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Intracellular zinc in insulin secretion and action: a determinant of diabetes risk?

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Zinc is an important micronutrient, essential in the diet to avoid a variety of conditions associated with malnutrition such as diarrhoea and alopecia. Lowered circulating levels of zinc are also found in diabetes mellitus, a condition which affects one in twelve of the adult population and whose treatments consume approximately 10 % of healthcare budgets. Zn²⁺ ions are essential for a huge range of cellular functions and, in the specialised pancreatic β-cell, for the storage of insulin within the secretory granule. Correspondingly, genetic variants in the *SLC30A8* gene, which encodes the diabetes-associated granule-resident Zn²⁺ transporter ZnT8, are associated with an altered risk of type 2 diabetes. Here, we focus on (i) recent advances in measuring free zinc concentrations dynamically in subcellular compartments, and (ii) studies dissecting the role of intracellular zinc in the control of glucose homeostasis *in vitro* and *in vivo*. We discuss the effects on insulin secretion and action of deleting or over-expressing *Slc30a8* highly selectively in the pancreatic β-cell, and the role of zinc in insulin signalling. While modulated by genetic variability, healthy levels of dietary zinc, and hence normal cellular zinc homeostasis, are likely to play an important role in the proper release and action of insulin to maintain glucose homeostasis and lower diabetes risk.

Diabetes: Zinc: Insulin: Imaging: Zinc transporter 8

Reflecting its importance for human health⁽¹⁾, zinc is present in all human tissues. The total amount of zinc in the human body is 2–3 g, with pancreas and bone having a particularly high content (Table 1). Total plasma zinc concentrations are 10–20 μM (Table 1) with free concentration in the blood in the nM range⁽²⁾.

Remarkably, almost 3000 mammalian proteins bind Zn²⁺ ions⁽³⁾, some 10 % of the proteome. Consequently, zinc plays a role in almost all aspects of cellular physiology. Aside from the structural role for tightly bound zinc in many proteins (e.g. transcription factors of the zinc finger family), these ions also modulate protein

function dynamically⁽⁴⁾. Indeed, over 300 enzymes are dependent on zinc for catalysis⁽⁴⁾.

Binding of zinc to regulatory sites on proteins means that tight control of cellular zinc concentrations over a limited range is crucial for the balance between health and disease: below a certain level, zinc will be limiting, while high zinc concentrations are likely to be toxic. A plethora of proteins is consequently involved in the control of intracellular zinc in man and other mammals. Cellular homeostasis is achieved firstly by ten members of the zinc transporter (ZnT; *SLC30A*) family⁽⁵⁾, which export Zn²⁺ ions from the cytosol to intracellular

Abbreviations: KO, knockout; T2D, type 2 diabetes; ZnT, zinc transporter.

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Table 1. Total concentrations of zinc in various human tissues

Tissue	Zinc concentration per tissue wet weight ($\mu\text{g/g}$)
Pancreas	140
Bone	100
Liver	58
Kidney	55
Muscle	51
Skin	32
Heart	23
Brain	11

Source: <http://www.iospress.nl/book/zinc-in-human-health/>.

organelles or the extracellular space. A further fourteen members of the zinc importer (*SLC39A*) family⁽⁶⁾ import zinc from these compartments into the cytosol (Fig. 1). Soluble metallothioneins (1–4) buffer and translocate zinc within the cytosol (see later and Fig. 4). Together, these systems achieve resting free Zn^{2+} concentrations in the cell cytosol in the range of 600 pM–1 nM^(7,8) (see next section). In some cell types, levels of free Zn^{2+} increase in response to stimuli⁽⁹⁾, to 1 nM or above^(10,11); at the site of release from intracellular stores, free zinc concentrations might be higher. However, with some exceptions^(12,13), changes in cytosolic zinc are slower and smaller than those seen e.g. for calcium ions where a signalling role is very clearly defined⁽¹⁴⁾.

None of the targets of the released Zn^{2+} ions has so far been structurally characterised. Zinc could in theory modulate protein function in co-catalytic sites and/or by binding to and inhibiting enzymes. A well characterised zinc target is the metal responsive element transcription factor-1, which senses increased zinc concentrations and mediates zinc-dependent expression of genes such as the metallothioneins⁽¹⁵⁾. Moreover, it has been postulated that zinc ion transients control protein–protein interactions (<http://www.iospress.nl/book/zinc-in-human-health/>, Chapter 4).

Imaging free Zn^{2+} in living cells

While total zinc levels in cells are in the mM range (Table 1)⁽⁴⁾, labile i.e. free concentrations are many orders of magnitude lower (pM–nM) in the range likely to regulate physiological targets. However, these concentrations are likely to differ between cell types, different intracellular organelles and in response to environmental perturbation or stimulation.

A deeper understanding of the role of zinc in cell biology and cell signalling has required the development of sensitive and non-invasive tools which provide both spatial and temporal resolution. Fluorescence microscopy is usually adopted and uses one of two probe types⁽¹⁶⁾. First, low molecular weight compounds which display a massive change in fluorescence intensity upon zinc chelation can be loaded into the cell. The second class are genetically-encoded zinc sensors, usually expressed from an introduced cDNA and which bind Zn^{2+} ions

