

Molecular genetic regulation of *Slc30a8*/ZnT8 reveals a positive association with glucose tolerance.

Ryan K. Mitchell^{1#}, Ming Hu^{1#}, Pauline L. Chabosseau¹, Matthew C. Cane¹, Gargi Meur¹, Elisa A. Bellomo¹, Raffaella Carzaniga², Lucy M. Collinson², Wen-Hong Li³, David J. Hodson^{1*}, and Guy A. Rutter^{1*}

¹Section of Cell Biology and Functional Genomics, Division of Diabetes, Endocrinology and Metabolism, Imperial Centre for Translational and Experimental Medicine, Imperial College London, Du Cane Road, London W12 0NN. ²Electron Microscopy Unit, Francis Crick Institute, Lincoln's Inn Fields, London, WC2A 3LY. ³ Department of Cell Biology & Biochemistry, UT Southwestern Medical Center, Dallas, Texas, USA.

Joint First Authors

Abbreviated Title: Impact of ZnT8 modulation on glucose homeostasis

Key terms: ZnT8; diabetes; insulin

Word count: 5876

*To whom correspondence and requests for reprints should be addressed,

Professor Guy A. Rutter

ICTEM, Imperial College London

Hammersmith Hospital

Du Cane Road, London W12 0NN, U.K.

Tel +44(0)20 759 43340

g.rutter@imperial.ac.uk

or Dr David Hodson, address as above

d.hodson@imperial.ac.uk

Disclosure statement: The authors have nothing to disclose

Abstract

Zinc Transporter 8 (ZnT8), encoded by *SLC30A8*, is chiefly expressed within pancreatic islet cells where it mediates zinc (Zn^{2+}) uptake into secretory granules. Whilst a common non-synonymous polymorphism (R325W), which lowers activity, is associated with increased type 2 diabetes (T2D) risk, rare inactivating mutations in *SLC30A8* have been reported to protect against T2D. Here, we generate and characterise new mouse models to explore the impact on glucose homeostasis of graded changes in ZnT8 activity in the β cell. Firstly, *Slc30a8* was deleted highly selectively in these cells using the novel deleter strain, Ins1Cre. The resultant Ins1CreZnT8KO mice displayed significant ($p < 0.05$) impairments in glucose tolerance at 10 weeks of age *versus* littermate controls and glucose-induced increases in circulating insulin were inhibited *in vivo*. Whilst insulin release from Ins1CreZnT8KO islets was normal, Zn^{2+} release was severely impaired. Conversely, transgenic ZnT8Tg mice, over-expressing the transporter inducibly in the adult β cell using an insulin promoter-dependent Tet-On system, showed significant ($p < 0.01$) improvements in glucose tolerance compared to control animals. Glucose-induced insulin secretion from ZnT8Tg islets was severely impaired, whereas Zn^{2+} release was significantly enhanced. Our findings demonstrate that glucose homeostasis in the mouse improves as β cell ZnT8 activity increases and, remarkably, these changes track Zn^{2+} rather than insulin release *in vitro*. Activation of ZnT8 in β cells might [therefore](#) provide the basis of a novel approach to treating type 2 diabetes.

Introduction.

The regulation of insulin secretion by glucose involves the uptake and metabolism of the sugar by pancreatic β cells (1), stimulation of mitochondrial oxidative metabolism (2), Ca^{2+} influx (3) and the exocytosis of the hormone from dense core secretory granules (4,5) where it is stored in a near-crystalline form alongside Zn^{2+} and Ca^{2+} ions (6). Although it is increasingly accepted that impaired insulin secretion underlies the development of type 2 diabetes (T2D) (7), a disease affecting more than 8 % of the adult population worldwide (8), the mechanisms involved remain poorly understood (9). Nonetheless, disease risk is strongly influenced by both genetic (10) and environmental (11) factors.

