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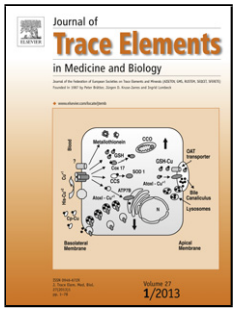

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Prolonged stimulation of insulin-release from MIN6 cells causes zinc depletion and loss of β-cell markers

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Abstract

Zinc is integral for the normal function of pancreatic β-cells in glycaemic control. Large amounts of zinc are secreted from β-cells following insulin exocytosis and regulated replenishment is required, which is thought to be mediated by the ZIP family of zinc importer proteins. Within type 2 diabetic patients, β-cells are stressed through prolonged stimulation by hyperglycaemia and this is thought to be a major factor contributing to loss of β-cell identity and mass. However, the consequences for the β-cell zinc status remain largely unexplored. We used inductively coupled plasma mass spectrometry (ICP-MS) to show that 24 h treatment of MIN6 cells with potassium chloride, mimicking hyperglycaemic stimulation, reduces the total cellular zinc content 2.8-fold, and qPCR to show an increase in mRNA expression for metallothioneins (*Mt1* and *Mt2)* following 4 and 24 h of stimulation, suggestive of an early rise in cytosolic zinc. To determine which ZIP paralogues may be responsible for zinc replenishment, we used immunocytochemistry, western blot and qPCR to demonstrate initial ZIP1 protein upregulation proceeded by downregulation of mRNA coding for ZIP1, ZIP6, ZIP7 and ZIP14. To assign a biological significance to the decreased total cellular zinc content, we assessed expression of key β-cell markers to show downregulation of mRNA for *MafA*, *Mnx-1*, *Nkx2*.2 and *Pax6*. Our data suggest hyperglycaemia-induced zinc depletion may contribute to loss of β-cell identity and promote β-cell dedifferentiation through disrupting expression of key transcription factors.

Keywords

Zinc

β-cells

ZIP transporters

Transcription factors

Gene expression

Dedifferentiation

Abbreviations

Zn^{2+}: zinc ions, MT: metallothionein, ZIP: Zrt- and Irt-related protein, ZnT: zinc transporter protein, GSIS: glucose-stimulated insulin secretion, K_{ATP} channels: ATP-sensitive potassium channels, L-VGCC: L-type voltage-gated calcium channels, Ca^{2+}: calcium ions, TRP: transient receptor potential, qPCR: quantitative PCR, ICP-MS: inductively-coupled plasma mass spectrometry, MODY: maternity-onset diabetes of the young, MRE: metal response element.

Introduction

Zinc is an essential micronutrient in mammalian physiology and human health. Links between zinc and diabetes were first established in 1938 [1], and a hypozincaemic status have since been described in some type 2 diabetic patients [2,3] and in animal models of diabetes [4,5]. Roles of both environmental and genetic contributors to Type 2 Diabetes pathogenesis and progression have been extensively documented, and estimates for heritability range from 30-70% [6,7].

Zinc is a structural, catalytic, and regulatory component of proteins within and outside cells [8]. Even at very low concentrations the free zinc ions (Zn^{2+}) exhibit potent effects [9,10]. Intracellular Zn^{2+} concentrations are therefore tightly regulated to mediate physiological targets whilst avoiding cross-metal interference [11]. Cells maintain intracellular Zn^{2+} concentrations through buffering and muffling (time-dependent redistribution through intracellular compartmentalisation) dynamics, which vary dependent on cellular state [12]. Buffering is mediated by metallothionein (MT1-4) zinc-binding proteins. MT mRNA levels are rapidly increased in response to a rise in cytosolic Zn^{2+}, primarily driven by activation of the Zn^{2+}-sensing transcription factor MTF1, which regulates expression of genes involved in intracellular zinc homeostasis [13]. Translocation of Zn^{2+} across biological membranes is mediated by the Zrt- and Irt-related protein (ZIP; SLC39A) and the zinc transporter protein (ZnT; SLC30A) families of zinc transporters, which move zinc into and out of the cytosol, respectively. In humans and rodents, 14 ZIP and 10 ZnT paralogues exist [14,15], varying in terms of tissue distribution, cytolocation, zinc-affinities, transporting efficiencies and regulatory mechanisms to tightly and specifically deliver zinc to different cell-types and organelles [14,16].

Type 2 Diabetes is a chronic metabolic disease characterised by hyperglycaemia proceeding to loss of insulin-secreting pancreatic β-cells through apoptosis and dedifferentiation to a progenitor-like state [17-19]. Critically, zinc is integral for β-cell function in glycaemic control [20,21]. β-cells maintain total zinc concentrations at 150-200μM, of which 70% is contained within insulin secretory granules at much higher concentrations [22]. Zinc is loaded into granules via the predominantly β-cell-specific zinc transporter 8 (ZnT8) [9,23], where two Zn^{2+} bind insulin hexamers, essential for proper insulin processing and granule maturation [24]. High concentrations of extracellular glucose (16.7-22.2mM [25]) stimulate signalling cascades resulting in Zn^{2+} and insulin co-release from β-cell granules [26,27], known as glucose-stimulated insulin secretion (GSIS). The GSIS pathway involves metabolism of glucose through glycolysis to elevate the ATP/ADP ratio, which agonises ATP-sensitive potassium ion (K^+) channels (K_{ATP} channels) to promote membrane depolarisation, activation of L-type voltage-gated calcium channels (L-VGCC), resulting in calcium ion (Ca^{2+}) and Zn^{2+} influx [28], membrane fusion of granules and insulin exocytosis [29]. ZnT8 is a recognised Type 2 Diabetes risk locus at which an arginine to tryptophan substitution polymorphism confers a ~14% increase in risk per arginine risk allele [30,31]. Rare loss-of-function ZnT8 polymorphisms are protective against Type 2 Diabetes [32], highlighting the complex relationship between zinc trafficking and β-cell function. An adequate zinc status is further important for maintaining the intracellular redox balance, signalling pathways linked to β-cell mass [33], and is associated in zebrafish with expression of transcription factors (*neurod1*, *hnf1ß*, *hnf4α*, *foxa1*, *nkx2.2*, and *pax6*) required for ß-cell differentiation and endocrine function [34-37]. A recent study using human insulin-secreting β-cell like stem cells additionally showed zinc supplementation induces up-regulation of the β-cell markers PDX1 and PAX4 [38]. Thus, there might be a link between zinc dysregulation in β-cells during diabetes and loss of β-cell phenotype and associated endocrine function.