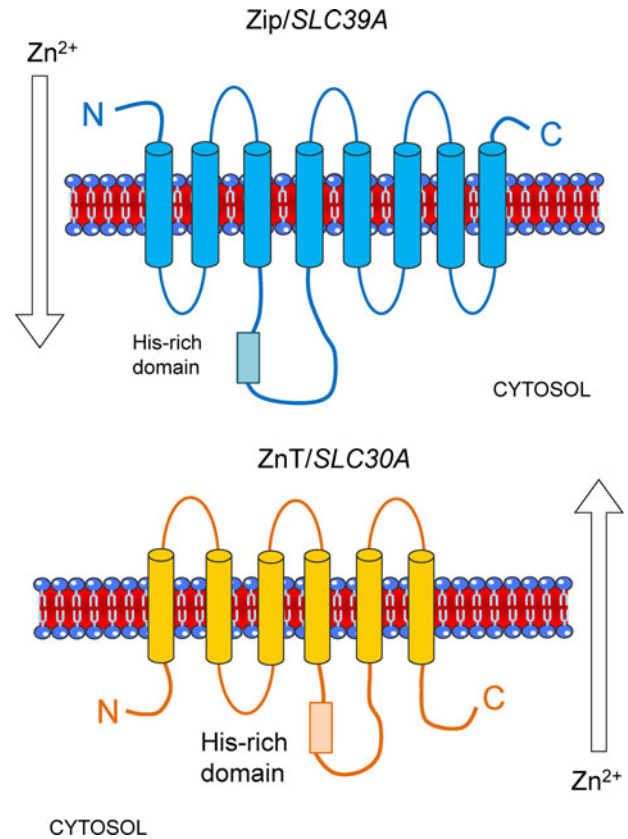


Fig. 1. (Colour online) Likely membrane topology and roles of the zinc importer (ZiP) and transporter (ZnT) families.

through a defined metal-binding protein domain to influence the fluorescence of protein-based fluorophores to which it is fused. These zinc sensors/probes differ according to their affinity for zinc (or sensitivity, reflected by the dissociation constant K_d), their selectivity for zinc against other metals ions and their dynamic range i.e. the change in fluorescence intensity triggered by zinc binding.

Chemically synthesised, low molecular weight probes can be divided in two categories: intensity-based and ratiometric (the latter will not be described here). Intensity-based probes are ‘turn-on’ fluorophores, and display a chelation-dependent increase in fluorescence intensity of up to 100-fold. Most of these probes are based on the modulation in the photon-induced electron transfer phenomenon^(16–18). Briefly, the probes are composed of a fluorophore, a spacer domain and an electron-rich metal chelate. Photoexcitation of the fluorophore is relaxed through electron transfer with the chelate and this fluorescence quenching is suppressed upon metal–ion binding. A variety of fluorescent probes with varying affinities and excitation/emission wavelengths have been described⁽¹⁹⁾. The first generation, including Zinquin⁽²⁰⁾, was derived from quinoline, a UV-excitable fluorophore. To resolve problems related to UV-excitation the next generation ZinPyr family (from ZP1 to ZP10), ZnAF, etc. was developed using fluorophores excitable with visible light. The widely-used FluoZin-3, originally based on a Ca^{2+} probe⁽²¹⁾, displays a high affinity for zinc

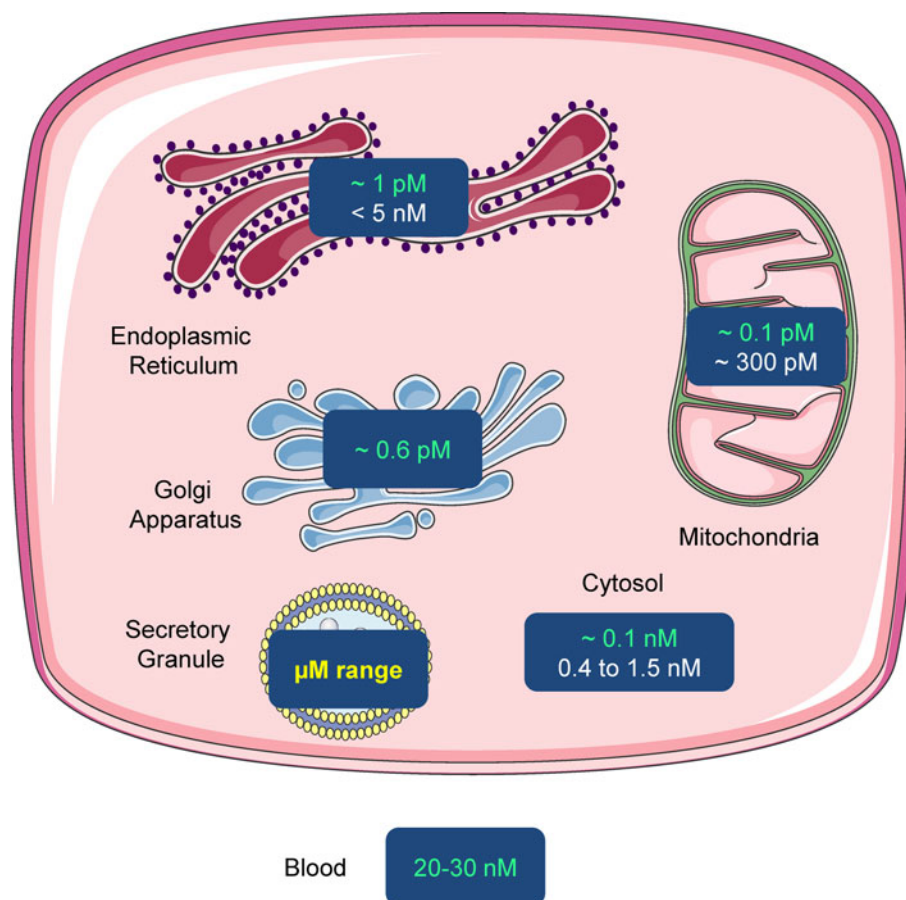


Fig. 2. (Colour online) Measurement of free Zn²⁺ concentrations in subcellular compartments in mammalian cells using genetically-encoded sensors. Green: ZapCY1/2⁽³¹⁾, white: eCALWY-4⁽⁷⁾, yellow: eZinCh-1⁽⁷⁾.

