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Sensors for measuring subcellular zinc pools

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Zinc homeostasis is essential for normal cellular function, and defects in this process are associated with a number of diseases including type 2 diabetes (T2D), neurological disorders and cardiovascular disease. Thus, variants in the SLC30A8 gene, encoding the vesicular/granular zinc transporter ZnT8, are associated with altered insulin release and increased T2D risk while the zinc importer ZIP12 is implicated in pulmonary hypertension. In light of these, and findings in other diseases, recent efforts have focused on the development of refined sensors for intracellular free zinc ions that can be targeted to subcellular regions including the cytosol, endoplasmic reticulum (ER), secretory granules, Golgi apparatus, nucleus and the mitochondria. Here, we discuss recent advances in Zn^{2+} probe engineering and their applications to the measurement of labile subcellular zinc pools in different cell types.

1. Introduction

Zinc is one of the most abundant transition metals in the body and ~10% of human proteins require zinc ions for their structure and/or function.[1] In man, loss of zinc homeostasis has been associated with numerous metabolic diseases,[2,3] including diabetes.[4] Thus, pancreata from patients with diabetes have a zinc content which is reduced by 75% compared to non-diabetic cadavers.[5] Whole zinc body status is also modified in diabetes mellitus, as serum zinc levels are significantly decreased[6–8] and urinary zinc loss is increased.[9]

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In cells, concentrations of free ionized Zn^{2+} are buffered and regulated by three main protein families: zinc transporters (ZnT), zinc importers (Zrt, Irt-like protein – ZIP) and metallothioneins (MTs) (Fig. 1). MTs are intracellular, low molecular weight, cysteine-rich proteins that are ubiquitous in eukaryotes. MTs have unique structural characteristics and possess redox and metal-binding capabilities. Among the four classes described, MT1 and MT2 are the most widely expressed isoforms in mammalian cells.[10,11] MTs bind Zn^{2+} with high affinity but can also function as Zn^{2+} donors to other Zn^{2+}-binding proteins. ZIP transporters (ZIP1 to ZIP14) are localised to the plasma membrane (in the case of most family members) and/or on organellar membranes including those of the nucleus, endoplasmic reticulum (ER) and Golgi apparatus (ZIP7, ZIP13, ZIP9), as well as lysosomes

Pauline Chabosseau

Doctor Pauline Chabosseau (1983) did her PhD in Molecular and Cellular Biolоby at Institut Curie in Paris. She is now a post-doctoral Research Associate in the Cell Biology and Functional Genomic section at Imperial College London. Her current focus is to study Zn^{2+} cellular homeostasis in the beta cell, using notably a large range of FRET-based Zn^{2+} sensors, to further investigate the link between zinc and type 2 diabetes.

Jason Woodier

Jason Woodier (1992) studied medicine at the University of St Andrews and the University of Glasgow, graduating in 2016. He is currently working as a junior doctor in Aberdeen. His undergraduate honours dissertation examined the effects of intracellular zinc on the cardiac ryanodine receptor, and he retains a deep interest in the interplay between zinc homeostasis and disease. Although his focus currently is on finishing his training in clinical medicine, he believes in the foundation of clinical practice in the sciences, and one day hopes to return to the scientist's workbench.

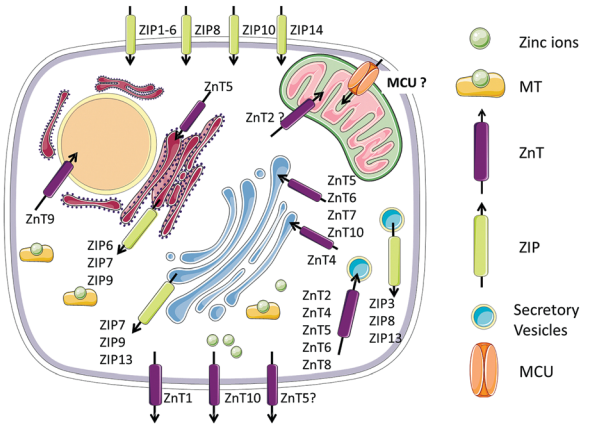


Fig. 1 Protein families involved in cellular zinc homeostasis. ZIP and ZnT localise at the plasma or organelles membranes, catalysing respectively zinc import/export to/from the cytosol to control and maintain Zn^{2+} subcellular concentrations.