Hyperglycaemia is universal in all type 2 diabetic patients and contributes to loss of β-cell mass through promoting β-cell dedifferentiation [18]; prolonged stimulation of the GSIS pathway is therefore characteristic of Type 2 Diabetic pathology. β-cells exhibit an exceptionally high turnover of zinc to maintain homeostatic control over zinc concentrations following insulin granule exocytosis, and replenishment is required to maintain intracellular homeostasis [39]. High glucose stimulation of mouse islets for 24 h has previously been shown to double cytosolic free Zn^{2+} concentrations and to up-regulate genes involved in cellular zinc homeostasis [40] including ZIP6 and ZIP7 [41,42]. However, the consequences of chronic hyperglycaemia for β-cell zinc content remains poorly understood and experimental exploration of the ZIP paralogues in β-cell function remains limited to a few studies [40,43-46]. We have previously shown through a systematic review complemented with experimental data that ZIP6, ZIP7, ZIP9 and ZIP14 in human and rodent, and ZIP1 in rodent are potentially biologically important for β-cell zinc trafficking [46]. Here, we postulated these ZIP paralogues are involved in intracellular zinc homeostasis during β-cell stimulation. We also hypothesised that prolonged stimulation may lead to cellular zinc loss through enhanced zinc/insulin co-secretion, and that this may affect expression of β-cell markers, which as a consequence could influence β-cell phenotype. Thus, the aim of the present study was to characterise the consequences of prolonged stimulation for β-cell zinc content and expression of key ZIP transporters and zinc-responsive β-cell markers.

We show that prolonged stimulation decreases the total zinc content of MIN6 cells but increases mRNA expression for *Mt1* and *Mt2*, indicative of an early surge in cytosolic Zn^{2+} followed by increased buffering capacity as MT1 and MT2 proteins are being produced [47]. Unexpectedly, this effect on MT was coupled with a decrease in *Mtf1* mRNA. Using a

combination of quantitative PCR (qPCR), immunocytochemistry and western blot, we observed an increase in ZIP1 and a decrease in ZIP7 protein, down-regulation of mRNA for *Slc39a1*, *Slc39a6*, *Slc39a7*, *Slc39a14*, and down-regulation of multiple zinc-responsive β-cell markers. Our data demonstrate that prolonged stimulation depletes β-cells of zinc, presumably as a result of a decrease of multiple ZIP transporters along with loss of gene expression of several markers of β-cell differentiation.

Methods and Materials

Cell line and culture

The adherent insulinoma β-cell line MIN6 (*Mus musculus*) was a kind gift from Dr. Jun-ichi Miyazaki [48]. Cells were maintained within 25mM glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% foetal bovine serum (FBS), 4mM L-glutamine, 50μM β-mercaptoethanol, 100μg/ml streptomycin and 100 units/ml penicillin (both Thermo Fisher) at 37^{o}C in a humidified atmosphere of 95% air and 5% CO_2. Cells were cultured for 24 h prior to experimentation. *For cell stimulation:* growth media was replaced with glucose-free DMEM containing 15% FBS, 4mM L-glutamine, 50μM β-mercaptoethanol, 100μg/ml streptomycin and 100 units/ml penicillin supplemented with either 3mM glucose (control) or 3mM glucose and 40mM potassium chloride (KCl) for 4 h or 24 h. KCl was used to depolarise MIN6 cells to induce insulin secretion since the cells were not glucose-responsive. *For zinc depletion:* FBS was depleted of metal ions through incubation with 5% (w/v) Chelex-100 (Sigma) for 1 h, and sterile-filtered through a 0.22μm syringe filter. Chelex-100 treated FBS was diluted 1:100 in 0.5% nitric acid (HNO_3) and heavy metal analysis carried out through inductively-coupled plasma mass spectrometry (ICP-MS) [49]. Chelex-100 treated FBS was supplemented with Ca^{2+}, K^+ and Na^+ to original concentrations. Growth media were replaced

with 25mM glucose DMEM containing 15% Chelex-100 treated-FBS, 4mM L-glutamine, 50μM β-mercaptoethanol, 100μg/ml streptomycin and 100 units/ml penicillin, supplemented with $ZnCl_2$ to total concentrations of 1μM or 8μM (control) Zn^{2+} for 24, 48 or 96 h.

Determination of total zinc content

Cells were lysed in hot 0.2% sodium dodecyl sulfate (SDS). Samples were diluted 1:100 with 0.5% HNO_3 (trace element grade) and the zinc concentration determined through ICP-MS. The total zinc content was normalised to protein content, determined through Bradford assays (Bio-Rad) [50], which were standardised to dilutions of bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Statistical significance of differences in cellular zinc content was examined through unpaired t-tests.

RNA extraction, cDNA synthesis and quantitative PCR

Total RNA was extracted using TRIzol Reagent (Thermo Fisher), reverse transcribed to cDNA using the high capacity RNA-to-cDNA kit (Thermo Fisher), and diluted ≥1:10 prior to experimentation. Quantitative PCR (qPCR) assays were designed using the online Universal Probe Library (UPL) assay design tool (Roche). Assay designs are provided in Additional file 1. PCR plates were loaded using the Biomek FX liquid handling robot (Beckman Coulter) and reactions (20-40ng cDNA, 0.1μM UPL probe, 0.2μM forward primer, 0.2μM reverse primer and 1X TaqMan Fast Advanced Mastermix (Applied Biosystems) amplified using the Prism7900HT sequence detection system (Applied Biosystems) and analysed with SDS (sequence detection systems) 2.4 software. All gene expression values were normalised to the housekeeping gene ubiquitin C, and relative expression calculated using the ΔΔCT method [51]. The efficiencies for all primers ranged from 81 – 120%. Significance of differences in

mRNA expression were examined through unpaired t-tests. Data show an average of three biological repeats.

