	Expression in T2D	Glucose effect on expression ex vivo	Effect of deletion/KD on GSIS	Effect of overexpression on GSIS
Mouse	*Mt1*	↓	↑	↓ (G2–G30)	↑	↓
	Mt2	↓	↑	↓ (G2–G30)	↔	ND
Human	*MT1E*	ND	↔	↓ (G2.2–G11.1)	ND	ND
	MT1X	ND	↑	↓ (G2.2–G11.1)	ND	ND
	MT2A	ND	↔	↓ (G2.2–G11.1)	ND	ND

↑, increased; ↓, decreased; ↔, no change; G*n*, *n* mmol/l glucose; KD, knockdown; ND, not determined; T2D, type 2 diabetes

The two glucose concentrations given between parentheses indicate the range within which the downregulation occurs

metabolism between human and mouse islets and may involve the activation of glucotoxicity-related pathways [4]. We have previously shown that rat islet *Mt1* expression is markedly induced by exogenous H_2O_2, the sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump inhibitor thapsigargin, the cytokine IL-1β and hypoxia [37]. However, the islet expression of genes induced by oxidative stress, ER stress and inflammatory stress are observed in the prediabetic stage in *db/db* mice [17], suggesting alternate mechanisms. On the other hand, evidence from several studies implicates a possible role of hypoxia: *Mt1* mRNA expression is upregulated by hypoxia in mouse and human islets (ESM Fig. 11 and [38]), and a clear temporal in vivo association is observed between MT expression and an hypoxic gene expression signature in islets of *db/db* mice [39, 40].

Limitations of the study and perspectives In this study, we used global KO animals. Since *Mt1-Mt2* deletion may affect other metabolic tissues, one may argue that the observed secretory phenotype involves the effect of systemic factors. However, this is unlikely as we systematically precultured isolated islets for 1 week before GSIS tests. We also combined different models to demonstrate that *Mt1* negatively regulates insulin secretion, i.e. by confirming the secretory phenotype after MT1 knockdown in MIN6 cells. Additionally, the attenuation of GSIS by *Mt1* overexpression further supports our hypothesis. Although the Tg-*Mt1* model is also global, it presents two important advantages: (1) the overexpression of the mouse *Mt1* gene rather than human *MT2A* gene [41]; and (2) the control of *Mt1* by its natural promoter rather than the insulin promoter, thereby avoiding the ER stress and oxidative stress observed in *MT2A*-transgenic mice [41]. Indeed, our Tg-*Mt1* islets displayed normal expression of ER stress-response genes (Fig. 8). Nevertheless, development of an *Mt1*-floxed mouse model is warranted for further exploration of these novel roles of *Mt1* in beta cell biology.

Conclusion *Mt1* negatively regulates insulin secretion. Downregulation of islet *Mt1* in obesity may, thus, contribute to beta cell compensation, and its upregulation in type 2 diabetes may contribute to beta cell failure. Inhibition of *Mt1* may, therefore, represent an attractive therapeutic target to preserve and restore insulin secretion in type 2 diabetes.

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Data availability All data points generated or analysed during the current study are shown in the figures of this published article (and its supplementary information files). Tabulated datasets are available from the corresponding author on reasonable request.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement MB conceived the study and designed experiments, acquired and analysed most of the data and wrote the first draft of the manuscript. JCJ conceived the study and designed experiments, analysed data and revised the manuscript. YCS, JYC, DRL, HC, MAS, EGP and HET designed experiments, acquired and analysed data and critically reviewed the manuscript. PG contributed to the analysis and interpretation of the data and critically reviewed the manuscript. All authors approved the final version of the manuscript. MB is the guarantor of this work.

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