and secretory vesicles (ZIP13, ZIP3, ZIP8). ZIPs transport Zn^{2+} ions into the cytoplasm from the extracellular space or from intracellular organelles that act as zinc stores.[12,13] Conversely, ZnT transporters are responsible for zinc efflux from the cytosol into organelles or towards the extracellular space.[14] Ten members have been described (ZnT1 to ZnT10), the majority of the latter family existing as homodimers.[11,15] However, it has been shown that ZnT5 and ZnT6 can also form heterodimers in the early secretory pathway,[16] as well as ZnT10 with ZnT2, ZnT3 and ZnT4 in endolysosomal compartments.[17] Additionally, using bimolecular fluorescence complementation, a study[18] demonstrated that ZnT1, ZnT2, ZnT3, and ZnT4 form stable heterodimers in intracellular compartments, some of which are completely different from their homodimer localization.[18]

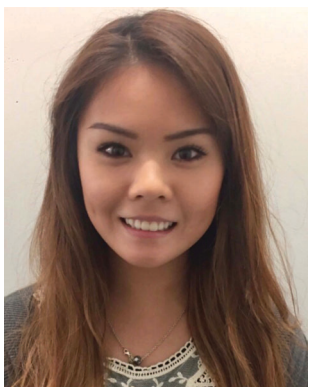
Only ZnT1 is exclusively localised on the plasma membrane. ZnT2–8 are expressed in intracellular compartments with some of the transporters, such as ZnT5 and ZnT10, also located on the plasma membrane.[11,17,19,20]

Levels of expression of ZnT transporters are tissue-dependent and, in the specific case of the vesicle-localized ZnT8, confined almost exclusively to pancreatic islet β and α cells.[21] It has been shown recently that ZnT8 also localised at the cell surface in the rat β cell line INS-1E, with an increased surface display under glucose stimulation.[22]

Until very recently, very little was known of the concentrations of free Zn^{2+} ions in discrete subcellular locations. However, studies in the past few years have demonstrated that organelles, including the ER, can sequester Zn^{2+} ions at high concentrations. Of note, release of Zn^{2+} from this, and potentially other intracellular stores, may allow these ions to act as intracellular second messenger.[23–26] A number of genetic disorders are caused by mutations in the genes encoding ZIP and ZnT transporters, further underlining the importance of maintaining Zn^{2+} homeostasis for normal cellular function.[15] Very interestingly, genome-wide association studies (GWAS) have demonstrated a link between type 2 diabetes (T2D) development and variants in the *SLC30A8* (ZnT8) gene. Thus, a non-synonymous variant (rs13266634) in the *SLC30A8* gene is enriched in diabetic patients.[27] Other examples include the recent identification of ZIP12 as a regulator of Zn^{2+} influx into the pulmonary endothelium, and the risk of hypertension,[28] whilst ZnT9 (*SLC30A9*) is implicated in neurological degeneration in cerebro-renal syndrome.[29] The specific case of zinc homeostasis in pancreatic β cells, and the link between the vesicular Zn^{2+} transporter ZnT8 and T2D risk, will be discussed in more detail later in this review.

2. Genetically-encoded zinc sensors

A deeper understanding of the role of Zn^{2+} ions in cell biology and cell signalling has required the development of sensitive and non-invasive sensors which provide both spatial and temporal resolution. The recent design and deployment of genetically-encoded sensors has been provided a particularly



Rebecca Cheung

Rebecca Cheung obtained her undergraduate degree in Biochemistry at King's College London (2016) and most recently completed her MRes in Diabetes & Obesity from Imperial College London (2017). The topic of her masters research project was CRISPR/Cas9 mediated engineering of insulin-secreting cells with type 2 diabetes risk variants of ZnT8. She is currently a PhD student at the same university under the supervision of Dr Aida Martinez-Sanchez working on AMPK-mediated regulation and roles of miR-125b and miR-184 in pancreatic β-cell function.

Guy A. Rutter

Professor Rutter is the Head of Cell Biology and Functional Genomics at Imperial College London. With funding from the Wellcome Trust, MRC, EU and others, his chief goals are to develop new means to enhance insulin secretion in Type 2 diabetes by studying the fundamental signalling pathways through which glucose act on the pancreatic β cell. He deploys knowledge flowing from genome-wide and other genetic studies for this disease, and state-of-the-art technologies ranging from mouse models through genome editing, optogenetics and photopharmacology.

precious set of tools for allowing these measurements *in cellulo*. Different families of probes have been developed by various laboratories, including Förster Resonance Energy Transfer (FRET)-based Zn^{2+} sensors, and more recently, Zn^{2+} sensors based on a single fluorescent protein.[30–33]