Immunocytochemistry

MIN6 cells were seeded onto sterile glass coverslips and treated as described above. Cells were fixed using 4% paraformaldehyde for 15 min, permeabilised in 0.25% (v/v) Triton X-100 for 10 min and blocked in 3% (w/v) BSA/PBS for 1 h. Cells were immunolabelled through incubation with primary antibodies targeting ZIP1 (SAB3500592; Sigma Aldrich), ZIP6 (PA5-21071; Thermo Fisher), ZIP7 (19429-1-AP; Proteintech), ZIP9 (PA5-21074; Thermo Fisher) and ZIP14 (PA5-21077; Thermo Fisher) overnight at 4^{o}C, followed by incubation with Alexafluor 595-linked secondary antibodies (A11012; Thermo Fisher) for 1 h at room temperature. Cells were extensively washed in PBS between each step. Coverslips were mounted onto coverslides using VECTASHIELD Antifade Mounting Media containing 4',6-diamidino-2-phenylindole **(**DAPI) (VECTOR Laboratories) and imaged using an AR1 confocal microscope (Nikon). We quantified the average Alexafluor 595 fluorescence intensities of six images taken across each slide using Fiji, ImageJ software [52], calculated as a ratio relative to DAPI. Significance of differences in protein abundances were examined through unpaired t-tests.

Western blotting

Protein lysates were prepared in 2x loading dye (Thermo Fisher) and boiled (10 min, 72^{o}C). Lysates (20µg) were separated on 12% acrylamide SDS-PAGE gels using SDS-PAGE buffer (both Thermo Fisher) supplemented with 0.1% (w/v) SDS (200V, 40 min). Proteins were transferred onto nitrocellulose membranes [GE Healthcare Life Sciences (1 h, 100V)] using transfer buffer (25mM Tris, 192mM glycine, 10% methanol (pH 8.3)), and membranes blocked

through incubation in 5% (w/v) BSA/Tris-buffered saline [TBS (50mM Tris-Cl pH 7.5, 150mM NaCl)] and 0.05% (v/v) TWEEN-20 [TBST (1 h, 25ºC)]. Membranes were incubated with primary antibodies targeting ZIP1 [SAB3500592; Sigma Aldrich (1:400)] or α-Tubulin [T9026, Sigma (1:15,000)] diluted in 1% BSA/TBST (16 h, 4ºC), followed by HRP-linked secondary antibodies [α-mouse: Amersham, NA931V (1:20,000); α-rabbit: Amersham, NA934V (1:15,000)] diluted in 1% BSA/TBST (1 h, 25ºC). Membranes were extensively washed with TBST between each step. Membranes were treated with ECL Western Blotting Detection Reagent (GE Healthcare Life Sciences) and visualised on X-ray film (GE Healthcare Life Sciences) using a film imager. For membrane stripping, immunoblots were washed in TBST, incubated in stripping buffer [Thermo Fisher (15 min, 25ºC)] and washed with TBST (10 min, 25ºC). Membranes were blocked with 5% (v/v) BSA/TBST (1 h, 25ºC) before further antibody incubation.

Proliferation assays

The rate of cell proliferation was determined using the Cell Proliferation ELISA, BrdU (colorimetric) assay (Roche), following manufacturer's instructions.

Apoptosis assays

The rate of cell apoptosis was determined through Caspase-Glo® 3/7 assays (Thermo Fisher), following manufacturer's instructions.

Transcription factor network analysis

MetaCore (GeneGo) Bioinformatics software from Thomson Reuters (https://portal.genego.com/) was used to create a network from qPCR data of transcription factor expression after zinc depletion for 48 h. The experimental data were uploaded to

MetaCore version 6.32 build 69020 and analysed through the 'Build Network' function, generating sub-networks enriched with the seed nodes from the qPCR data using canonical pathways and limiting the number of nodes in a network to 50. The top network was pruned to remove all objects with less than three links to analysed genes, leaving only FOXA2 (HNF-3B) in addition to the analysed genes. Data showing the basis of this network are provided in Additional file 2.

Results

Prolonged stimulation decreases the total zinc content of MIN6 cells

Zinc is co-released with insulin from β-cells during stimulation with glucose- or KCl–induced depolarisation. However, the consequences of prolonged stimulation for β-cell zinc content remain poorly understood. We stimulated MIN6 cells for 24 h and found a 2.8-fold depletion in total cellular zinc (**Fig 1A**). Total cell zinc depletion was associated with elevated mRNA expression of the metallothioneins *Mt1* and *Mt2* (1.3-fold and 2.1-fold, respectively, at 4 h and 2.9-fold and 1.9-fold, respectively, at 24 h), indicative of an increase in cytosolic Zn^{2+} and consequent MTF1-mediated transactivation [53]. We further noted down-regulation of mRNA for *Mtf1* 1.4-fold at 4 h and 1.8-fold at 24 h (**Fig 1B**).

Prolonged stimulation disrupts expression of multiple β-cell ZIP transporters

Increased zinc uptake during β-cell stimulation is likely influenced by changes in activity and/or abundance of ZIP and ZnT paralogues important for β-cell function. We therefore explored protein and mRNA expression of ZIP paralogues we previously highlighted as important for β-cell function [46] and mRNA expression for ZnT1 and ZnT8. We identified

up-regulation of ZIP1 protein (1.5-fold) and a small but statistically significant down-regulation of ZIP7 protein (1.1-fold) through immunocytochemistry following 24 h sustained stimulation (**Fig 2A-B**). ZIP1 protein up-regulation was verified using western blot (p=0.04) (**Fig 2C**). Whilst we did not observe any differences in the mRNA abundances for these paralogues at 4 h, mRNA for *Slc39a1* and *Slc39a7* showed statistically significant down-regulation (*Slc39a1*: 6.8-fold, *Slc39a7*: 2.0-fold) along with those for *Slc39a6* (2.3-fold), and *Slc39a14* (2.2-fold) at 24 h (**Fig 2D**). We further observed statistically significant down-regulation of mRNA for *Slc30a8* (1.8-fold) at 24 h, but mRNA for *Slc30a1* remained unchanged. These data suggest prolonged β-cell stimulation induces initial up- and down-regulation of ZIP1 and ZIP7 proteins, respectively, followed by downregulation of mRNA for ZIP1, ZIP6, ZIP7, ZIP14 and ZnT8.