($K_d = 15$ nM). Trappable versions based on the intracellular cleavage of acetoxymethyl esters⁽²²⁾ and improved in terms of brightness and photostability, have also been developed⁽¹⁹⁾.

Compartmentalisation of these probes is not readily controlled and can vary depending on the cell type^(19,23,24). Biological targeting is, however, possible e.g. to mitochondria with the addition of a thiamine pyrophosphate group⁽²⁵⁾. Attachment to the cell surface to allow sampling of extracellular Zn²⁺ has also been achieved, and used to measure co-release of Zn²⁺ alongside insulin^(26,27). The latter ‘click-SnAr-click’ strategy was also used to add targeting moieties to other intracellular localisations (e.g. mitochondria and lysosomes)⁽²⁷⁾.

Genetically-encoded zinc sensors use Förster Resonance Energy Transfer as the sensing modality and consist of a donor and acceptor fluorescent protein linked by a zinc-binding peptide sequence⁽²⁸⁾. Two families have been designed by the Palmer group based on zinc fingers: Zif- and Zap-sensors. The low (μ M) affinity Zif family is derived from the mammalian transcription factor Zif268, and contains either a wild type zinc fingers (ZifCY1), or a mutated (ZifCY2) domain^(29,30). The Zap sensors, based on the *Saccharomyces cerevisiae* transcriptional regulator Zap1, have a very high (pM)

affinity for zinc⁽³⁰⁾. The first member of the family ZapCY1 showed a K_d of 2.5 pM, and was saturated when expressed in HeLa cells. ZapCY2 has a decreased affinity ($K_d = 811$ pM) and is suitable for *in cellulo* measurements.

The eCALWY sensors⁽⁷⁾ developed by Merckx and colleagues, consist of two cysteine-containing metal binding domains (ATOX1 and WD4) connected by a long, flexible glycine-serine linker and flanked by modified cerulean and citrine fluorescent proteins. Shortening of the linker length between the metal binding domains and/or mutation of one of the metal binding cysteines in the WD4 domain yielded a series of sensor variants showing affinities (pM–nM)⁽⁷⁾.

Taking advantage of simple fusion with a targeting sequence, several zinc sensors have been addressed to different organelles such as the mitochondria (mito-eCALWY-4, mito-ZapCY1)^(8,31), the endoplasmic reticulum (ER-eCALWY-4, ER-ZapCY1)^(8,23), Golgi apparatus (golgi-ZapCY1)⁽³⁰⁾ nucleus (NLS-Zaps)⁽³²⁾ and insulin-secreting vesicles (vamp2-eCALWYs, vamp2-eZinCh1)⁽⁷⁾.

A summary for the results obtained with these probes is presented in Fig. 2. While similar values were returned for all probes when located in the cytosol, i.e. 0.1–1.5 nM^(7,8,28,30,33) (Fig. 2), in line with results using

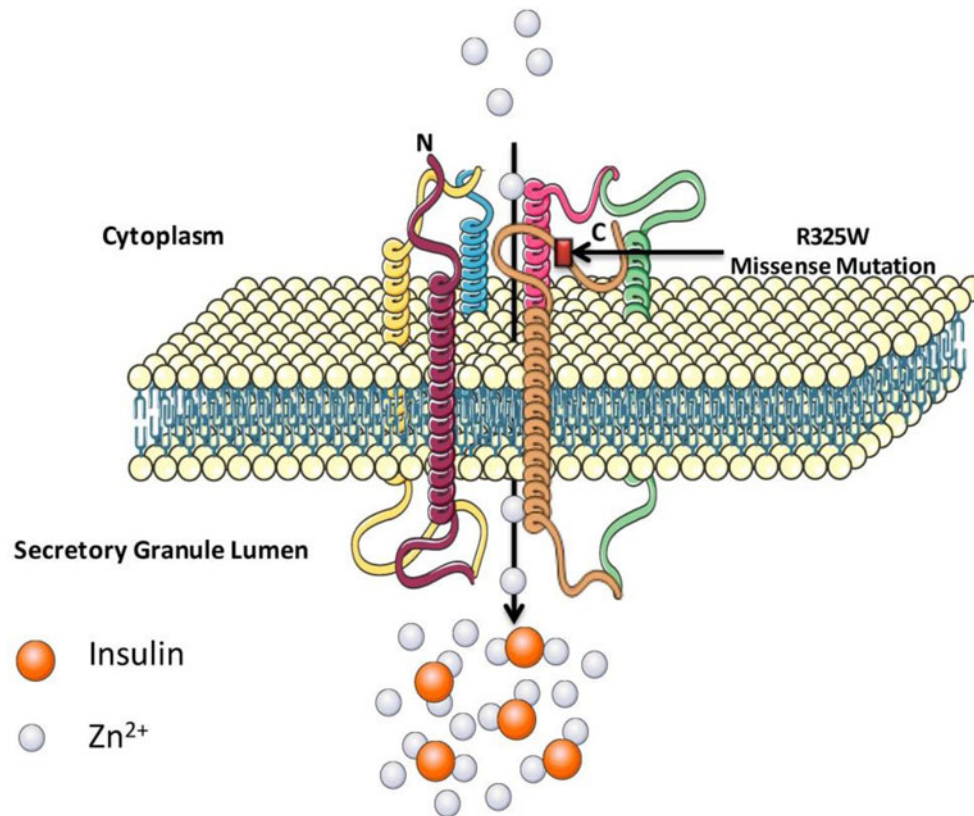


Fig. 3. (Colour online) Schematic of the genome-wide association studies (GWAS)-identified zinc transporter ZnT8 and localisation of the risk associated polymorphic amino acid R/W 325.

