REVIEW

SLC30A8 mutations in type 2 diabetes

Guy A. Rutter · Fabrice Chimienti

Abstract *SLC30A8* encodes the secretory granule-resident and largely endocrine pancreas-restricted zinc transporter ZnT8. Interest in this gene product was sparked amongst diabetologists in 2007 when the first genome-wide association study for type 2 diabetes identified polymorphisms in *SLC30A8* as affecting disease risk. Thus, the common polymorphism rs13266634 was associated with lowered beta cell function and a 14% increase in diabetes abundance per risk (C) allele. This non-synonymous variant encodes a tryptophan-to-arginine switch at position 325 in the protein's intracellular carboxy-terminal domain, resulting in reduced zinc transport activity and, consequently, decreased intragranular zinc levels. Whereas insulin secretion from isolated islets is most often increased in mice inactivated for *Slc30a8*, null animals usually show impaired glucose tolerance and lowered circulating insulin. Since *Slc30a8* null animals display little, if any, zinc secretion from islets, the lower plasma insulin levels could be explained by increased hepatic clearance as a result of lowered local zinc levels, or less efficient insulin action on target tissues. Despite the emerging consensus on the role of ZnT8 in glucose homeostasis, a recent genetic study in humans has unexpectedly identified loss-of-function *SLC30A8* mutants that are associated with *protection* from diabetes. Here, we attempt to reconcile these apparently contradictory findings, implicating (1) differing degrees of inhibition of ZnT8 activity in carriers of common variants vs rare loss-of-function forms, (2) effects dependent on age or hypoxic beta cell stress. We propose that these variables conspire to affect both the size and the direction of the effect of *SLC30A8* risk alleles in man.

Keywords Diabetes genetics · GWAS · Insulin secretion · Review · SLC30A8 · Zinc transport · ZnT8

Abbreviations
GWAS	Genome-wide association study
LoF	Loss-of-function
MT	Metallothionein
PC	Prohormone convertase
SNP	Single nucleotide polymorphism
ZnT	Zinc transporter
ZiP	Zinc importer

Introduction

Tight control of intracellular zinc concentrations is vital in all cell types, with adequate levels required to maintain the activity of the zinc-dependent enzymes and binding proteins (which number ~3,000, or >10% of the proteome), including many involved in anti-oxidant processes [1]. Conversely, Zn^{2+} levels in the cytosol must remain below those that would become toxic, for example as a result of interference with thiol-dependent redox systems or by chelation of essential anions (phosphate, ATP, etc.).

In pancreatic beta cells, zinc homeostasis assumes particular importance because of the requirement, in most mammalian species, for zinc in the crystallisation of insulin within secretory granules [2]. Thus, proinsulin forms a zinc-containing hexamer soon after synthesis [3]. Six monomers are arranged around two central zinc ions, complexed by

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histidine 30 in the insulin B-chain, leading to a huge concentration of zinc in the granule (~30 mmol/l, of which <0.1 mmol/l is free) [4, 5]. As discussed below, this accumulation of zinc by granules is essential for the normal packing and the crystalline character of insulin [6], and probably for the stability of the organelle itself.

It has long been known that dysregulation of zinc at the whole body level occurs in both type 1 and type 2 diabetes [7]. However, whether the observed zinc deficiency is a cause or a consequence of the disease has been difficult to establish. A potentially causal role for changes in zinc homeostasis in the pancreatic beta cell was suggested in 2007 with the identification of an association between type 2 diabetes risk and polymorphisms in the *SLC30A8* gene [8]. This gene encodes a recently identified zinc transporter, ZnT8, whose expression is largely confined to the endocrine pancreas within beta (and to a lesser extent alpha) cells [9]. In beta cells, ZnT8 is the most abundantly expressed member of an extended family of zinc transporters (ZnT1–13, encoded by *SLC30A1–13*) responsible for the efflux of zinc from the cytosol [10]. These oppose the actions of zinc importers (ZiPs; *SLC39*), which allow zinc influx into the cytosol, and zinc binding proteins, notably metallothionein (MT) 1–3. The interplay between these sets cytosolic-free zinc levels in the appropriate intracellular range, now thought to be 10^{-9} mol/l (~1 nmol/l) [5, 11]. Given that this value is a least 10,000 times lower than that in the secretory granule, it is evident that the regulation of zinc accumulation into the latter is likely to be important, not just for insulin storage but in the maintenance of cytosolic zinc in the range compatible with normal cellular functions. In this regard, the insulin granule could be seen as a 'sink' for zinc ions, mostly present as zinc complexes, which may facilitate the accumulation of high amounts of zinc against a huge gradient.