Prolonged stimulation disrupts expression of zinc-responsive β-cell markers

β-cell proliferation and function are controlled by multiple transcription factors, many of which are required to maintain the β-cell phenotype. Changes in the expression/function of important β-cell proteins are suggestive of loss of cellular identity and indicate mechanisms contributing to β-cell dedifferentiation [18,54]. We examined the expression of the transcription factors *Foxa1, Foxo1, Hnf1b, Hnf4a, MafA, Mnx1, Neurod1, Nkx2.2, Nkx6.1, Pax4, Pax6* and *Pdx1* in MIN6 cells following stimulation with KCl for 24 h and found significant up-regulation of mRNA expression for *Hnf1b* 2.9-fold, and down-regulation of mRNA expression for *Foxa1* (1.8-fold), *MafA* (3.8-fold), *Mnx-1* (2.6-fold), *Nkx2*.2 (2.6-fold) and *Pax6* (2.7-fold) (**Fig 3A**). Although not statistically significant, mRNA for *Hnf4a* showed 3.1-fold numerical increase ($p<0.2$) and *Neurod1* 2.0-fold decrease ($p<0.06$). These data indicate an association between

prolonged stimulation and changes in expression of key β-cell transcription factors with the predominant trend being down-regulation.

The transcription factors NEUROD1, HNF1B, HNF4A, FOXA1, NKX2.2 and PAX6 have previously been identified as zinc-responsive [34-37]. Since prolonged stimulation decreases intracellular zinc content, we asked whether changes in β-cell transcription factor expression may be consequential of cellular zinc depletion. We treated MIN6 cells with growth medium containing low (1μM) zinc for 24 h to show no differences in mRNA expression for these key transcription factors compared to control cells cultured in growth medium with 8μM zinc (**Fig 3B**). However, after 48 h we observed statistically significant down-regulation of mRNA for *Hnf1b* (3.3-fold), *Hnf4a* (2.9-fold), *MafA* (1.8-fold), *Mnx-1* (3.6-fold), *Nkx2.2* (1.2-fold), *Nkx6.1* (2.5-fold) *Pax4* (1.5-fold) *Pax6* (3.2-fold) and *Pdx1* (2.7-fold) (**Fig 3C**). These data demonstrate loss of *Hnf1b, Hnf4a, MafA, Mnx-1, Nkx2.2, Nkx6.1, Pax4, Pax6* and *Pdx1* in response to zinc depletion in mammalian β-cells and suggest changes in expression following chronic stimulation may be a result of zinc depletion caused by prolonged zinc secretion in combination with down-regulation of zinc importers.

Suppression of β-cell markers is suggestive of loss of β-cell identity and mass. To explore the biologically relevant consequences of zinc depletion-induced transcription factor down-regulation we examined mRNA expression for *Arx*, which is activated to induce β-to-α-cell reprogramming [55], and MIN6 cell survival. We recorded a comparable increase in *Arx* mRNA abundance for cells cultured with 40mM KCl for 24 h and low (1μM) zinc for 48 h (4.0-fold and 2.9-fold, respectively). Although we did not observe any differences in MIN6 cell proliferation or apoptosis following 48 h zinc depletion (data not shown), by 96 h, cells exhibited a 1.3-fold decrease in proliferation ($p<0.05$) and 1.4-fold increase in apoptosis

($p<0.005$). These data support the contribution of chronic stimulation-induced MIN6 cell zinc depletion to loss of β-cell identity and decreased survival.

Discussion

Zinc depletion of MIN6 cell causes loss of β-cell markers

Zinc is an inter- and intracellular signalling component, regulating almost all aspects of cellular function including gene expression. In the current study, we demonstrate the transcription factors *Hnf1b, Hnf4a, MafA, Mnx-1, Nkx2.2, Nkx6.1, Pax4, Pax6* and *Pdx1* are down-regulated by zinc depletion in mammalian β-cells. These transcription factors have important roles in maintaining β-cell identity and their deregulation impacts cellular phenotype and function, including insulin pathway regulation, β-cell mass and islet architecture (**Fig 4**). PDX1 is considered the key transcription factor in mature β-cells mediating β-cell survival and mass [56] and controlling a large array of genes [57,58]. PDX1 [59], HNF4A [60], MAFA [61] and PAX6 [62,63] required for maximal insulin production. NKX6.1 stimulates islet cell proliferation [64], and MNX1 facilitates proliferation in aged cells [65]. Similarly, PAX4 stimulates proliferation and promotes survival of mature β-cells through protecting against stress-induced apoptosis [66,67]. NKX2.2 acts upstream of GLUT2, the transporter responsible for glucose uptake into rodent β-cells, and is necessary for proper β-cell clustering and islet architecture [68], and HNF1B regulates pathways mediating glucose signalling in β-cells [69]. Critically, monogenetic mutations in Hnf1b, Hnf4a, Pdx1 and Pax4 result in 'maternity-onset diabetes of the young' (MODY) [70] and, recently, mutations in Nkx2.2 and Mnx-1 have additionally been linked to the neonatal disease [71]. Consistent with our results, *hnf1ß*, *hnf4α, nkx2.2,* and *pax6* exhibit zinc-responsive expression in zebrafish [34-37], and PDX1 and PAX4 show up-regulation in response to zinc in human exfoliated deciduous tooth stem cells induced into insulin-secreting β-cell like stem cells [38]. Importantly, β-cell and glucagon-

secreting α-cells are derived from common progenitors and share expression of many transcription factors at differing equilibrium, many of which are specific to the endocrine pancreas; suppression of important β-cell transcription factors induces β-cell dedifferentiation and transdifferentiation into α-cells. PDX-1, NKX2.2 and NKX6.1 are essential for maintaining β-cell phenotype in early β-cell development, proceeded by a prominent role of PAX4, MAFA and the related transcription factor MAFB. In mature β-cells, loss of PDX-1 and NKX2.2 causes complete loss of β-cell identity and PAX4, NKX6.1 and PDX-1 inhibit glucagon expression to prevent α-cell transdifferentiation [54,72]. Concurrently, we demonstrate MIN6 cell zinc-depletion increases mRNA expression for *Arx*, which is indicative of loss of β-cell identity and α-cell reprogramming, and reduces MIN6 cell mass through both reduced proliferation and increased apoptosis. Our data further suggest the systemic hypozincaemia present in some type 2 diabetic patients may contribute to loss of β-cell mass through promoting β-cell dedifferentiation or transdifferentiation into α-cells.