FRET-based sensors are based on an energy transfer phenomenon occurring between a donor and acceptor fluorescent protein after excitation of the donor. Cyan (CFP) and yellow (YFP) fluorescent proteins, derived from green fluorescent protein (GFP), or the equivalent blue/yellow fluorescent proteins pairs, are usually used. The donor and acceptor are linked by a peptide sequence containing a zinc-binding domain, and change of conformation upon zinc chelation induces a modification in the FRET energy transfer and impacts the emitted fluorescence intensity ratio YFP/CFP.[32] Two families, based on zinc fingers have been developed by Palmer and colleagues: Zif- and Zap-sensors.[34,35] Zif sensors are derived from the mammalian transcription factor Zif268 and contain either a wild type zinc finger (ZifCY1) or a mutated domain (ZifCY2).[34,35] However, these probes have a relatively low affinity for free Zn^{2+} ions (μM range) and are not well suited for measurements in the cytosol where the concentration has been estimated to be in the hundreds of picomolar range.[36] Zap sensors, based on the *Saccharomyces cerevisiae* transcriptional regulator Zap1, provide higher affinity, and ZapCY2 (K_d = 811 pM) has been used successfully for Zn^{2+} measurements in the cytosol and in subcellular compartments[35] (Table 1). Both Zap- and Zif-sensors display an increased FRET ratio upon zinc binding, *i.e.* the maximum fluorescence intensity ratio is obtained upon zinc saturation conditions as 100% of the sensor population is in a closed conformation; the minimum FRET ratio is achieved after zinc depletion. The eCALWY sensors, developed by Merkx and colleagues and ourselves, are based on cerulean/citrine protein pairs and include two cysteine-containing metal binding domains (ATOX and WD4) connected by a long flexible glycine–serine linker. Each domain provides two cysteines to form a single tetrahedral zinc binding pocket. In the absence of Zn^{2+} ions, an intramolecular complex is formed between cerulean and citrine, due to the introduction of mutations (S208F and V224L) on the surface of both fluorescent domains, and which results in high FRET. Conversely, when Zn^{2+} binds between ATOX and WD4, the interaction between cerulean and citrine is disrupted, lowering the citrine/cerulean fluorescence intensity ratio. Mutations and/or shortening of the linker length led to the development of a series of six sensors with affinities ranging from the picomolar to the micromolar range.[37]

Deployed in a variety of cell types, eCALWY-4 (K_d = 630 pM) has proved to be a reliable sensor for measuring cytosolic Zn^{2+} in most cases.[23,37] More recently, Merkx and colleagues have developed an alternative zinc sensor containing, innovatively, a Cys2His2 binding pocket that was created on the surface of the donor (cerulean) and acceptor (citrine) fluorescent domain. Named eZinCh-2, the sensor provides an affinity for Zn^{2+} that is similar to eCALWY-4 (K_d = 1 nM at pH 7.1), but displays a slightly larger change in the fluorescence intensity ratio, and has been used successfully in different cell types.[33,38]

FRET sensors are, by definition, ratiometric sensors, as Zn^{2+} concentration changes are monitored by the ratio of the emitted fluorescence intensity of the acceptor *versus* the donor. This ratio reflects sensor occupancy and is dependent on Zn^{2+} concentration but not sensor expression, provided cellular autofluorescence is low.[30,32] However the sensors' dynamic range, *i.e.* changes in fluorescence intensity ratio upon Zn^{2+} binding, is often limited. In the case of eCALWY sensors, the dynamic range has been improved greatly by the introduction of mutations on the surface of both of the GFP-based fluorophores.[37] Palmer and colleagues have also developed a zinc sensor[31] based on a single fluorescent protein as an alternative option presenting a great dynamic range. This sensor, named Green Zinc Probe 1 (GZnP1 – K_d = 58 pM, pH 7.4), was generated by attaching two zinc fingers from the yeast transcription factor Zap1 (ZF1 and ZF2) to the two ends of a circularly permuted green fluorescent protein (cpGFP). Upon Zn^{2+} binding, the formation of two zinc finger folds results in a conformational change of cpGFP leading to an increase of fluorescence intensity.

Very recently, Merkx and colleagues[39] have developed an alternative type of genetically encoded sensor for intracellular zinc ions based on Bioluminescence Resonance Energy Transfer (BRET), an energy transfer phenomenon occurring between a donor luciferase and an acceptor fluorescent domain. The advantages of BRET-sensors include an absence of photobleaching and of phototoxicity induced by excitation light, and no background autofluorescence during measurement. In the recent report,[39] the luciferase NanoLuc domain was fused to the cerulean domain of eCALWY-1 and eZinCh-2, to create BLCALWY-1 and BLZinCh-1 and -2 sensors, with a preserved affinity and specificity towards zinc ions with respect to the parent probes. Additionally, a chromophore-silencing mutation was introduced in the cerulean domain of BLZinCh-1 to create a BRET-only sensor

Table 1 Main families of genetically-encoded zinc sensors, and their targeting to organelles