The C variant of *SLC30A8* at single nucleotide polymorphism (SNP) rs13266634 was shown by Sladek and colleagues [8] to be enriched in individuals with type 2 diabetes, suggesting that this variant affected diabetes risk. C allele carriers express a form of ZnT8 in which tryptophan 325 in the C-terminus of the protein is replaced by an arginine residue (R325W). C allele carriers showed lowered insulin secretion during intravenous glucose infusion [12], although genotype-dependent differences in insulin secretion were not observed in isolated islets [13]. Whilst this may reflect some underpowering of the latter study to detect small differences, it may also suggest that the predominant effect of the common human *SLC30A8* variants may be to affect Zn^{2+} release, and hence the subsequent metabolism of insulin (see following sections). Carriers also show increased proinsulin:insulin ratios [14], though whether this is due to altered insulin processing in beta cells (not apparent after ZnT8 deletion in mice) [15, 16] or altered hepatic metabolism of these products [17] is unclear.

It should also be noted that although ZnT8 has also emerged as an autoantigen for type 1 diabetes, the common type 2 diabetes-associated variants do not affect type 1 diabetes risk [18]. Interestingly, the major humoral epitope in ZnT8 includes the arginine residue at position 325, similar to the common polymorphism rs13266634 [19].

Effects of R325-W substitution on ZnT8 function

Purification of ZnT8 in sufficient quantities to allow crystallisation has yet to be achieved. Nonetheless, amino acid 325 is predicted, by comparison [15, 20] with the three-dimensional structure of the bacterial homologue YiiP [21], to lie at the 'tip' of the ZnT8 molecule at the interface between monomers in a dimeric structure, but facing away from the interface itself [20]. Whilst a W to R substitution at this site is thus not predicted to affect either dimerisation or zinc binding at the active site [15], both allosteric (intramolecular) and protein–protein (intermolecular) interactions (e.g. with a putative zinc-binding protein responsible for delivering zinc to the active site) are conceivable, and may consequently affect zinc transport activity. It is also possible that introducing positive charges at the 'tip' of the protein may impair Zn^{2+} transport through the protein. These possibilities have so far been assessed by overexpression of either variant in beta cells and imaging [15] or radioisotope-based studies on isolated granules [22]. In both cases, it was inferred that the W325 form was several-fold more active than the R325 form as a zinc transporter. Thus, W325-ZnT8 expressing cells displayed higher levels of fluorescence than the R325-ZnT8-expressing cells when assessed with zinc probes (FluoZin-3 and Zinquin), showing at least partial co-localisation with insulin-containing granules. However, both approaches are subject to some limitations including (1) uncertainties as to the precise subcellular localisation of the fluorescent zinc probes used [15], and (2) potential clonal effects in studies of organelles isolated from cell lines [22]. Moreover, overexpression studies lead to at least some, albeit limited, incorporation of ZnT8 into the plasma membrane [15], and it has been speculated [23] that the W variant may be leakier in these assays, and thus a *less* active pump into the granule lumen. Of note, overexpression of either version in the clonal INS-1 (832/13) cell line led to an increase in cytosolic zinc concentrations [11] when these were assessed with the recombinant probe eCALWY-4 [5], known to be tightly restricted to this (cytosolic) compartment. These and other experiments raise the important question of whether ZnT8 can act bi-directionally depending on the circumstances, as demonstrated for ZnT5 [24]. In any case, assays of ZnT8 activity in heterologous cells or purified systems such as proteoliposomes [25] are urgently required to address these questions afresh.