Prolonged stimulation of MIN6 cells causes zinc depletion and deregulation of β-cell markers

Chronic hyperglycaemia elevates the requirement for zinc replenishment through extending the duration of secretory pathway activation and β-cell zinc loss. Following prolonged MIN6 cell stimulation, we observed down-regulation of *Foxa1*, *MafA*, *Mnx-1*, *Nkx2*.2 and *Pax6*. We also found that a massive amount of the cellular zinc was lost during this treatment, probably reflecting the zinc lost from insulin granules, which has been previously observed following chronic exposure of rat INS-1E cells to high extracellular glucose [73]. Since depletion of zinc from the culture medium caused down-regulation of mRNA for nine out of the 12 β-cell markers measured, including *MafA*, *Mnx-1*, *Nkx2*.2 and *Pax6*, suppressed expression of these genes following prolonged stimulation may be a direct result of zinc depletion. The effects on β-cell markers were apparent following 24 h of KCl-induced depolarisation, but only after 48

h of treatment with reduced zinc concentration in the culture medium. This difference in time-course of the effect might be explained by temporal differences in depleting the cellular zinc content, which was very rapid during KCl treatment. However, treatment with a low zinc concentration in the culture medium did not completely mimic the effect triggered by prolonged stimulation using KCl. Interestingly, we show that depletion of zinc from the medium down-regulates *Hnf1b*. However the mRNA for this transcript was up-regulated following 24 h KCl stimulation; a similar relationship was observed for *Hnf4a* which was downregulated by zinc depletion for 48 h but not by the 24 h KCl-induced stimulation. Like most β-cell markers, expression of both *Hnf1b* and *Hnf4a* is tightly regulated by multiple factors, and an alternative pathway likely dominates regulation during cellular stimulation. Nevertheless, since major differences in *Hnf1b* and *Hnf4a* expression profiles are reported between healthy human and rodent cells [74], these results may not be easily translatable to the human Type 2 Diabetes.

The role of zinc in maintenance of key transcription factors in MIN6 cells

Complex coordination and cross-regulation exist between transcription factors to tightly regulate β-cell phenotype and function. Based on published interactions between these transcription factors and our experimental data showing zinc depletion-mediated changes in gene expression, we are able to speculate zinc might target this network at MAFA, NEUORD1 or NKX2.2 (**Fig 5**). Zinc may influence changes in gene expression via MTF-1 or another zinc-signalling pathway such as via the zinc-finger transcription factors KLF4 [75] or ZNF658 [76]. Alternatively, the results may stem from altered transcription factor phosphorylation affecting protein activation and/or localisation, which has been previously shown for MAFA [77] and NEUROD1 [78]. Pinpointing the molecular action of zinc in this network requires further

investigation and could prove important for understanding loss of β-cell identity and insulin secretory function during hyperglycaemia-induced β-cell dedifferentiation.

Fluctuations in markers for cytosolic Zn^{2+} during β-cell stimulation

Cellular zinc depletion was accompanied by up-regulation of mRNA expression for the metallothioneins *Mt1* and *Mt2*, which is suggestive of an early surge in cytosolic Zn^{2+} [40] followed by increased buffering capacity as MT1 and MT2 proteins are produced [79]. An early surge in Zn^{2+} uptake might be explained by increased zinc trafficking to insulin granules. We further demonstrated a decrease in mRNA expression for MTF1 within MIN6 cells, which cannot be readily explained. Zn^{2+} binds MTF1 at six Cys_2His_2-type zinc-finger domains, which exhibit differing binding affinities. Zn^{2+} binding promotes translocation of MTF1 from the cytosol to the nucleus, where MTF1 binds metal-response elements (MRE) in the promoters of zinc-regulated genes [80]. Although MTF1 transcript and protein levels increase with zinc treatment, MTF1 activity is mainly believed to be regulated post-translationally through nuclear-cytoplasmic shuttling, DNA-binding, phosphorylation and interaction with other transcriptional co-activators [13]. The observed increases in *Mt* mRNA could be a result of epigenetic regulation, increased MTF1 post-translational activation, or MTF1-independent mechanisms governing MT gene expression [81].

Stimulation-induced increases in cytosolic Zn^{2+} are consistent with a previous study on mouse islets [40], however these authors observed MT1 and MT2 decrease and did not assess the total cellular zinc content. This variation could be explained by differences in the experimental design, including use of glucose rather than KCl to induce cellular stimulation or the use of primary islets containing 70-80% β-cells rather than a β-cell line which exist as a monolayer. We acknowledge that it is possible these differences in results could be an artefact of tissue

culture rather than a physiological response, but our observations would still lend valuable information for interpreting future data on changes to the zinc status of β-cells. Furthermore, the variation may only represent temporal differences between conditions, perhaps influenced by the time required to induce intracellular zinc depletion. These observations need to be explored further to distinguish between physiological state and the pathological state proceeding loss of β-cell identity and β-cell dedifferentiation.

Regulation of zinc transporter expression during prolonged stimulation of MIN6 cells

L-VGCCs are activated during stimulation of the insulin secretory pathway [28] but are not thought to play the pivotal role in cellular zinc uptake [40], indicating important roles for the ZIP transporters. Our results suggest that an early post-transcriptional or post-translational up-regulation of ZIP1 protein may be integral in the physiological response to increased stimulation in murine MIN6 cells. ZIP1 is a plasma membrane-bound paralogue which we previously identified as highly expressed and potentially biologically important for rodent but not human β-cells [46]. It is therefore possible human β-cells encompass a different mechanism from rodents to adapt to increased zinc replenishment during elevated insulin secretion, perhaps through up-regulating expression of transporters functionally substituting in β-cells for the rodent ZIP1. Contrary to our expectation of what would happen following KCl-induced cellular zinc depletion, we observed down-regulation of *Slc39a1*, *Slc39a6*, *Slc39a7* and *Slc39a14* mRNA after 24 h KCl stimulation. This could be in response to elevations in cytosolic Zn^{2+}, either representing a protective mechanism to prevent cytosolic Zn^{2+} accumulation to pathological concentrations or reflecting zinc-responsive changes to mRNA transcript levels. Down-regulation of mRNA for ZIP4, ZIP5, ZIP6, ZIP7 and ZIP10 has previously been identified as a mechanism to protect against cytosolic zinc overload [41,42,82,83]. ZIP7 is localised to the Golgi apparatus [42], which stores zinc at high concentrations [84], and

functions to release zinc spatially and temporally in response to stimuli [42]; ZIP7 down-regulation could be indicative of zinc depletion from the Golgi apparatus or represent a protective mechanism to prevent further depletion. Furthermore, ZIP14 facilitates import of non-haem iron in addition to zinc [85,86], indicating stimulation-induced ZIP14 downregulation could be a protective mechanism against accumulation of iron to cytotoxic concentrations. It is possible the observed ZIP suppression is simply a pathological response to prolonged stimulation rather than serving a physiological purpose. Since ZIP6, ZIP7 and ZIP14 additionally exhibit potent roles in stimulating cellular proliferation [87-92], chronic stimulation is likely to be detrimental to β-cell proliferation, and this may help to explain the loss of β-cell mass observed in type 2 diabetic patients.