	K_d (pH 7.1)	Red variant	Targeted to organelles	Ref.
eCALWY				
CALWY	0.2 pM			30
eCALWY-l	2 pM			37
eCALWY-2	9 pM			37
eCALWY-3	45 pM			37
eCALWY-4	630 pM		ER, mitochondria	23 and 37
eCALWY-5	1850 pM			37
eCALWY-6	2900 pM		ER, mitochondria	23 and 37
redCALWY-1	12.3 pM	Yes		30
redCALWY-4	234 pM	Yes		30
Zap				
ZapCYl	2.5 pM		Golgi, ER, mitochondria	35
ZapCY2	811 pM			35
ZapOC2	—	Yes	Nudeus	40
ZapCmR2	—	Yes	Nudeus	40
ZapCmRl	—	Yes	Nucleus	40
eZinCh				
eZinCh-1	8.2 nM		Vesicles	37
eZinCh-2	1 nM		ER, mitochondria, vesicles	33

(BLZinCh-3) that displayed a 50% increased BRET response associated with an unpredicted 10-fold increase in Zn^{2+} affinity. BRET/FRET and BRET only variants of ZinCh-2 allowed monitoring of Zn^{2+} in plate-based assay as well as in BRET-based single cell imaging experiments.[39]

One of the great advantages of genetically-encoded sensors is that they are easily targetable to subcellular structures by the inclusion of a signal peptide or other targeting sequence at either end of the coding sequence. So far, eCALWY-4, eZinCh-2 and the zap-sensors have been successfully targeted to different organelles, including mitochondria, ER, secretory granules and Golgi apparatus.[23,33–35,38,40,41]

3. Low molecular weight and hybrid probes

Low molecular weight (LMW) probes are tailored by chemical synthesis and are composed of a chelating agent and a fluorophore (for reviews see ref. 32 and 36). If some LMW probes are permeant to cell membranes, the presence of charged moieties prevents the entry of others into the cell. A now currently used strategy to allow cell membrane transit was first developed by Roger Tsien, and consists of the addition an acetoxymethyl (AM) ester group.[42] The AM group is then hydrolysed once within the cell, trapping the charged probe inside.

Most LMW probes for zinc ions are non-ratiometric, and are based on the principle of photo-induced electron transfer (PET) occurring between the fluorophore and the chelating moiety, quenching fluorescence. Upon zinc binding, PET is disrupted and the probes display greatly enhanced fluorescence emission (for reviews see ref. 32 and 36).

One of the first probes developed, Zinquin,[43] was successfully used to measure labile zinc but had the disadvantage of being excitable in the UV range, hampering measurements in live cells due to phototoxicity and susbtantial background autofluorescence. Subsequently, a large number of probes have been designed that are excitable in the visible range. An exhaustive list of LMW probes has previously been provided by others[32] and only the most-frequently used will be described here.

The Zinpyr (ZP) probe family (ZP1 to ZP10)[32,44,45] is based on fluorescein, with excitation wavelengths above 490 nm. Most of the members of this family are naturally cell permeant, and several designs have been developed to improve pH sensitivity, affinity for Zn^{2+}, background fluorescence and fluorescence dynamic range upon zinc binding. ZP probes have a K_d in the hundreds of picomolar range, except for ZP9 and ZP10 which have lower affinities. The ZnAF probe family[46–48] are also fluorescein-based and have the great advantage of displaying extremely low background fluorescence in the ion-free state, associated with an augmented response to Zn^{2+} binding. If the original ZnAF probes were not membrane permeant, this issue was solved by the addition of diacetyl derivatives to mask the negative charge on the probes.[48] ZnAF probes have a great affinity for Zn^{2+}, with K_d from the nanomolar to the micromolar range. Fluorescein-based Newport Green DCF and PDX[49] display a lower affinity to zinc (K_d = 1 μM and 40 μM respectively). FluoZin-3,[49] now one of the most used probes to measure labile Zn^{2+}, was based on an existing calcium probe BAPTA, originally developed by Roger Tsien.[50] FluoZin-3 (K_d = 15 nM) has an excitation peak at 495 nm, shows a 200-fold fluorescence increase upon zinc binding and is cell permeant when associated with an AM group (FluoZin-3-AM).