A non-synonymous variant in the *SLC30A8* gene associated with elevated T2D risk was identified by genome-wide association studies (GWAS) in 2007 (12). Expressed almost exclusively in pancreatic β and α cells (13-15), *SLC30A8* encodes a secretory granule-resident zinc transporter, ZnT8, implicated in the accumulation of zinc within these organelles and thus in insulin storage (16). Given these likely roles, *SLC30A8*/ZnT8 has been mooted as a potentially tractable new target for personalised disease therapy.

Subsequent functional studies on the expressed ZnT8 protein (14,17) demonstrated that the risk (R325) variant is a less active zinc transporter than the protective (W) form. Consequently, possession of risk alleles seems likely to impair insulin crystallisation and storage. Supporting this view, mice inactivated globally (14,18) or selectively in the β cell (15,19) for *Slc30a8* revealed striking abnormalities in the formation of dense cores within insulin granules. Surprisingly, however, measurements of insulin release from isolated islets from *Slc30a8* null mice revealed either no change (18) or improved (14,19) glucose-stimulated insulin secretion from isolated islets or the perfused pancreas, and unchanged insulin content. Despite this, glucose homeostasis and circulating insulin levels were both lowered in ZnT8 null animals. Providing a possible explanation for this conundrum, Tamaki and colleagues (19) demonstrated that the enhanced release of Zn^{2+} ions alongside insulin in W-variant carriers suppresses insulin clearance (and presumably non-productive insulin signalling) by the liver, favouring insulin action on this, as well as other tissues (notably adipocytes and skeletal muscle). An observed increase in C-peptide:insulin ratio in human R-carriers supported this model, since the mature hormone, but not proinsulin, is expected to be cleared by the liver. Moreover, *Slc30a8* elimination from the mouse has no effect on insulin

processing (14,18), arguing against a β cell-autonomous action of the variant on the release of mature *versus* partially processed forms. Together, the above findings have stimulated the search for activators of the transporter which, by favouring Zn^{2+} accumulation by β cell secretory granules, may eventually prove useful in the clinic.

However, and challenging the above view, a recent study based largely on Swedish, Finnish and other Northern European populations, but also including individuals from elsewhere, identified rare ($< 0.1\%$ of the population) nonsense (truncating) or mis-sense mutations in the *SLC30A8* gene. Unexpectedly, the carrier population showed a ~ 3 -fold enrichment for healthy individuals *versus* those with T2D, implying a *protective* role for the mutant transporter. Although only a small number of carriers was involved (345 in total of $\sim 150,000$ subjects sequenced) a range of structurally distinct variants was found in cohorts with differing ancestry, providing evidence that the *SLC30A8* mutations, rather than other polymorphisms in the same linkage disequilibrium (LD) block, were likely to explain the changes in disease risk.

The above findings are nonetheless difficult to reconcile with the observed increase in T2D risk in carriers of the common risk alleles. Although an activating effect of the identified mutants on the remaining allele cannot be excluded absolutely, an alternative explanation (20) is that a complex interplay between insulin storage and Zn^{2+} release by β cells, and downstream effects on target tissues including the liver, results in a bimodal (bell-shaped) dependence of T2D risk on ZnT8 activity. Thus, modest decreases in β cell ZnT8 activity, as observed in carriers of the common risk (R) variants, may act chiefly by lowering β cell Zn^{2+} secretion, thus enhancing insulin clearance by the liver. On the other hand, a more substantial lowering of ZnT8 activity, engendered by rare loss-of-function alleles, may lead to a more dramatic increase in insulin release from the pancreas, an effect outweighing impaired Zn^{2+} release and altered insulin clearance.

The impact of deleting ZnT8 from the β cell in mice has also been the subject of some debate. Thus, one recent study (21) reported that global knockout on a pure C57BL6 background exerted no effects on glucose tolerance, in contrast to findings on more mixed backgrounds (14,18). Moreover, several previously-reported β cell-selective deletion models are complicated by deletion in other tissues, including the brain, when *Cre* deleter strains (notably

RIP2Cre and Pdx1), with activity in these tissues (22), are used. Correspondingly, RIP2Cre:ZnT8 mice gain more weight *versus* controls on a high fat diet than observed with globally deleted animals (23). The latter findings argue for a role for ZnT8 in a small number of neuronal cells in which the *Pdx1* or *Ins2* promoter may be at least transiently active during development or at later stages. On the other hand, the mouse insulin promoter 1 *Cre* (MIP Cre) used in (19) may also be affected by the co-expression of growth hormone encoded by the cDNA included in this transgene (24).