FluoZin-3⁽¹¹⁾, much more variation exists for mitochondria: (0.1 pM for mito-ZapCY1⁽³¹⁾ and 300 pM for mito-eCALWY-4⁽⁸⁾) and endoplasmic reticulum (about 1 pM⁽³⁰⁾ and above 5 nM⁽⁸⁾). The reasons for these variations are unclear and may involve differences in intracellular pH on which the probes are steeply dependent. Red-shifted variants have been created for Zap and eCALWY sensors^(32,34).

Hybrid probes include both genetically-encoded and small molecular elements. These include a probe based on a carbonic anhydrase variant covalently bound to a chemical fluorophore or fused with a red fluorescent protein^(35,36).

The above zinc sensors and probes are thus precious tools with which to decipher the link between changes in intracellular zinc levels, diabetes risk and pathogenic mechanisms.

Zinc and diabetes

A role for zinc in diabetes aetiology has been known since 1930, when the zinc concentration was reported to be reduced by about 50 % in the pancreas of diabetic compared with non-diabetic cadavers⁽³⁷⁾. Epidemiological studies also suggest that whole body zinc status might be associated with diabetes^(38,39). Studies on patients with type 2 diabetes (T2D) revealed that the serum concentration of zinc was decreased compared with healthy control subjects^(40,41), a finding associated with increased urinary zinc loss⁽³⁸⁾.

Adequate levels of Zn²⁺ are essential not only to ensure appropriate synthesis, storage and structural stability of insulin⁽⁴²⁾ but also to protect against oxidative stress in T1 and T2 diabetes and their associated pathologies⁽⁴³⁾. Thus, zinc is a pro-antioxidant, and a cofactor of superoxide dismutase (isoforms 1 and 3), that regulates the expression of metallothioneins and glutamate-cysteine ligase, thus affecting glutathione levels⁽⁴³⁾.

The idea that zinc supplementation might improve the symptoms of T2D have been examined not only in animal models but also in diabetic patients. Studies on obese *ob/ob* mice showed that high zinc supplementation attenuates fasting hyperglycaemia and hyperinsulinaemia⁽⁴⁴⁾. Later studies provided similar results, and also revealed reduced body weight following treatment with lower zinc concentrations, in *db/db* mice⁽⁴⁵⁾. Moreover, recent studies on streptozotocin-induced diabetic rats revealed that zinc supplementation improves symptoms such as polydipsia and high HDL cholesterol levels⁽⁴⁶⁾. Amelioration of the diabetic phenotype in T2D patients has also been observed upon zinc supplementation (see Table 2. Data reviewed in⁽⁴⁷⁾).

Zinc imaging to assess beta cell mass *in vivo*?

Pancreatic β -cells have an exceptionally high total Zn²⁺ content (about 10–20 mM), with the majority of β -cell Zn²⁺ being located within dense core insulin secretory

Table 2. Zinc supplementation studies in Type 2 diabetic patients (modified from⁽⁴⁷⁾)

Zinc compound tested	Dose	Physiological effects
Zinc sulphate (ZnSO ₄)	30 mg/d – 12 weeks	Decrease in HbA _{1c} levels
Zinc sulphate (ZnSO ₄)	22 mg/d – 16 weeks	Decrease in HbA _{1c} levels Decrease in total cholesterol Decrease in cholesterol/HDL ratio
Zinc sulphate (ZnSO ₄)	30 mg/d – 12 weeks	Reduced serum Homocysteine Increased folate and vitamin B ₁₂
Zinc sulphate (ZnSO ₄)	660 mg/d – 6–8 weeks	No beneficial effects
Zinc gluconate	240 mg/d – 12 weeks	No beneficial effects

HbA_{1c}, Glycosylated haemoglobin A (a time-averaged measure of blood glucose).

granules⁽⁴⁸⁾. Zinc ions are relatively less abundant in the exocrine pancreas and in other tissues⁽⁴⁹⁾. Although type 1 diabetes involves immune-mediated destruction of insulin-secreting pancreatic β -cells, T2D usually involves defects in both insulin release, and in hormone action⁽⁵⁰⁾. Both impaired glucose sensing by the β -cell⁽⁵¹⁾, and a loss of overall β -cell mass⁽⁵²⁾, are involved in the decline in β -cell function in T2D, though the relative contributions of each are contested⁽⁵³⁾. Classical *in vivo* imaging approaches such as magnetic resonance and positron emission tomography are challenging due to the relative paucity of islets within the pancreas (about 1 % of the total pancreatic volume) and the absence of suitable contrast reagents, which are thus eagerly sought. Dual-modal zinc probes, based on transition metal chelates capable of binding these ions, may therefore be of clinical value as imaging tools in the future⁽⁵⁴⁾.

Zinc transporter 8 in insulin secretion and type 2 diabetes risk

During its biosynthesis in β -cells insulin is initially packaged as Zn²⁺-coordinated hexamers⁽⁵⁵⁾ before its concentration and crystallisation into dense cores within the secretory granule. The hormone is subsequently released into the circulation upon stimulation by glucose⁽⁵⁶⁾. The latter process involves enhanced mitochondrial metabolism⁽⁵⁷⁾, closure of ATP-sensitive K⁺ channels⁽⁵⁸⁾, Ca²⁺ influx⁽⁵⁹⁾ and granule fusion at the plasma membrane⁽⁶⁰⁾.