Whilst the chemical probes generally have a greater dynamic range, in terms of fluorescence responses, than genetically-encoded sensors, their subcellular localisation can be hard to predict and control.[32] LMW probes may, for example, spontaneously accumulate in organelles such as mitochondria, Golgi, endosomes, secretory vesicles, *etc.* It has been shown with Ca^{2+}-responsive probes that both molecular charges and lipophilicity parameters play a role in determining accumulation into subcellular organelles,[51] and it is not uncommon for a probe to exhibit different localisation depending on cell types.[52]

Several successful attempts have been made to target LMW probes specifically to a given organelle. For instance, mitochondrial targeting can be achieved by the addition of a positively charged group. As an example, the mitochondrially-targeted zinc sensor RhodZin-3[53] was developed from FluoZin-3 by replacing the fluorescein moeity with a rhodamine fluorophore, whose positive charge directs accumulation into the mitochondria (negative inside). Another example of molecular design for targeting is our zinc probe ZIMIR (Zinc Indicator for Monitoring Induced exocytotic Release).[54] ZIMIR comprises a fluorophore based on fluorescein, a Zn^{2+} binding motif derived from dipicolylamine and a moiety composed of a pair of dodecyl alkyl chains for cell membrane tethering. In order to provide a simplified synthetic process towards a probe with similar properties, we have recently developed a "click-SNAr-click" approach.[55] Another strategy is to target LMW probes to organelles by the addition of a peptide targeting unit. This way, ZP1 and Zinquin have been both successfully anchored to the extracellular side of the plasma membrane.[56]

Hybrid sensors have also been described that are based on FRET principles, in this case occurring between a genetically-encoded component coupled to a small molecule. For zinc hybrid sensors, the ion detection domain was based on a carbonic anhydrase (CA) variant fused with red fluorescent protein RFP[57] and the cell-permeable fluorescent co-factor dapoxyl sulphonamide added to the cells will bind to an open coordination position on the zinc ion when bound to the sensor, allowing then FRET between the dapoxyl sulfonamide and the fluorescent protein domain. Intracellular concentrations measured in mammalian pheochromacytoma PC12 cells were in the low picomolar range and thus lower than those above measured in other mammalian cell types with probes and sensors described.[57] While hybrid sensors can also be targeted to organelles, they still require the addition of the synthetic cofactor to allow zinc imaging.[57]

4. Zinc ions in the endoplasmic reticulum

Despite the well-defined presence of Zn^{2+} transporters/importers on the ER membrane[11–13] free Zn^{2+} concentrations in the

ER have been the subject of controversy. The first *in cellulo* measurement using a genetically-encoded FRET sensor was performed using ER-targeted ZapCY1 which had a very high affinity for zinc ions.[35] Using this sensor, the authors estimated a Zn^{2+} concentration of 0.9 pM in HeLa cells, a concentration a hundred times lower than that measured in the cytosol. However, a later study by ourselves[23] using eCALWY-4 targeted to the ER showed that the concentration could be much higher as the probe used in this case was saturated with zinc ions. Concentrations higher than 5 nM were found in all cell types tested including HeLa cells. The latter result is in line with the hypothesis that the ER may act as a mobilizeable zinc store and that, in certain cell types, Zn^{2+} may be released from this organelle to act as a second messenger. Using Newport green as a probe, Yamasaki *et al.*[24,25] showed that mast cells stimulated through the high-affinity IgE receptor rapidly release intracellular Zn^{2+} from the endoplasmic reticulum (ER), a phenomenon called a "Zinc wave".

Recent measurements using eZinCH-2 targeted to the ER in HeLa cells returned a value of 0.8 nM, with a very high cell-to-cell variability, as the concentration range was from 0.3 nM to 1.5 nM.[33] These results thus also indicate a high ER Zn^{2+} concentration compared to the cytosol. Very interestingly, previous work by Taylor and colleagues[58] showed increased expression of ZIP7 in tamoxifen-resistant TamR cells compared to wild type MCF-7 cells. ZIP7 is a Zn^{2+} importer located almost exclusively on the ER membrane, and its phosphorylation was shown to result in the release of Zn^{2+} from the ER into the cytosol. By deploying ER-eZinCH-2 in these cells, it was found accordingly that TamR cells have a slightly increased Zn^{2+} ER content (0.54 ± 0.27 nM for MCF-7 *versus* 0.75 ± 0.49 nM for TamR cells).[33,38]

Recently, our own laboratory has examined the localization of ZIP7 and ZnT7 in cardiomyocytes, studies which also revealed the presence of both proteins on the sarco(endo)plasmic reticulum (S(E)R).[59] Very interestingly, markedly increased mRNA and protein levels of ZIP7 were observed in cardiomyocytes from diabetic rats or high-glucose-treated H9c2 cells while ZnT7 expression was lowered in these models relatively to controls. It was also shown that hyperglycemia induced a marked redistribution of cellular free Zn^{2+}, increasing cytosolic free Zn^{2+} and lowering free Zn^{2+} in the S(E)R. These changes involved alterations in ZIP7 phosphorylation. Thus, subcellular free Zn^{2+} redistribution in the hyperglycemic heart, resulting from altered ZIP7 and ZnT7 activity, may contribute to cardiac dysfunction in diabetes.[59]

