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Letter to the editor

Free zinc determines the formability of the vesicular dense core in diabetic beta cells

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ABSTRACT

During the progression of type 2 diabetes, total body zinc deficiency disrupts the formability of the electron-dense core in beta-cell vesicles, but the mechanism is unclear. Using fluorescence imaging, transmission electron microscopy and pharmacokinetics assays, we established a strong link between an increasing concentration of free zinc and the formability enhancement of the dense core electron density. Thus, our results highlight a mechanism by which zinc supplementation enhances the maturation of dense cores and restores the secretion of insulin in two diabetic mouse models both *in vitro* and *in vivo*. This study provides a potential research direction for investigating the etiology and nutrition of zinc in the management of type 2 diabetes.

Dear Editor,

Zinc deficiency in beta cells is strongly correlated with the progression of type 2 diabetes (T2D) (Chabosseau and Rutter, 2016). Over the past 15 years, more than 300 papers have mentioned or focused on the islet-specific zinc transporter ZnT8, which is involved in the crystallization and storage of insulin in secretory vesicles in beta cells (Germanos et al., 2021). We previously constructed a zinc fluorescence energy resonance transfer (FRET) biosensor, ZnT72R, to measure the zinc concentration inside vesicles reflecting zinc efflux transported by ZnT8 and found free zinc deficiency in vesicles from diabetic *db/db* mice (Xian et al., 2020). However, determining the degree to which free zinc in vesicles contributes to diabetes and whether zinc supplementation can prevent and even reverse T2D is difficult. Here, we investigated the contribution of free zinc to vesicle maturation and developed a potential zinc supplementation strategy for the treatment of T2D.

Because the secretary vesicle consists of two parts, a dense core with zinc-insulin crystals and a lumen with free zinc (Foster et al., 1993), we first designed a vesicle perforation and imaging assay. Live beta cells were costained sequentially with two zinc dyes. Fluozin1 was distributed in the cytoplasm near the cell membrane; in contrast, ZRL1 labeled zinc in insulin vesicles (Fig. 1A). When a vesicle was perforated, the fluorescence of ZRL1 suddenly disappeared, and the same vesicle was then stained with Fluozin1 (Fig. 1B). Fluozin1 ($K_d = 7.8$ μmol/L) has a higher zinc affinity than ZRL1 ($K_d = 73$ μmol/L) and more easily captures zinc after vesicle opening. As a result, the fluorescence intensities of ZRL1 and Fluozin1 exhibited opposite change trends (Fig. 1C). The tau value represented a period in which the fluorescence intensity dropped to 1/e and was used to evaluate the time required to dissolve zinc-insulin crystals. Fluozin1 fluorescence normalized to the maximum fluorescence and the dissolution time showed a positive exponential correlation (Fig. 1D). This result indicated that Fluozin1 labeled crystal zinc and required dissolution time to release insulin. The Fluozin1 fluorescence normalized to the maximum fluorescence, and ZRL1 fluorescence normalized to the maximum fluorescence showed a positive linear correlation (Fig. 1E). Thus, vesicular free zinc showed a positive correlation with zinc-insulin crystals.

To further verify that the concentration of free zinc is the main driving force of the formation of the dense core, we identified the transmission electron microscope (TEM) characteristics of dense-cores in the beta-cell vesicles from guinea pigs (GPs) that cannot bind zinc and represent the physiological limit of zinc deficiency in vesicles (Fig. S1A). GPs had a larger dense core area and lower electron density than C57 mice (Fig. S1B). Because GPs evolved via a different route than other species, insulin mutations in GPs accompanied by a series of metabolic compensatory changes do not result in hyperglycemia or diabetes (Chan et al., 1984). Furthermore, the groups treated with the vesicle-permeable zinc chelator N, N, N′, N′-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) had a larger dense core area and lower electron density than the TPEN-untreated groups (Fig. S1C, D). However, the *db/db* mice had a smaller dense core area and lower electron density than C57 mice (Fig. S1E, F). The normalized results of the above three groups (Fig. S1G, H) showed that the decrease in the concentration of free zinc led to a lower dense core electron density in beta-cell vesicles, which indicated the immaturity of the vesicles.