ZnT transporters likely function co-operatively with the ZIPs to mediate β-cell zinc trafficking in response to chronic stimulation. A recent study has reported appreciable expression of all ZnT exporters in pancreatic β-cells [93]. Of potential notable importance, ZnT1 is detected with high abundance at the plasma membrane and ZnT8 at the insulin granule membranes. We demonstrate *Slc30a8* mRNA downregulation in MIN6 cells following 24 h KCl stimulation. ZnT8 downregulation has been suggested to increase β-cell survival [94,95]; however, studies using rat INS-1E cells demonstrate repression reduces cellular insulin content and GSIS[96] and overexpression stimulates increase in total intracellular zinc and protects against zinc-depletion induced cellular death [97], indicating loss of ZnT8 reduces β-cell identity and survival. Accordingly, there is currently no clear consensus on the role of ZnT8 for β-cell phenotype and function. There was no observed difference in mRNA expression for ZnT1. ZnT1 is regulated by MTF1 [98] and dietary zinc [99] at the transcriptional, post-transcriptional and post-translational level, including through presumed subcellular redistribution [100], and knockdown increases cadmium influx and toxicity [101]. We are therefore unable draw

conclusions about ZnT1 regulation in response to chronic MIN6 cell stimulation from our data without first exploring the abundance and subcellular localisation of the expressed protein.

Conclusion

In this study, we explored the consequences of prolonged β-cell stimulation, characteristic of the hyperglycaemia universal in type 2 diabetic patients, for cellular zinc status and markers of β-cell function. We show sustained stimulation depletes β-cells of zinc and down-regulates multiple ZIP transporters of importance for β-cell function [46]. Potentially important for understanding human Type 2 Diabetes development, we demonstrate key β-cell transcription factors disrupted by prolonged stimulation are zinc-responsive in mammalian β-cells and their mRNA levels depressed by zinc depletion. We further anchored these findings to β-cell function, by showing that zinc depletion decreases proliferation and increases apoptosis. Critically, our results reveal a plausible causative link between hyperglycaemia-induced zinc depletion of pancreatic β-cells and loss of expression of transcription factors of importance for β-cell differentiation, proliferation and endocrine function.

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Declarations of interest

None

Figure legends

Fig 1. MIN6 cell zinc content following prolonged stimulation. (A) Total cellular zinc content after 24 h treatment with 40mM KCl determined through ICP-MS and normalised to protein content. N=4. **(B)** mRNA expression of *Mt1*, *Mt2* and *Mtf1*, assessed through qPCR. Expression was calculated as a ratio compared to cells cultured under the same conditions but without KCl (3mM glucose; control). N=3. Error bars show ±SEM. *p<0.05**,** **p<0.005, ***p<0.001.

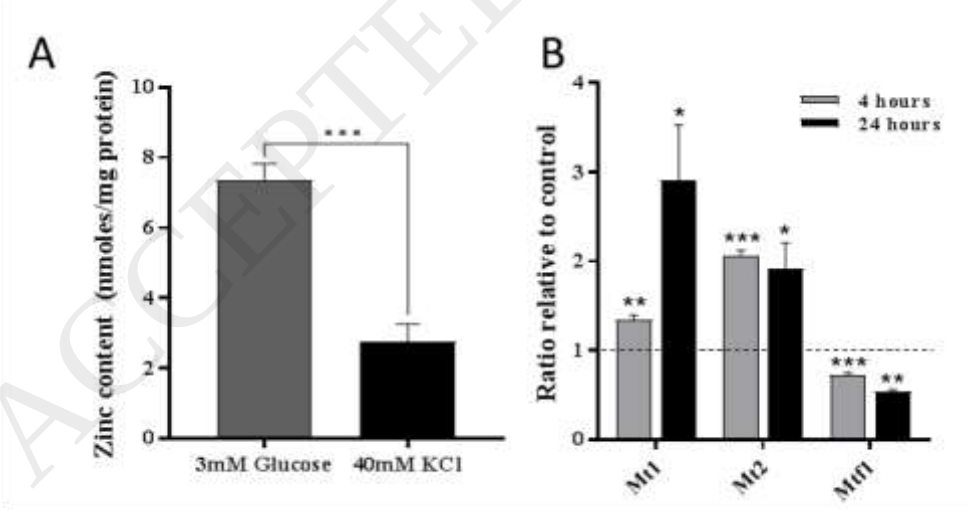


Fig 2. Changes in ZIP/*Slc39a* expression in MIN6 cells following prolonged stimulation. (A-C) ZIP protein abundances following culture with 40mM KCl for 24 h, imaged by immunocytochemistry. **(A)** Representative immunocytochemistry images for ZIP1 and ZIP7

after 24 h. Scale bar: 100µm. **(B)** Average changes in ZIP protein abundances after 24 h. **(C)** Western blot for ZIP1 protein abundance after 24 h. **(D)** *Slc39a* mRNA abundances following culture with 40mM KCl for 4 h (grey) and 24 h (black), assayed through qPCR. Expression was calculated as a ratio compared to cells cultured under the same conditions but without KCl (3mM glucose; control). N=3. Error bars show ±SEM. *p<0.05, **p<0.005, ***p<0.001.