5. Zinc ions in the nucleus

Zap sensors were the first and so far only nucleus-targeted genetically-encoded FRET sensors.[40] Palmer and colleagues developed alternately Zap sensors by switching the YFP and CFP fluorescent domain with different green-red or orange-red donor/acceptor pairs. These sensors were targeted to the nucleus with a nuclear localisation sequence (NLS). The green-red NLSZapSR2 sensor was co-expressed in HeLa cells with the yellow-blue ZapCY2 sensor targeted either to the cytosol or to the ER, the mitochondria or the Golgi, allowing simultaneous monitoring of zinc uptake by the nucleus and different subcellular compartments. Thus, upon an increase in cytosolic Zn^{2+} concentration, nuclear zinc rises quickly, whereas the ER, Golgi and mitochondria showed a slower a delayed zinc increase. Additionally, nuclear Zn^{2+} was buffered at a higher level than cytosolic Zn^{2+}. These data suggest the interesting possibility the nucleus may serve as a zinc reservoir, at least in some cell types and circumstances.

LMW probes have also been used to monitor free zinc in the nucleus in other studies. Using the UV-excitable TSQ probe, Cherian and colleagues[60] showed that, in myoblasts, zinc, alongside MT, was concentrated mainly in the cytoplasm but was translocated into the nucleus in newly-formed myotubes during early differentiation. Of note, the changes in subcellular localization of MT and Zn^{2+} were accompanied by increased apoptosis in these cells, consistent with a role in the latter process.

6. Zn^{2+} concentrations in intracellular vesicles

Pancreatic β cells are extremely rich in zinc with around 70% of Zn^{2+} ions being located within the insulin secretory vesicles (also termed insulin secretory granules or ISGs). There, the total concentration of Zn^{2+} reaches 10–20 mM.[61,62] Indeed, in these cells, Zn^{2+} ions are involved in the processing and storage of insulin, and in insulin hexamer formation. The hexamerization process reduces insulin solubility and triggers its crystallization, increasing then the storage capacity of the vesicles.[63,64] When insulin is secreted into the extracellular medium during exocytosis, hexamers are rapidly converted into active monomers, concomitantly liberating significant concentration of Zn^{2+} into the circulation. These may potentially exert actions independent to those of insulin.[63,65]

Zinc ions are concentrated within the insulin secreting vesicles *via* the zinc transporter ZnT8.[66,67] As discussed above, ZnT8 expression is almost entirely limited to the pancreas, specifically to α and β cells in both human and mouse.[68,69] ZnT8 is the only ZnT isoform showing such a dramatic tissue-specific expression pattern and its expression level is the highest of all transporters expressed in both α and β cells, making it the most strongly expressed transporter in the islet.[21]

The importance of vesicular zinc transport in the function of the β cell has been highlighted by recent GWAS studies. Indeed, a first GWAS for T2D demonstrated an association between disease risk and ZnT8 variants, since a non-synonymous single nucleotide polymorphism (SNP) (rs13266634) in the *SLC30A8* coding sequence is enriched in diabetic patients.[27] This SNP leads to the replacement of an arginine by a tryptophan at position 325 (R325W) at the C-terminus of the transporter, positioned on the cytosolic surface of the granule. The risk variant (R325) is associated with a 17% increase in disease risk per allele[70] and R325 variant carriers show significantly impaired insulin secretion during intravenous glucose tolerance tests, and lower β cell function by HOMA-B assessment.[71,72]

Of note, subsequent studies also identified five other SNPs, all located in exon13 of *SLC30A8* and with one of them causing an amino acid replacement at position 325 (for review ref. 66 and 73).

It is still not clear exactly how, or if, the rs13266634 polymorphism impacts transporter activity. In a study performed in our laboratory,[74] we measured Zn^{2+} accumulation in the vesicle interior using Zinquin after overexpression of either isoform in a β cell line. We showed that, in the presence of supraphysiological Zn^{2+} concentrations, zinc accumulation into granules was significantly higher in cell overexpressing the W- *versus* the R-form. Thus, the risk variant R325 appeared from these studies to possess lower transport activity than the protective variant, W325.[74] Similar data were also obtained by Kim *et al.* using isolated secretory granules and radiotracers (^{65}Zn).[75]

However, these results were difficult to reconcile with a study performed in 2014[76] in which Flannick and colleagues identified 12 rare protein-truncating variants statistically associated with a 65% decrease in T2D risk. Among the variants, the two most common sequences failed to express a stable protein. Thus haploinsufficiency, leading to a lower expression of the active transporter and consequently to a reduced zinc transport activity, appeared to be protective against T2D. Aligning with the latter finding, in a recent study from Merriman and colleagues,[77] R325 was found to be more active than the W325 form following induced expression in HEK293 cells. Additionally, purified ZnT8 variant activity was assessed in proteoliposomes and, over a broad range of permissive lipid compositions, the R325 variant exhibited accelerated zinc transport kinetics compared to the W325 form.[77]