ZnT8 (encoded by the *SLC30A8* gene), a member of the zinc transporter family (see Fig. 1 and Introduction) is highly and almost exclusively expressed in the β - and α -cells of the endocrine pancreas, where it facilitates the import of cytosolic Zn²⁺ into secretory granules (Fig. 3)⁽⁶¹⁾. Autoantibodies to ZnT8 have been found in individuals with type 1 diabetes and are a relevant prognostic feature of the disease⁽⁶²⁾. Importantly, genome-wide association studies have provided a potential link between ZnT8 activity and T2D development. Thus, Sladek *et al.*⁽⁶³⁾ found that an SNP, rs13266634, within exon 13 of the *SLC30A8* gene was enriched in individuals with T2D. The variant at rs13266634 results in a missense mutation whereby an arginine residue is replaced by a tryptophan at position 325 (R325W). Risk allele carriers (R325) present with impaired insulin secretion during an intravenous glucose tolerance

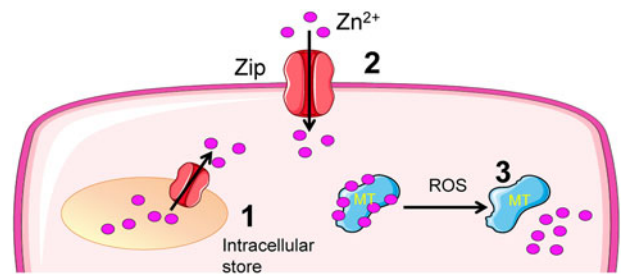


Fig. 4. (Colour online) Possible sites of insulin action on intracellular Zn²⁺ homeostasis. Intracellular free zinc concentration could be impacted by: – action on zinc transporter (Zip and ZnT) on the release and uptake, respectively, of zinc ions from intracellular stores (1) or the extracellular medium (2) or by modulating the buffering of Zn²⁺ by metallothioneins (3)⁽⁴¹⁾.

test⁽⁶⁴⁾ increased proinsulin: insulin ratio⁽⁶⁵⁾ and lower β -cell function (as assessed through Homeostatic model assessment-B⁽⁶⁶⁾), suggestive of impairments in both insulin secretion and processing. Four SNP located in the 3'-untranslated region of *SLC30A8*, two of which are in strong linkage disequilibrium with rs13266634, have also been associated with an increased risk of developing T2D. However, there are conflicting reports as to whether or not possession of these SNP is associated with changes in certain parameters of glucose homeostasis (e.g. fasting blood glucose, glucose tolerance etc.)^(64–68). Interestingly, the effects of dietary zinc supplementation to lower T2D risk are dependent on *SLC30A8* genotype⁽⁶⁹⁾. More recent studies in human populations have reported twelve rare loss-of-function SNP, resulting in the production of a truncated protein that is associated with a 65 % decrease in T2D risk⁽⁷⁰⁾. This result was unexpected given the action of the common variant, which is likely to lower ZnT8 activity (see later), to increase disease risk.

Providing a link between increased T2D risk and rs13266634 inheritance has proved challenging. Unlike most genome-wide association studies identified SNP to date, rs13266634 is located in a coding region (exon) of the genome and, therefore, affects the primary sequence of ZnT8 and potentially the tertiary structure of the protein and its function (Fig. 3). However, the crystal structure of ZnT8 is yet to be elucidated and structure–function relationships, as well as the effects of SNP

Table 3. Glycemic phenotype of ZnT8 null mouse lines

Deletion (Cre)	Deletion strategy	Genetic background	Zinc content	Granule structure	GSIS <i>in vivo</i>	GSIS <i>in vitro</i>	Insulin sensitivity	Glucose tolerance	Reference*
Global	Exon 3	Mixed	Reduced	NR	NR	Reduced	NR	No change	Pound (mixed)
Global	Exon 3	C57BL6	Reduced	No change	NR	No change	No change	IGT	Pound (BL6)
Global	Exon 1	Mixed	Reduced	Altered	Reduced	No change	No change	IGT	Nicolson
Global	Exon 1	Mixed	Reduced	Altered	NR	No change	No change	NR	Lemaire
B-cell (RIP2Cre)	Exon1	Mixed	Reduced	Altered	No change	Reduced	NR	IGT	Wijesekara
B-cell (RIPCRe)	Exon 5	C57BL6	Reduced	Altered	Reduced	Increased	No change	IGT	Tamaki
B-cell (Ins1Cre)	Exon 1	C57Bl6	Reduced	Altered	Reduced	No change	No change	IGT	Mitchell

RIP2Cre, rat insulin promoter 2-driven Cre recombinase; GSIS, glucose-stimulated insulin secretion; IGT, impaired glucose tolerance; NR, not recorded (unpublished results).

*Pound (mixed)⁽⁷⁶⁾, Pound (BL6)⁽¹⁰⁹⁾, Nicolson⁽⁷²⁾, Lemaire⁽⁷⁵⁾, Wijesekara⁽⁷⁸⁾, Tamaki⁽⁷⁹⁾, Mitchell⁽⁸⁶⁾.

upon these, have been modelled on the bacterial homologue YiiP, which only shares 51.8% sequence homology⁽⁷¹⁾. Position 325 is located towards the ‘tip’ of the ZnT8 molecule within regions involved in homodimerisation, but as both R- and W-side chains point away from the dimerization interface itself, it is unlikely that R325W substitution would have an effect on ZnT8 dimerisation or Zn²⁺ binding^(72,73). It is nonetheless conceivable that the positive charge of the R-side chain may hinder inter- and intra-molecular interactions, for example with presently unidentified Zn²⁺-binding proteins which deliver the ions to the mouth of the channel to facilitate Zn²⁺ transport. Providing evidence for this are zinc uptake studies^(72,74), which showed that R325 is a less active Zn²⁺ transporter than the non-risk W325 in β -cells.