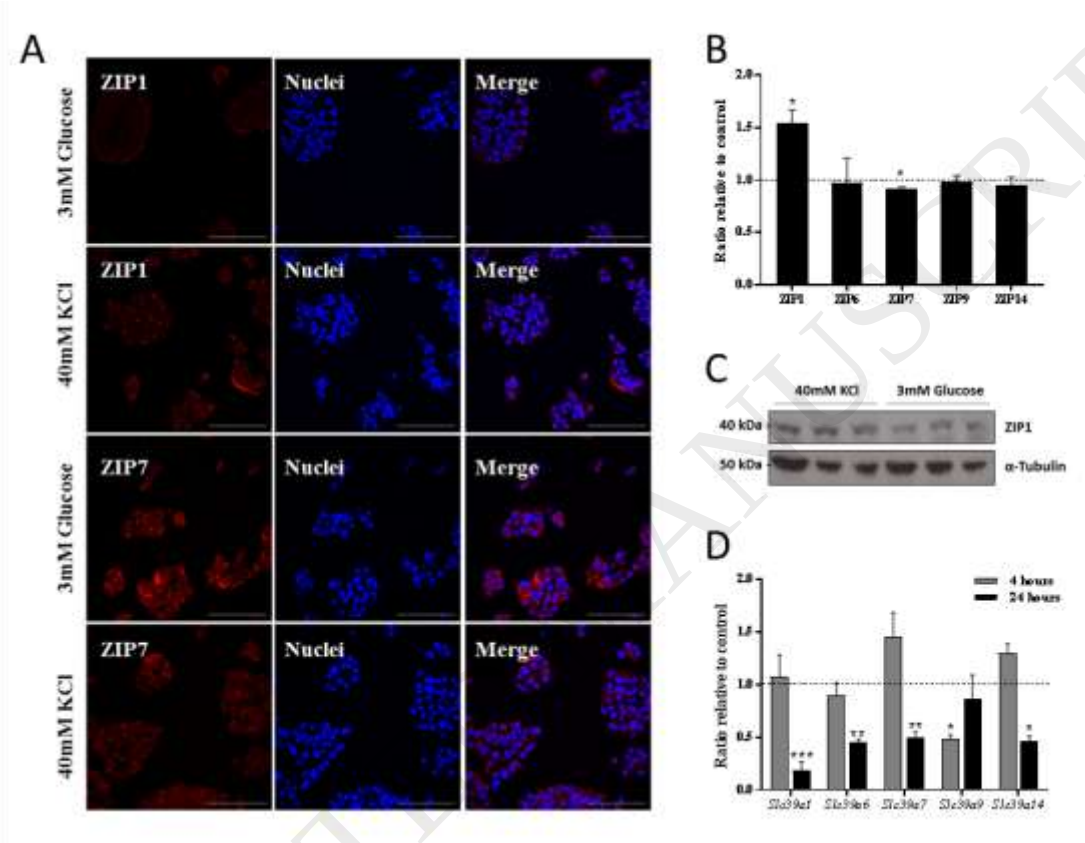


Fig 3. Changes in mRNA expression of transcription factors in MIN6 cells. (A) Expression in response to stimulation with 40mM KCl for 24 h. Expression was calculated as a ratio compared to cells cultured under the same conditions but without KCl (3mM glucose; control). **(B-C)** Expression in response to extracellular zinc depletion for **(B)** 24 h or **(C)** 48 h. Expression was assayed through qPCR and calculated as a ratio compared to cells cultured with 8µM extracellular zinc (control). N=3. *p<0.05**,** **p<0.005.

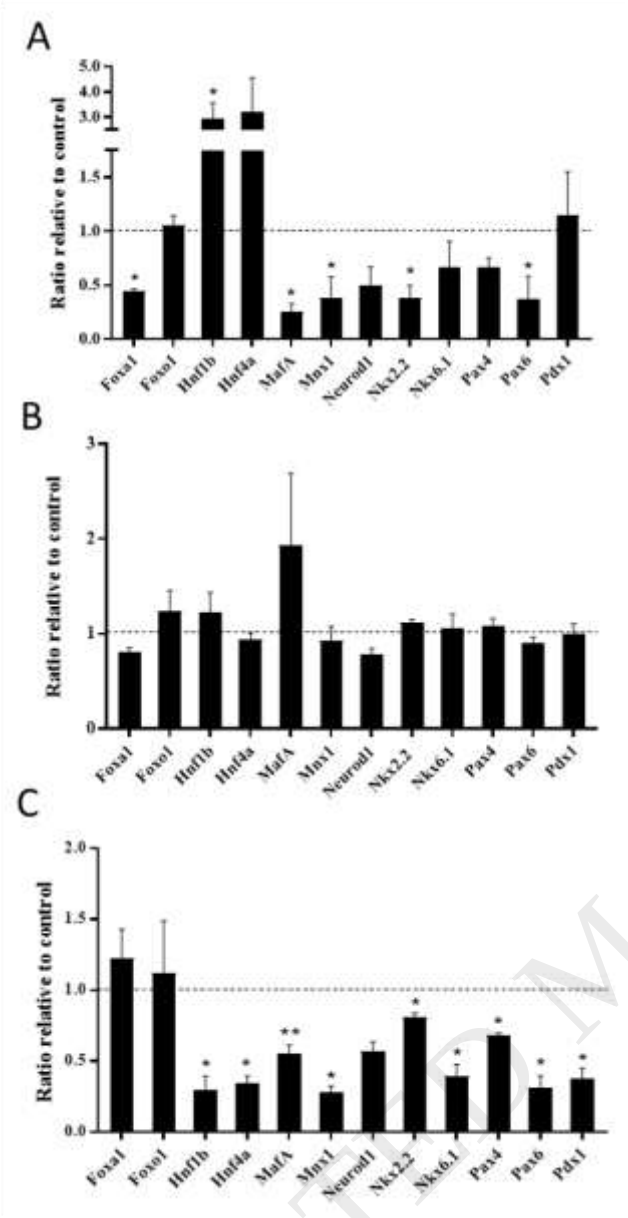


Fig 4. Schematic illustrating key roles of the zinc-responsive transcription factors in mature β-cells. All transcription factors shown are down-regulated in response to zinc depletion in MIN6 cells. Transcription factors highlighted in orange show down-regulation in response to prolonged stimulation with KCl and those highlighted in green show up-regulation in response to prolonged stimulation with KCl. The arrows represent known signal transduction pathways between the transcription factors in β-cells.