Further paradoxes exist when considering the above results and *in vivo* data, especially those obtained in mouse models. To investigate the role of ZnT8 in the maintenance of glucose homeostasis, several groups, including ours, have developed ZnT8 null mice, either with global deletion or cell type-specific deletion restricted to β or α cells. Although variations are observed between models, global and β cell-selective null mice display either unchanged glucose metabolism or glucose intolerance. We are aware of no study to date that has reported an improvement in this metabolic parameter (see for review ref. 78). Additionally, we generated in the laboratory a mouse line overexpressing human W325 ZnT8 variant in β cells. These animals showed improved glucose tolerance, possibly attributable to increased Zn^{2+} secretion, as insulin secretion was not improved compared to control littermates.[79]

In summary, observations in man of lower ZnT8 activity being associated with disease protection in carriers of rare variants, and data obtained in mice, need to be reconciled. Interestingly, we observed in the laboratory that ZnT8 deletion has an impact on intracellular zinc homeostasis, as primary β cells from ZnT8-KO mice display a lower free zinc concentration.[79,80]

To resolve these apparent discrepancies, measurement of free Zn^{2+} concentration in subcompartments (notably the cytosol and secretory granule interior) of the living β cell has become essential. A first attempt to develop vesicle-targeted zinc sensors used eCALWY sensors fused to a vesicle-targeting vesicle-associated membrane protein 2 (VAMP2) sequence. Although efficient localisation to the vesicular interior was achieved, VAMP2-eCALWY sensors were not responsive in this environment to treatments inducing zinc depletion (*N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine: TPEN) or zinc saturation ($ZnCl_2$ plus pyrithione: Zn/Pyr).[37] More recently, the eZinch2 sensor was similarly targeted to vesicles (VAMP2-eZinCh2): the new targeted sensor was, in contrast, responsive to TPEN or Zn/Pyr and returned a free vesicular Zn^{2+} concentration at steady state of ~120 nM.[33]

Alternatively, ZIMIR,[54] membrane-targeted non-cell permeant zinc probe, has been successfully used to measure secreted zinc during glucose-stimulated insulin secretion of β cells and has proven to be a useful tool to assess zinc content in the vesicles.[54,79] Confirming the efficiency of ZnT8 inactivation in mouse cells, ZIMIR indicated the complete blockade of Zn^{2+} secretion in the absence of the transporter.[54,79]

Other cell types display high vesicular zinc content, including T-cells. In the murine cytotoxic T-cell line CTLL-2, Zinquin loading resulted in a uniform staining while FluoZin-3 exclusively labelled vesicular structures, called "zincosomes" which sequestered levels of zinc.[81,82] In CTTL-2 cells, "zincosomes" co-localized with a fluorescent lysosome tracker. After stimulation with IL-2, vesicular Fluozin-3 decreased while Zinquin fluorescence increased, indicative of an intracellular translocation of Zn^{2+}.[82] It was further demonstrated that Zn^{2+} functions as an ionic signalling molecule after T cell activation[83] as cytoplasmic zinc concentrations increased within 1 min of T cell receptor activation, in particular in the subsynaptic compartment.

In another example of the use of LMW probes to follow the subcellular localisation of Zn^{2+}, Haase and Bayermann[84] used Zinquin[43] to monitor the sequestration of Zn^{2+} into intracellular vesicles in rat C6 glioma cells.

In the brain, ZnT3 is required for the accumulation of zinc ions inside the synaptic vesicles of glutamatergic neurons.[85,86] During ischemia, simulated by deprivation of oxygen and glucose (OGD), followed by reperfusion[87] use of a cell impermeant form of Newport Green allowed the demonstration of marked increases in extracellular Zn^{2+}.

The deployment of zinquin demonstrated that dietary deficiency of omega-3-polyunsaturated fatty acids increased ZnT3 expression and concomitantly increased free Zn^{2+} in the brain:[88] a corollary of these findings is that increased consumption of oily fish may act in this way to lower the risk of Alzheimer's disease and dementia.