To further elucidate the role of ZnT8 in glucose homeostasis, several groups including our own have produced animal models harbouring either global ZnT8 deletion^(72,75–77) or deletion restricted to the β -cell^(78,79), with some recombination in the hypothalamus⁽⁸⁰⁾. Each mouse model shows variations in certain phenotypic traits (see Table 3)⁽⁸¹⁾, which are attributed to differences in genetic background, deletion strategy and housing conditions. While the majority of animal models displayed impairments in glucose tolerance upon ZnT8 deletion, albeit with subtle age and sex-differences between the colonies, no investigators reported improvements in glucose tolerance upon ZnT8 deletion. Using electron microscopy, marked changes were seen in insulin granule morphology, with a large proportion of insulin secretory granules lacking a dense core of crystallised insulin, but containing either ‘empty’ granules or granules possessing ‘rod like’ structures. Islet Zn²⁺ was also decreased in both global and β -cell specific ZnT8 null animals^(72,78). Although circulating insulin levels were significantly lowered in ZnT8 null mice compared with controls, glucose-stimulated insulin release was unchanged or slightly increased in islets isolated from ZnT8 null mice. Providing an elegant explanation for these apparently contradictory results, Tamaki *et al.*⁽⁷⁹⁾ recently demonstrated a role for Zn²⁺ co-secreted with insulin from granules in regulating the rate of hepatic insulin clearance mediated by clathrin-dependent internalisation of the insulin receptor. As Zn²⁺ does not affect the uptake of C-peptide or proinsulin, this mechanism could

potentially explain the impairments in both circulating insulin and the increased proinsulin:insulin ratio seen in risk allele carriers⁽⁸²⁾. Interestingly, cytosolic free Zn²⁺, as measured with the eCALWY4 probe, was reduced in the global knockout (KO) mouse for ZnT8⁽⁷⁷⁾, alongside granule zinc concentrations as estimated by the release of zinc during exocytosis⁽²⁶⁾. These findings imply a more complex role for this transporter in the regulation of zinc fluxes than has previously been appreciated.

Exposing ZnT8 null animals to the diabetogenic effects of a high-fat diet led to varying results between global and β -cell specific mouse models. High-fat diet-fed β -cell specific null mice displayed similar bodyweights but displayed impaired secretion and were glucose intolerant compared with littermate controls whereas global deletion of ZnT8 resulted in increased weight gain and subsequently displayed higher levels of insulin resistance⁽⁸³⁾.

Notably, ZnT8 is also expressed at low but detectable levels in rat-insulin promoter-expressing neurons residing within hypothalamic appetite centres. Therefore, ectopic deletion in both global and rat-insulin promoter2Cre-driven ZnT8 mouse models may explain the phenotype observed in some of these mouse models. To overcome this issue, as well as other issues with Cre strains that express a human growth hormone minigene⁽⁸⁴⁾, we deleted ZnT8 using a highly-specific β -cell Ins1Cre driver line, which produces no detectable recombination in the hypothalamus⁽⁸⁵⁾. Confirming previous results, Ins1Cre-mediated ZnT8 deletion results in impaired glucose tolerance, abnormal insulin granule morphology and reduced cytosolic Zn²⁺ concentrations⁽⁸⁶⁾. This model also shows reduced liberation of Zn²⁺ from isolated islets, supporting the view of Tamaki *et al.* that effects of Zn²⁺ on the liver may limit the levels of bioavailable insulin to impair glucose tolerance. Furthermore, we have recently generated a mouse line in which the protective variant (W325) of human ZnT8 is expressed selectively in the β -cell driven by an insulin promoter-controlled tetracycline responsive promoter⁽⁸⁷⁾. In contrast to both global and β -cell-specific ZnT8 null mice, increased ZnT8 expression significantly improved glucose tolerance compared with wild type littermate controls, as expected. However, the improved glucose tolerance did not appear to be due to enhanced insulin secretion, which was reduced in islets isolated from these animals. This is likely instead to be

secondary to elevations in secreted Zn^{2+} , acting to inhibit insulin secretion through an autocrine/paracrine loop^(86,88). Whether the enhanced Zn^{2+} secretion has any effects on neighbouring α -cells, or on hepatic insulin clearance⁽⁷⁹⁾, is yet to be investigated.

Genome-wide association studies and animal data have provided conflicting reports regarding the role of ZnT8 in T2D risk (see⁽⁸²⁾ for a detailed discussion). Collectively, these data would suggest that both inheritance of rs13266634 and deletion of ZnT8 in mice are detrimental in maintaining glucose homeostasis. Given the highly-restricted expression profile of ZnT8, there may be some promise in therapeutically-targeting ZnT8 in the treatment of T2D.

A role for zinc transporter 8 in glucagon secretion?

In addition to expression in β -cells, there is evidence that ZnT8 is also present in α -cells, at least in mouse and human pancreata. Its expression in these cells was confirmed by immunocytochemistry in pancreatic slices and dissociated islets and by gene expression analysis on a fluorescence-activated cell sorting purified mouse population⁽⁷²⁾. Similarly to the β -cell, ZnT8 appears to be the most highly expressed member of the ZnT family in the α -cell. In the porcine pancreas, however, ZnT8 is exclusively expressed in the β -cell⁽⁸⁹⁾ implying clear species differences and suggesting that in pig islets zinc homeostasis is regulated differently from human and rodent islets. Knockdown of ZnT8 in glucagonoma-derived α TC1-9 cells resulted in an increase of glucagon mRNA, and decreased regulated glucagon secretion by 70%. Overexpression of the ZnT8 R325 or W325 variants in these cells reciprocally led to reduced glucagon content and 50% lower glucagon secretion⁽⁹⁰⁾.