Our first goal here was therefore to explore the impact of deleting ZnT8 more specifically in the β cell, and on a pure C57BL6 background, using a new deleter strain in which the *Ins1* promoter, which is inactive in brain and other tissues (22) drives expression of Cre after introduction into the endogenous locus (“knock-in”) (25,26). Importantly, *Ins1*Cre mice do not express the growth hormone (GH) minigene, unlike both RIP2Cre (24) and MIPCre mice (27), and mice bearing the transgene alone display no abnormalities in glucose tolerance (22) ([G.R. unpublished results](#)).

Up to now, there have been no attempts to examine the effect of *over*-expressing ZnT8 selectively in the β cell, thus mimicking one of the likely actions of agents capable of stimulating the activity of the transporter. Our second goal here was therefore to generate a series of transgenic mouse lines in which ZnT8 expression is under the control of rat insulin promoter Tet-On system (28).

We demonstrate that highly selective deletion of ZnT8 in the β cell leads to dense core granule mis-formation and glucose intolerance. By contrast, overexpression of the transporter in the β cell in adults leads to improved glucose tolerance but reduced insulin secretion, whilst Zn^{2+} release is markedly enhanced. A positive relationship thus pertains between β cell ZnT8 expression (and Zn^{2+} secretion), and glucose tolerance. If reflective of human physiology, these results lend weight to the view that ZnT8 activation might prove beneficial in the context of T2D.

Results.

Impaired glucose tolerance and insulin secretion in Ins1Cre:ZnT8^{fl/fl} mice.

β cell-selective deletion of ZnT8 with a variety of Cre deleter strains (eg. RIP2 (15) and MIP (19)) display varying degrees of recombination at extrapancreatic sites, due to ectopic expression of Cre. By contrast, Ins1Cre knockin mice display no detectable expression of the recombinase in the brain, only very minor recombination in other islet cells (<3% of α cells *in utero*), but > 94% recombination in β -cells (25,29). We therefore used this model to inactivate ZnT8 selectively in β cells (Fig. 1A). Confirming efficient deletion of the endogenous ZnT8 alleles in the β -cell with Ins1Cre, islets from Ins1Cre^{+/-}:ZnT8^{fl/fl} (Cre⁺) mice showed >80 % reduction in ZnT8 mRNA levels (****p<0.001; two way ANOVA; Cre⁻ vs. Cre⁺; n= 3 & 4 respectively) compared to litter mate controls (Ins1Cre^{-/-}:ZnT8^{fl/fl}; Cre⁻), with no changes in the expression of other ZnT family members (Fig. 1B). Loss of ZnT8 immunoreactivity was seen specifically in the β , and not the α cell compartment, as demonstrated using immunohistochemical analysis of isolated islets, staining for insulin and glucagon respectively (Fig. 1C) and counting the number of ZnT8 positive cells colocalised with insulin and glucagon (Fig. 1D). A decrease in overall immunoreactivity of > 90 % for monomeric ZnT8 was shown using Western (immuno-) blotting analysis (Fig. 1E) compatible with an islet composition of ~70-80 % β cells (30) and levels of ZnT8 expression in α cells about 50% of those in β cells (Fig. 1C and (31)) .