Models of ZnT8 inactivation in mice

To date, there have been seven separate reports on the effects of ZnT8 deletion globally [15, 16, 26, 27] or selectively in the beta cell [17, 28, 29] in mice. There are many common features of these models, including dramatically lowered total zinc concentrations but conserved islet insulin content, dramatically impaired secretory granule morphology (i.e. a loss of dense cores and the appearance of rod-like cores) in most studies, and impaired glucose intolerance in four of the seven reports. Importantly, in none of the published studies was glucose tolerance *improved* by ZnT8 elimination. Nonetheless, significant differences were observed in the penetrance of the deletion as a function of age, sex and genetic background [30]. Even for global deletion, the inactivation strategies were not identical between studies. Thus, whereas those of Nicolson et al, Lemaire et al and Wijesekara et al [15, 16, 28] used animals in which exon 1 of the *Slc30a8* gene was deleted (either globally or conditionally), Pound et al [26] used floxed alleles of exon 3, encoding a transporter lacking two transmembrane domains. Tamaki et al [17] used animals where exon 5, predicted to harbour a zinc-binding domain, was missing. Strikingly, for animals in which the gene was deleted globally with the same strategy, marked differences in glucose tolerance, notably in age of onset, emerged for mice maintained at different sites [15, 16, 28], suggesting effects of environmental factors such as diet or bacterial flora. There was also substantial variation in the effects on glucose-stimulated insulin secretion from isolated islets, with increased [15–17], unchanged [27] and decreased [26, 29] secretion reported in different models. Again, differences may reflect altered genetic background [26] or deletion selectively in the beta cell and a subset of neurons [28].

A lowering of intragranular zinc may be predicted to impair proinsulin processing, since the maturation of zinc processing enzymes prohormone convertase-1 and -2 (PC1 and PC2) is dependent on the zinc metalloenzyme carboxypeptidase E. Although such a mechanism might explain the clinical finding of elevated circulating proproinsulin:insulin ratios in R allele carriers [14], other mechanisms may also be at play (see below). Consistent with this view, complete deletion of ZnT8 in *Slc30a8* null mouse islets had no observable effect on proinsulin processing to mature insulin in isolated islets [15, 16].

How might normal or enhanced insulin secretion in vitro (as observed in most, though not all studies, see above) be compatible with lowered circulating insulin levels in vivo? In a recent and very elegant study with beta cell-selective null mice [17], Tamaki et al provided one potential mechanism by demonstrating increased clathrin-mediated uptake of mature insulin, and hence hepatic clearance of the hormone, as a result of lowered pancreatic zinc release (Fig. 1a). Since zinc did not affect uptake of C-peptide or proinsulin, this
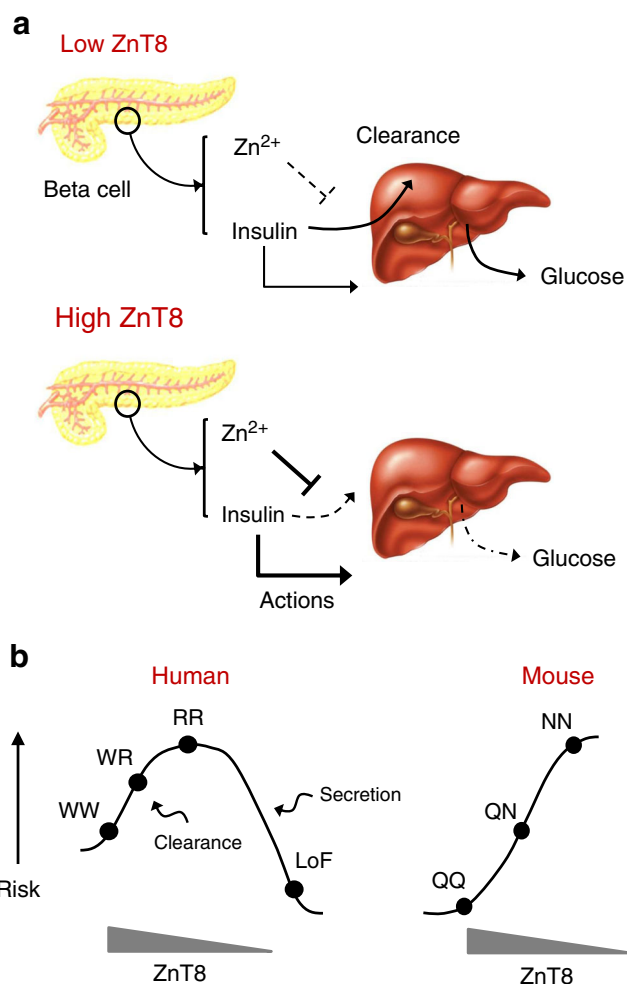