To elucidate the relationship between free zinc and mature insulin in vesicles in *db/db* mice, we performed the first three-channel imaging of live beta cells (Fig. 1F, the details were described in the supplementary information), and the calculated results from a single vesicle showed that the concentration of free zinc in vesicles from *db/db* mice was lower than that in vesicles from C57 mice (Fig. 1G). The linear fitting results showed a positive correlation between the expression of NPY-ZnT72R and the concentration of free zinc (Fig. 1H). Subsequently, we fixed the same batch of live cells immediately after imaging and further used anti-proinsulin to evaluate the maturity of the vesicles. We next performed the second three-channel imaging (Fig. 1I, the details were described in the supplementary information). The fluorescence intensity of mRuby2 indicated the NPY-ZnT72R expression level in immature dense core vesicles (DCVs) containing proinsulin and mature DCVs without proinsulin. In both C57 mice and *db/db* mice, the mature DCVs (-proinsulin) contained more NPY-ZnT72R than the immature DCVs (+proinsulin) (Fig. 1J and K). Moreover, the NPY-ZnT72R-nonexpressing vesicles contained more proinsulin (Fig. 1L, M). In summary, we confirmed that

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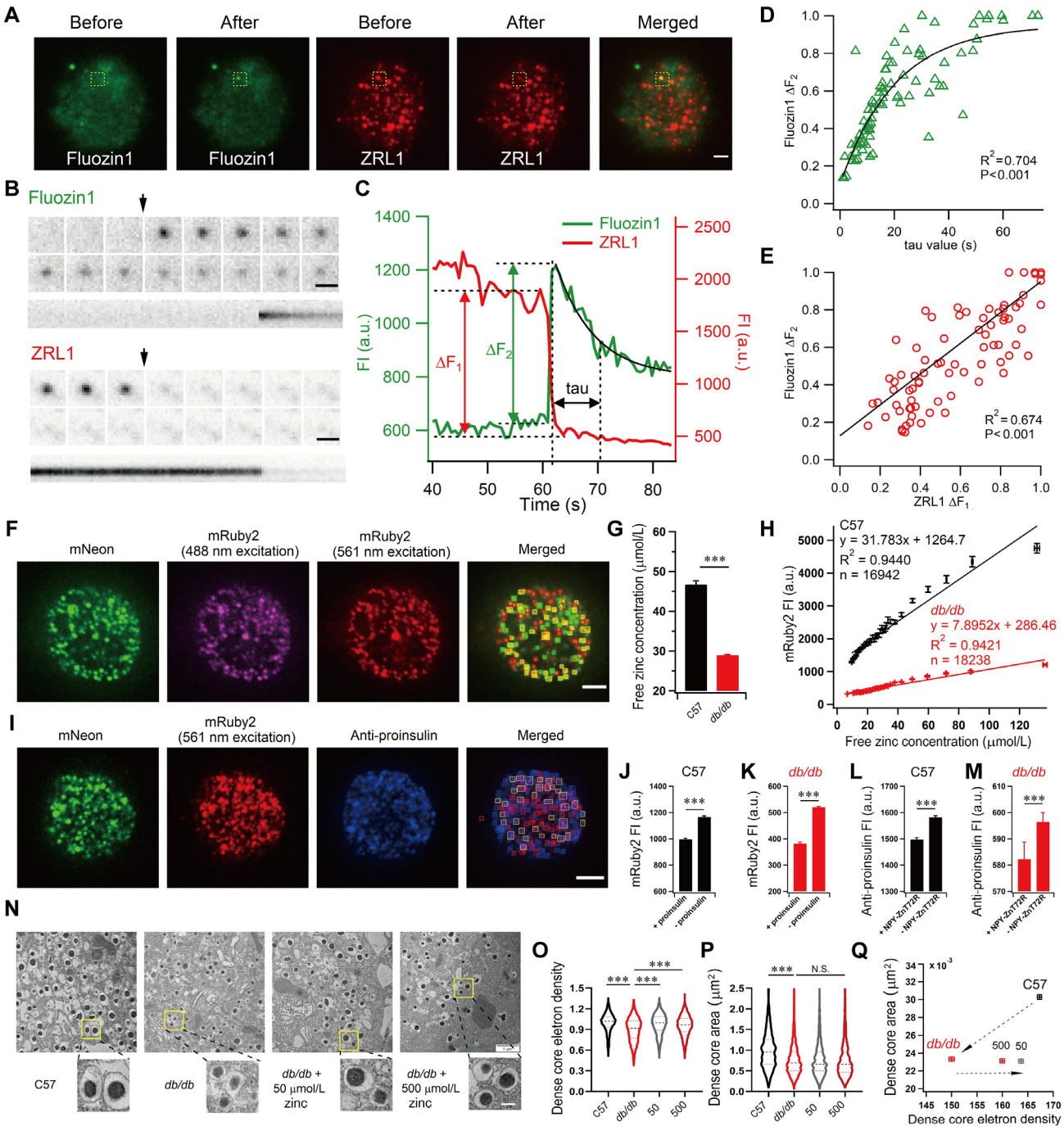