As a result of its co-secretion with insulin, zinc can reach high local concentrations within the islet, with the potential of acting as a mediator of paracrine signalling. Thus, in the perfused rat pancreas, the zinc chelator Ca^{2+} -EDTA led to a stimulation of secretion when using the mitochondrial substrate monoethyl-succinate as a secretagogue, an agent which normally acts as an inhibitor of glucagon release⁽⁹¹⁾. Correspondingly, zinc diminished pyruvate- or glucose-induced glucagon secretion from isolated rat α -cells through the reversible activation of K_{ATP} channels and a subsequent decrease in electrical activity⁽⁹²⁾. Likewise, studies in the mouse demonstrated an inhibitory effect of Zn^{2+} on glucagon secretion⁽⁹³⁾. However, in the latter species, this effect of zinc could not be attributed to K_{ATP} channel activation but to uptake of the ion by calcium channels and an alteration in redox state⁽⁹³⁾. Of note, fasting plasma glucagon levels were normal in global ZnT8 null mice and glucagon secretion from isolated islets was not higher in KO mice than controls. However, exogenous zinc did lead to a reduction of glucagon secretion under stimulatory conditions⁽⁹⁴⁾. Assuming that granular zinc was almost entirely depleted in the KO mice, these data suggest that zinc secreted from β -cells is not responsible for the inhibition of glucagon secretion at high glucose.

Although there have been extensive studies on the role of ZnT8 in the β -cell (see preceding section and references therein), its function in the α -cell has not been investigated in detail. One study looked briefly at an α -cell-specific ZnT8 KO and did not detect any differences in fasting plasma glucagon levels or glucose homeostasis compared with control mice⁽⁷⁸⁾. Further examination of islets from these mice is necessary as well as investigating changes at the single cell level.

In line with our earlier findings⁽⁷⁸⁾, we have more recently observed no effect of ZnT8 deletion selectively in α -cells on glucose homeostasis or fasting glucagon⁽⁹⁵⁾. However, we observed in these more recent studies that KO mice displayed enhanced responses to hypoglycaemia *in vivo* and increased glucagon secretion from isolated islets, implying cell-autonomous roles for ZnT8 in the α -cell.

Zinc and insulin action

Insulin binding to the α -subunit of the insulin receptor leads to enhanced intrinsic protein tyrosine kinase activity of the β -subunit and phosphorylation of the receptor on multiple tyrosine residues⁽⁹⁶⁾. The activated insulin receptor then phosphorylates several scaffolding proteins, including the insulin receptor substrates, which subsequently bind and activate other signalling proteins to trigger two main different pathways (Fig. 5): the activation of the serine/threonine kinases Ras and Raf by the SOS/Grb2/SHC complex leading to extracellularly-regulated kinases1/2 activation and cellular proliferation, and activation of phosphatidylinositol 3'-kinase⁽⁹⁷⁾. The latter triggers the translocation of phosphoinositide-dependent kinase-1 to the plasma membrane together with protein kinase B and activates downstream protein kinases such as p70 ribosomal S6 kinase. The latter stimulate glucose transport, glycogen synthesis, lipogenesis and other processes (Fig. 5).

An action of zinc on insulin-target tissue was described for the first time by Coulston and Dandona, when they observed that treatment of rat adipocytes with high zinc concentrations led to an increased rate of lipogenesis⁽⁹⁸⁾. At the cellular level, zinc was found to increase insulin receptor substrates-1 tyrosine phosphorylation in the presence or absence of insulin in skeletal muscle cells⁽⁹⁹⁾. Moreover, zinc was able to activate protein kinase B in several studies using 3T3-L1 and rat adipocytes: zinc treatment induced not only phosphorylation of the insulin receptor, but also of protein kinase B. While the former is possibly dependent on the inhibition by zinc of protein tyrosine phosphatase 1B⁽¹⁰⁰⁾, the latter is a consequence of phosphatidylinositol 3'-kinase inhibition⁽¹⁰¹⁾. Phosphatase and tensin homologue is responsible for the negative regulation of phosphatidylinositol 3'-kinase /protein kinase B activity and appears to be degraded in response to zinc⁽¹⁰²⁾ at physiological (about 600 pM) concentrations⁽¹⁰³⁾.

As mentioned earlier, one of the most studied targets of the insulin-mimetic effects of zinc is protein tyrosine phosphatase 1B and a recent study has elucidated the