Fig. 1 Interplay between ZnT8 expression, hepatic insulin clearance and action of the hormone. (**a**) Decreases in ZnT8 expression decrease insulin output from the beta cell, whilst reduced Zn^{2+} production favours clearance of the hormone by the liver [17]. (**b**) Possible relationship between ZnT8 expression, as determined by the number of risk (R, human) or null (N, mouse) alleles, and type 2 diabetes risk. In the mouse, increased risk of hyperglycaemia appears to be set chiefly by the control of hepatic glucose output, increasing with loss of ZnT8 function

mechanism may explain both the lowered absolute circulating insulin and decreased insulin:proinsulin ratios in human risk allele carriers at rs13266634. Whether lowered zinc levels also affect (improve?) insulin action on the liver by enhancing insulin receptor internalisation and signalling is unclear. However, evidence against this is provided by the finding that zinc-deficient animals are less sensitive to insulin [31]. Similarly, zinc has been shown to have an insulinomimetic effect on target organs such as adipocytes [32]. Thus, apart from insulin clearance by the liver, one can speculate that circulating zinc might also affect (improve) insulin action, mainly by enhancing insulin signalling through modulation of protein phosphatases [33]. Although extrapolation to the

human case is complex, reduced insulin:C-peptide ratios in human carriers of R325-ZnT8 [17] suggest that lowered zinc secretion from beta cells does indeed enhance hormone clearance by the liver.

Rare variants of ZnT8 in man

The above findings, based on diabetes risk for common human *SLC30A8* variants and studies in knockout mice, have thus enabled a model—if not yet a consensus view—to be developed concerning the role of ZnT8 in insulin secretion and glucose homeostasis. However, the modest effects of ZnT8 alleles (identified by genome-wide association studies, GWAS) on diabetes risk has fuelled the search for rarer, more penetrant variants. This search culminated in 2014 with the publication by Flannick et al [34] of 12 different rare variants that are not detected in many populations and with allele frequencies of much less than 1% in those populations where the mutations are present. Strikingly, of ~350 carriers found in total (of ~150,000 genotyped individuals), a highly significant ($p{\sim}10^{-6}$) excess of *SLC30A8* haploinsufficiency was observed in controls vs individuals with type 2 diabetes cases, implying a protective effect of this allele. This protective action appears unlikely to be explicable through an effect of neighbouring genes in linkage disequilibrium with the *SLC30A8* variants given the distinct ancestry of carriers (five different groups) and the rarity of the variants. However, linkage studies within the families of affected cohorts were not reported and would have provided further confidence for the direction of the effect.

How might the discrepancies with data from mouse mutants and more common *SLC30A8* variants be explored? Of note, most of the rare variants identified by Flannick et al [34] encode truncated (e.g. Arg138*) proteins, or frame shifts (Ser34fs*50) and are unlikely, therefore, to be active zinc transporters. Whether these may act as dominant-negative (or -positive) regulators of wild-type ZnT8 (or of other ion transporters that affect Zn^{2+} distribution across the granule membrane, including zinc importers of the *SLC39* family or H^+ carriers) has not been explored.

Most puzzling, perhaps, is the apparent difference between the protective actions on diabetes risk of human variants exerting a complete loss-of-function (LoF) [34] vs increased diabetes susceptibility for the common R variant with (presumably more modestly) decreased activity [8, 15]. Of note, diabetes risk increases linearly with copy number for the common R allele (with homozygous carriers having double the diabetes risk of heterozygotes) [8]. Similarly, SNP rs13266634 has been associated with protection from post-transplantation diabetes mellitus, the protective effect being cumulative between W/W, W/R or R/R genotypes [35]. Homozygous carriers of LoF variants were not identified, as