Fig. 1. Zinc supplementation enhanced the maturation of dense cores. (A) A representative example (dotted box) of vesicles labeled with Fluozin1 and ZRL-1 before and after vesicle perforation with digitonin. Scale bar, 2 μm. (B) Representative montage images of the same vesicle labeled with Fluozin1 and ZRL1 before and after vesicle perforation with digitonin. Scale bar, 1 μm. Kymographs, 1.5 μm high. The arrows indicate the time point when the vesicle started to lyse. (C) Time courses of Fluozin1 and ZRL1 signals from the example shown in (B). a.u., arbitrary unit. (D) The change in Fluozin1 fluorescence (ΔF_2) and crystal dissolution time (evaluated by tau value) showed a positive exponential correlation. n = 80 events from 6 cells. (E) The change in Fluozin1 fluorescence (ΔF_2) and the change in ZRL1 fluorescence increment (ΔF_1) showed a positive linear correlation. n = 85 events from 6 cells. (F) Representative three-color image of a single live beta cell, scale bar, 5 μm. (G) The free zinc concentration in vesicles in C57 (n = 12,376) and *db/db* (n = 11,568) mice. n = 13 cells for both C57 and *db/db* mice from 5 independent mouse preparations per group. (H) Free zinc concentration and NPY-ZnT72R expression in single vesicles show a positive linear correlation in normal C57 mice and diabetic *db/db* mice. n = 20 cells for both C57 and *db/db* mice, from 4 independent mouse preparations per group. (I) Representative three-color image shows the maturity of vesicles, scale bar, 5 μm. (J, K) Expression of NPY-ZnT72R in mature and immature DCVs from C57 (J) and *db/db* (K) mice. n = 13 cells for both C57 and *db/db* mice. (L, M) Expression of proinsulin in NPY-ZnT72R-expressing and NPY-ZnT72R-nonexpressing vesicles from C57 (L) and *db/db* (M) mice. n = 17 cells for both C57 and *db/db* mice. (N) Representative TEM images of nontreated beta cells from C57 mice and nontreated, 50 μmol/L zinc-treated and 500 μmol/L zinc-treated beta cells from *db/db* mice. Scale bar, 1 μm. Typical normal, symmetric and round vesicles are magnified at the bottom. Scale bar, 0.2 μm. (O, P) The normalized violin diagram shows a comparison of the distribution data of the dense core electron density (O) and dense core area (P). (Q) The dense core structure patterns of diabetic progression and rescue by zinc supplementation. FI, fluorescence intensity.

vesicles with higher concentrations of free zinc had more mature insulin.

We next assessed whether zinc supplementation could ultimately restore the electron-dense core and increase insulin secretion. Exogenous zinc supplementation increased the zinc content in blood (Fig. S2A, left), pancreases (Fig. S2A, right), islets (Fig. S2B-E) and beta-cell vesicles (Fig. S2G) in the time sequence in which the level of ZnT8 mRNA was decreased in *db/db* mice compared with C57 mice (Fig. S2F). TEM imaging showed that compared with that in the diabetic (nontreated *db/db* mice) control, the dense core electron density, but not the dense core area, was increased after zinc supplementation at concentrations of 50 μmol/L overnight and 500 μmol/L for 2 hours, returning to the level observed in the normal control (C57 mice) (Fig. 1N–Q). Finally, we assessed whether the structural recovery of the dense core could enhance insulin secretion. Zinc-treated and zinc-nontreated *db/db* mice with matched initial body weights and blood glucose levels were subjected to intraperitoneal glucose tolerance tests and glucose-stimulated insulin secretion evaluations *in vivo*. The results showed that zinc supplementation alleviated glucose intolerance (Fig. S3A, C) by increasing insulin secretion both *in vivo* (Fig. S3B, D) and *in vitro* (Fig. S3E, F). These results were also repeatedly verified in high-fat diet-induced mice (Fig. S3G-L). Encouragingly, our data provide a theoretical basis for modulating the formability of dense cores via zinc supplementation *in vivo* to quickly restore structural and functional vesicles and enhance insulin secretion.

In conclusion, this study demonstrated that the concentration of free zinc, another key factor downstream of vesicular zinc transporters, determined the formability of the dense core. These results further our understanding of the vesicular mechanism of T2D development and treatment.

Author contribution

Y.W. designed the research; Y.X., M.-X.Z., Y.-Z.H., J.L. and W.-Z.Z. performed the research; Y.W., Y.X. and M.-X.Z. analyzed the data; and Y.W. wrote the manuscript.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellin.2022.100020.

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