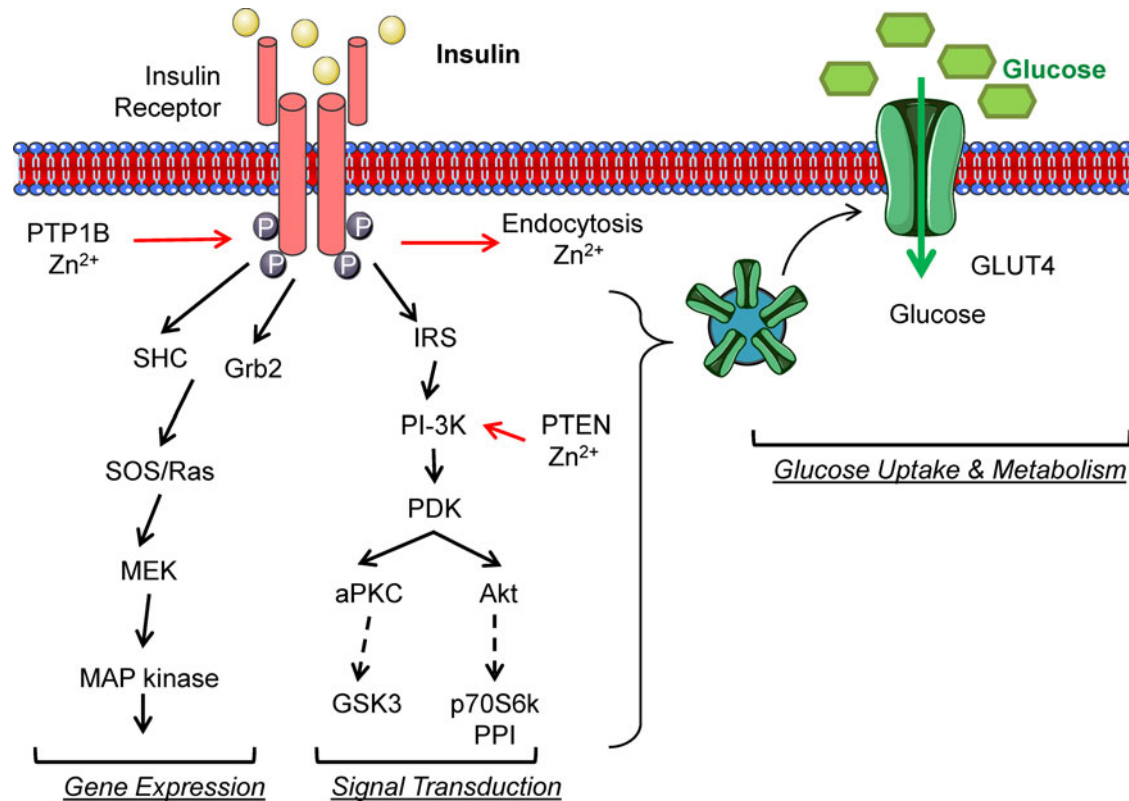


Fig. 5. (Colour online) Possible sites of action of Zn^{2+} on insulin signalling. PTP1B, protein tyrosine phosphatase 1B; SHC, Src-homology-2-containing; SOS, Son-of-sevenless; MEK, MAP/ERK kinase; MAP, mitogen-activated protein kinase; IRS, insulin receptor substrate; P1-3K, phosphatidylinositol 3' kinase; PTEN, phosphatase and tensin homologue; PDK, phosphoinositide-dependent kinase; aPKC, atypical protein kinase C; GSK, glycogen synthase kinase; PPI, protein phosphatase inhibitor-1; GLUT4, glucose transporter family member 4.

inhibition constant and the mechanisms of zinc binding to protein tyrosine phosphatase 1B^(100,104). Zinc also inhibits protein tyrosine phosphatase 1B with an apparent inhibition constant as low as 5 nM. By acting through these two targets, an increase in intracellular zinc signal can be predicted to enhance insulin signalling downstream of the insulin receptor. Zinc also mimics insulin action by triggering nuclear exclusion of the transcription factor FOXO1, and induces glycogen synthesis by lowering the phosphorylation of glycogen synthase kinase-3⁽¹⁰⁵⁾.

The effect of zinc on insulin signalling described above may also explain, at least in part, the impact of the metal ion in the pathogenesis of T2D. Although *in vitro* analyses showed that insulin leads to an increase in intracellular zinc⁽⁴¹⁾, the exact physiological mechanisms responsible are not entirely understood (Fig. 2). While the free concentration of Zn^{2+} within the endoplasmic reticulum is contested (see earlier) it has been suggested that zinc importer-7-mediated release from this compartment may be involved in the actions of epidermal growth factor, which like insulin acts through a receptor tyrosine kinase⁽⁹⁾. Moreover, ablation of zinc importer-7 in skeletal muscle cells displayed a reduction in GLUT4 protein expression and insulin-stimulated glycogen synthesis⁽¹⁰⁶⁾. As discussed earlier, zinc may also inhibit insulin

clearance by the liver, leading to elevated circulating levels of the hormone⁽⁷⁹⁾.

Finally, zinc may also contribute to the anti-oxidant actions of insulin, serving (i) as a cofactor of superoxide dismutase, (ii) to enhance the expression of metallothioneins and (iii) to stimulate glutamate-cysteine ligase and glutathione synthesis. Interestingly, supplementing diabetic rats with zinc increased superoxide dismutase activity⁽¹⁰⁷⁾. Decreased lipid peroxidation, concomitant with an increase in glutathione concentration, was also found in zinc treated diabetic rats⁽¹⁰⁸⁾. Potential sites of action of Zn^{2+} on insulin signalling are shown in Fig. 5.

Conclusions

Recent advances, stretching from human genetics through mouse models to the creation of new imaging modalities, have provided unexpected insights on the role of zinc in the release and actions of insulin. Importantly these new tools provide compelling evidence to indicate that these ions play an under-appreciated role to support the actions of the hormone on target tissues and suggest that Zn^{2+} should be considered as both an important extra- and intra-cellular signalling species.

Nonetheless, important controversies, including the role of ZnT8 in the glucose homeostasis and diabetes risk in man, remain to be resolved⁽⁸²⁾.

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Conflicts of Interest

None.

Authorship

G. A. R. designed and coordinated the writing and assembled the final manuscript. Chief contributors to the individual sections were: G. A. R., Abstract, Introduction, Conclusions; P. C., Zn²⁺ imaging; E. A. B., Introduction and insulin action; W. M., Introduction; R. K. M., D. J. H., A. S. and M. H., ZnT8.

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