Another recent and exciting development is that by Lippard and colleagues at MIT of diacetylated Zn^{2+} probes, such as DA-Zinpyr-1, which serve as reaction-based probes. Thus, elevated levels of intracellular zinc catalyse deacetylation, giving large increases in fluorescence signal. These have been used to excellent effect recently to demonstrate the loss of a synaptic zinc-rich layer of the dorsal cochlear nucleus in ZnT3 knockout mice.[89]

7. Zn^{2+} ions in the mitochondrial matrix

The ZnTs/ZiP family members responsible for the transport of Zn^{2+} across most intracellular membranes (ER, secretory

granules, *etc.*) are now reasonably well defined.[90] However, and although one study[91] showed that ZnT2 is localised to the mitochondrial inner membrane in murine mammary gland cells, no ubiquitously expressed ZnT has clearly been identified as yet as responsible for Zn^{2+} uptake into the mitochondrial matrix. Likewise, Zn^{2+} efflux mechanisms from mitochondria remain obscure. Importantly, free Zn^{2+} concentrations in the mitochondrial matrix must be tightly regulated to ensure adequate concentrations for Zn^{2+}-dependent processes whilst preventing accumulation to toxic levels. Thus, high [Zn^{2+}] inhibits the electron transport chain at the *bc*1 complex, resulting in inhibition of O_2 consumption and a lowering of the mitochondrial inner membrane potential ($\Delta\psi_m$).[92,93] Correspondingly, under ischemic conditions, zinc toxicity in neurons is due, at least in part, to mitochondrial Zn^{2+} uptake.[93–96] As for the ER, absolute free Zn^{2+}concentrations within the mitochondrial matrix remain a subject of controversy. Initial studies using genetically-encoded or hybrid sensors in HeLa and neuronal PC12 cells gave concentrations of <1 pM.[41,57] On the other hand, using the eCALWY-4[37] sensor targeted to the mitochondrial matrix (mito-eCALWY-4),[23] we have reported a value of $\sim$200 pM in multiple cell types. Additionally, a recent study using the mitochondria-targeted sensor mito-eZinCh-2 returned a value in the low picomolar range in HeLa cells.[33] Whilst the reasons underlying the variability between these results is not fully understood, it may reflect the varying sensitivity of the different probes to pH changes and alterations in dissociation constant (K_d) *in vivo versus* the value determined *in vitro*. Nonetheless, a labile zinc pool has also been detected in mitochondria using the low molecular weight probe RhodZin-3[93] in studies supporting a value for intramitochondrial Zn^{2+} in the picomolar range.

Additionally, the diacetyled form of the LMW probe Zinpyr-1 has been delivered to the mitochondria by a TPP targeting (DA-ZP1-TPP).[97] Exposure to Zn^{2+} triggers metal-mediated hydrolysis of the acetyl groups to afford a large zinc-induced fluorescence response. Using this probe, the authors observed a decreased zinc mitochondrial uptake in cancerous prostate cells compare to healthy cells.

Studies have suggested a role for the mitochondrial calcium uniporter (MCU) in mitochondrial Zn^{2+} uptake.[92,98] A ubiquitously-expressed protein in mammals, MCU is localised to the mitochondrial inner membrane and is primarily responsible for Ca^{2+} entry into the organelle.[99,100] Thus, the MCU complex, which also includes MICU1/2 and EMRE subunits, facilitates Ca^{2+}-stimulated ATP production[101,102] as well as the negative effects of prolonged or dysregulated Ca^{2+} entry such as membrane depolarisation and reactive oxygen species (ROS) production.[103,104]

Several studies have implicated MCU in mitochondrial Zn^{2+} entry, using the inhibitor ruthenium red to inhibit the transporter.[94,105] Notably, Malayandi and co-workers showed using isolated mitochondria that ruthenium red partially blocks Zn^{2+} entry in the organelle and suggested the existence of distinct mechanisms of Zn^{2+} uptake, one being MCU-dependent.[106] Studies exploring the role of MCU in mitochondrial Zn^{2+} transport in living cells are, however, lacking.

8. Conclusions

Recent findings have highlighted the crucial role of Zn^{2+} ions in the biology of many cell types and have served as an important spur for the development of new probes for these ions. The latter have proved vital tools in understanding the roles played by intracellular zinc importers and transporters in health and disease. In the future it is likely that some of these probes may also provide the basis of assays which allow the identification of regulators of these molecules allowing translation to the clinic of some of these exciting findings.

Abbreviations

ER	Endoplasmic reticulum
FRET	Förster resonance energy transfer
GWAS	Genome-wide association studies
ISG	Insulin secreting granules
MCU	Mitochondrial calcium uniporter
MT	Metallothionein
S(E)R	Sarco(endo)plasmic reticulum
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
T2D	Type 2 diabetes
TPEN	*N*,*N*,*N*′,*N*′-Tetrakis(2-pyridylmethyl)ethylenediamine
ZIP	Zinc importer protein
ZnT	Zinc transporter.

Conflicts of interest

There are no conflicts to declare.

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