Maintained on a regular chow diet, male Ins1Cre^{+/-}:ZnT8^{fl/fl} mice displayed normal glucose tolerance at 6 weeks of age (Fig. 2A; ns; repeated measures two-way ANOVA; n=8 Cre⁻ and 14 Cre⁺ respectively) but impaired responses to the sugar by 10 weeks (Fig 2B; 11.5 $\pm$ 0.59 mmol/L vs. 13.6 $\pm$ 0.74 mmol/L; Cre⁻ vs. Cre⁺; p<0.05; 30 min. time point; repeated measures two-way ANOVA; n= 8 and 11 respectively). These changes gradually resolved with age (Fig 2C; ns; repeated measures two-way ANOVA; n = 7 Cre⁻ and 10 Cre⁺). Female KO mice showed no evident abnormalities at either age (Supp. Fig 1A-F). Ins1Cre^{+/-}:ZnT8^{fl/fl} (aged 10 weeks) showed significantly higher glucose responses (Fig 2D, p<0.001 15 and 30 min. time point, repeated measures two-way ANOVA, n=9 Cre⁻ & 12 Cre⁺) but lower insulin responses (Fig 2E; 0.70 $\pm$ 0.073 vs 0.49 $\pm$ 0.072; Cre⁻ vs. Cre⁺; p<0.05; 30 min time point; repeated measures two-way ANOVA; n=14 Cre⁻ & 13 Cre⁺) in response to a 3g/Kg body weight

glucose injection, consistent with impaired insulin secretion or enhanced clearance of the hormone. Insulin sensitivity measured using an insulin tolerance test was unchanged in both male (Fig 2F) and female (Supp. Fig. 2) $Ins1Cre^{+/-}:ZnT8^{fl/fl}$ mice, as assessed at 10 or 8 weeks, respectively. These findings are in-line with those in global (14) or mouse insulin 1-promoter deleted animals (19).

Unchanged glucose-, incretin- and KCl-stimulated insulin secretion but altered Zn^{2+} dynamics in isolated $Ins1Cre^{+/-}:ZnT8^{fl/fl}$ islets

Islets isolated from 10 week old male $Ins1Cre^{+/-}:ZnT8^{fl/fl}$ mice showed no change with respect to control islets in *in vitro* insulin secretion in response to 16.7.mmol/L glucose, incretin or depolarisation induced with KCl (Fig 3A; ns; two-way ANOVA; $n=12-16$ replicates per genotype). Secretion in response to lower (8 mmol/L) glucose concentrations was also unchanged (Supp. Fig 3A) and, similarly, glucose-stimulated insulin release was not different between null and wild-type islets assayed during perfusion at 16.7 mmol/L glucose (Supp. Fig. 3B). Likewise, deletion of ZnT8 did not affect the amplitude (0.50 ± 0.05 vs. 0.47 ± 0.06 ; Cre^{-} vs. Cre^{+} ; ns; Student's t-test; $n=17$ & 9 islets respectively) or AUC (1132 ± 19.7 vs. 1107 ± 24.2 ; Cre^{-} vs. Cre^{+} ; ns; Student's t-test; $n=17$ & 9 islets respectively) of glucose (Fig. 3B) or KCl-stimulated intracellular free Ca^{2+} increases (Fig 3C; Amplitude 1.38 ± 0.11 vs. 1.23 ± 0.15 ; AUC 443 ± 9.71 vs. 450 ± 17.3 ; Cre^{-} vs. Cre^{+} ; ns; Student's t-test; $n=5$ & 13 islets respectively). Finally, β cell- β cell connectivity (32), known to contribute to the regulation of insulin release from intact islets, was unaltered in ZnT8 null islets (Fig. 3D,E).

We next used the recombinant Förster resonance energy transfer (FRET)-based probe eCALWY4 (33,34) to measure cytosolic Zn^{2+} concentrations. Consistent with findings in global ZnT8 null mice (35), $Ins1Cre^{+/-}:ZnT8^{fl/fl}$ β -cells showed a significant reduction in free cytosolic Zn^{2+} concentration (920.3 ± 261 pM vs. 212.8 ± 32.5 pM, $**p<0.01$, Cre^{-} vs. Cre^{+} , Student's t-test, $n=20$ & 11 islets, respectively) (Fig. 4A-C). Furthermore, use of the cell surface-targeted Zn^{2+} binding probe ZIMIR (36), to measure Zn^{2+} co-secreted from insulin granules, demonstrated that $Ins1Cre^{+/-}:ZnT8^{fl/fl}$ islets secreted substantially less Zn^{2+} compared to control islets after stimulation with glucose (Fig. 4D).