expected given the rarity of these alleles. Assuming the activity of the R form of ZnT8 is 30% lower than that of the W form [22], then Zn^{2+} accumulation into granules will be reduced by 15% and 30%, respectively, in heterozygotes and homozygote C allele carriers. This compares with a predicted 50% loss in heterozygotes for LoF variants. Although data from the mouse models (see above) would still predict a deleterious effect on glucose homeostasis in the latter group, it is conceivable that there are species-specific differences in the complex interplay between insulin storage, secretion and the actions of beta cell derived, co-secreted Zn^{2+} on hepatic insulin clearance [17] (Fig. 1a). Thus, it is possible that in humans the relationship between ZnT8 activity and diabetes risk follows a complex (e.g. bell-shaped) dose-response whereby relatively small decreases in ZnT8 activity (R form) may be deleterious whereas a more substantial decrease (LoF) may be protective, in a scenario whereby improved insulin secretion outweighs the deleterious effects of increased clearance (Fig. 1b). By contrast, in the mouse there may be a simpler relationship, whereby increased hepatic clearance as beta cell ZnT8 levels fall serves to progressively impair insulin action (Fig. 1b). Of note, mice possess a glutamine at position 325 in ZnT8, and animals bearing a humanised R or W form 'knocked in' at the murine *Slc30a8* locus have yet to be created or compared.

Another possibility is that small changes in ZnT8 activity may affect disease risk in opposite directions depending on age. This possibility is suggested first by the action of ZnT8 deletion in mice, which causes impaired glucose tolerance and the loss of normal insulin granules in younger (6–8-week-old) animals, a change gradually lost as animals age [15]. Second, we have recently observed a protective effect of ZnT8 deletion on beta cells towards hypoxia that becomes apparent only as animals age [11]. It might thus be possible that higher concentrations of zinc, which by themselves might, under certain conditions, induce cell death [36], impair cell survival during oxidative stress as a consequence of oxidative stress-induced zinc release from MTs [37]. The relevance of these findings in mice to the human disease remains, however, untested.

Conclusions

The identification by GWAS of genes that affect diabetes risk in a definable manner [8, 38, 39] has provided the exciting possibility of developing new therapies tailored towards individual patients based on genotype ('personalised medicine') [40]. Indeed, the identification of such variants ultimately holds the promise of addressing the aetiological causes of the disease, rather than simply its symptoms (e.g. hyperglycaemia and its complications) [41]. However, changes in disease susceptibility with risk allele load are usually small for GWAS-identified variants, and even the inheritance

Studies of the functional effects of a particular variant in humans are by definition *associative* (in the absence of highly specific drugs for a particular target), and highly dependent on identifying carriers of what may be very rare, more penetrant alleles (as in [34]). So whilst being far from perfect [44], as exemplified by the findings on ZnT8 described herein, studies of disease-associated genes in model cellular systems [45] or living organisms, including mice [46] usually remain essential. Importantly, the latter permit 'clean', interventions (i.e. gene silencing or tissue-specific inactivation) alongside in-depth physiological, morphological and functional analyses, providing the opportunity to establish *causality*. Nonetheless, differences, for example in basal metabolic rate [47], between small mammals and humans, as well as differences in organ size and inter-organ distances (relevant when considering the transit of zinc and insulin from pancreas to liver, for instance) mean that mouse models will always carry inherent limitations.

In the case of ZnT8, work in rodents and on variants in humans have served complementary roles in revealing both new biological insights, e.g. the vital role for zinc in insulin crystallisation [15, 16, 48] and the interplay between beta cell zinc and the liver [17], whilst highlighting the potential advantages and drawbacks of ZnT8 as a target for therapy. Although activators of ZnT8 have until now appeared to be the logical agents to develop, the new findings discussed here [34] suggest that the effects of both activators and inhibitors of this transporter will need to be examined in suitable models prior to clinical trials in human.

of several [42, 43] does not usually provide a clinically useful predictor of type 2 diabetes risk for a given individual. Furthermore, in the case of *SLC30A8*, evidence for a targetable variant-drug combination at rs13266634 has been described as 'low' by some estimates (www.pharmgkb.org, accessed 14 August 2014).

Duality of interest There is no duality of interest associated with this manuscript.

Contribution statement Both authors were responsible for the conception and design of the manuscript, drafting the article and revising it critically for important intellectual content. Both authors approved the version to be published.

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