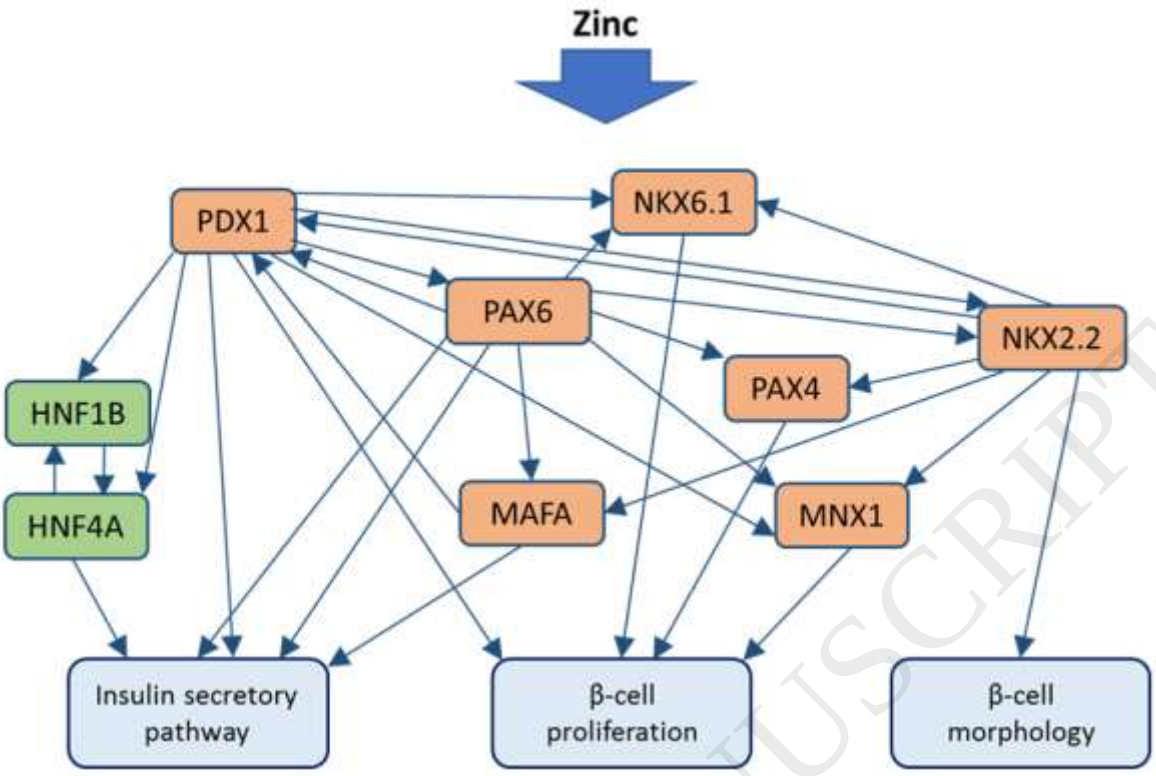
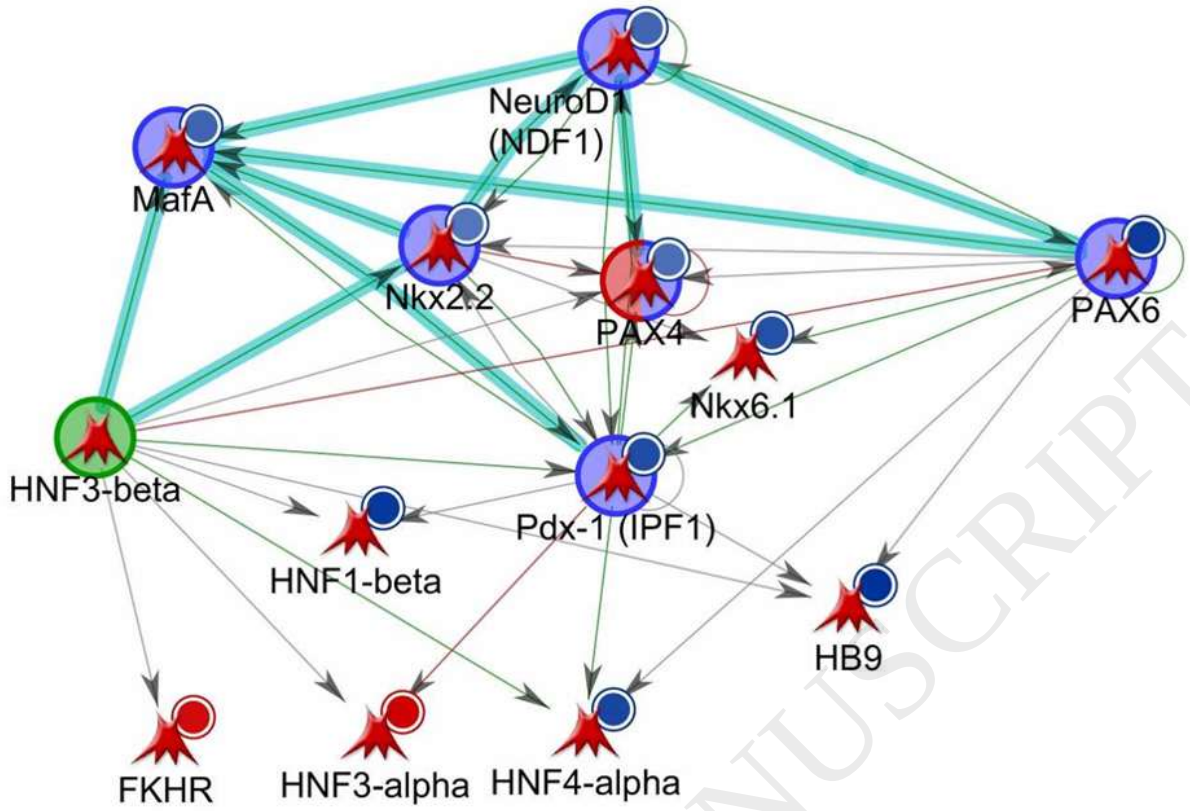


Fig 5. Network of interactions between the transcription factors FOXA1 (HNF3A), FOXA2 (HNF3B), FOXO1 (FKHR), HNF1B, HNF4A, MAFA, MNX1 (HB9), NEUROD1, NKX2.2, NKX6.1, PAX4, PAX6 AND PDX1 in β-cells. The pathways highlighted in blue are the 'nodes' considered to drive the network in response to zinc. The red circles indicate the respective gene is up-regulated and the blue circles indicate the respective gene is down-regulated in response to zinc. Created using GeneGo MetaCore Bioinformatics software from Thomson Reuters (https://portal.genego.com/). Data showing the basis of this network are provided in Additional file 2.



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