$Ins1Cre^{+/-}:ZnT8^{fl/fl}$ mice show altered insulin granule morphology but preserved β cell mass.

The above metabolic and cellular disturbances were accompanied by a drastic change in secretory granule morphology (Fig. 5A), with a near-complete loss of dense core granules in islets from $\text{Ins1Cre}^{+/-}:\text{ZnT8}^{\text{fl/fl}}$ mice, and the emergence of “atypical” granules possessing abnormal “rod-like” ($0.65 \pm 0.28\%$ vs $31.9 \pm 3.42\%$, Cre^- vs Cre^+ , $p < 0.0001$, $n=8$ β -cells/genotype) or “empty” ($36.3 \pm 2.48\%$ vs $85.6 \pm 1.37\%$, Cre^- vs Cre^+ , $p < 0.0001$, $n=8$ β -cells/genotype) cores (Fig. 5B). The total number of granules was unchanged (Fig. 5C; 129 ± 9.25 vs. 128 ± 11.9 ; Cre^- vs. Cre^+ ; ns; Student’s t-test; $n=8$ β -cells/genotype), but average granule diameter was increased (Fig. 5D, 377.47 ± 5.12 nm vs 328.09 ± 5.46 nm, Cre^+ vs Cre^- , $p < 0.001$, $n=204/226$ granules), likely reflecting increased osmotic stress resulting from free electrolytes in the ZnT8 null granule (18). Staining pancreatic slices for insulin and glucagon (Fig. 5E) showed no changes in total β cell mass (Fig. 5F; $0.513 \pm 0.07\%$ vs. $0.334 \pm 0.17\%$; Cre^- vs. Cre^+ ; ns; Student’s t-test; $n=3$ animals per genotype), α cell mass (Fig 5G; $0.055 \pm 0.015\%$ vs. $0.051 \pm 0.019\%$ Cre^- vs. Cre^+ ; ns; Student’s t-test; $n=3$ animals per genotype) or $\alpha:\beta$ cell ratio (Fig. 5H; 0.151 ± 0.011 vs 0.206 ± 0.025 ; Cre^- vs. Cre^+ ; ns; Student’s t-test; $n=3$ animals per genotype). These data are in line with previous findings using alternative deleter strains to eliminate ZnT8 from the β cell (14,18,37).

The above studies thus demonstrate that deleting ZnT8 selectively in the β cell leads to normal insulin, but abnormal Zn^{2+} secretion, *in vitro*, but markedly lower post-stimulation insulin levels and glucose tolerance *in vivo*.

Improved glucose tolerance in ZnT8 transgenic mice.

To explore the impact of increasing ZnT8 activity in β cells we next generated transgenic mice in which the expression of the transporter was under the control of a bi-directional tetracycline-inducible promoter (38). β cell-selective induction was then achieved by activation of a Tet-On transactivator expressed selectively in β cells under the control of the rat insulin 2 promoter (RIP7-rtTA) (Fig. 6A). Whilst 7 founders were produced, we selected two (#31, #23; copy numbers 5 and 13, respectively) for further analysis ([Supp. Fig 4](#)). Treatment with doxycycline of transgenic animals derived from founder #31 (5 copies) resulted in an approximate 4-fold induction of human *ZnT8* mRNA expression (Fig. 6B; 4.40 ± 0.08 vs. 16.9 ± 0.40 ; ZnT8 Tg- vs. ZnT8 Tg+; $p < 0.0001$; Student’s t-test; $n=3$ per genotype). This was accompanied by both an increase in luciferase mRNA expression (Fig. 6C; 4.78 ± 0.06 vs. 11.7 ± 0.25 ; ZnT8 Tg- vs. ZnT8 Tg+; $p < 0.0001$; Student’s t-test; $n=3$ each

genotype) and activity in isolated islets (Fig. 6D; 68.33 ± 2.85 vs. 1362 ± 86.9 ; ZnT8 Tg⁻ vs. ZnT8 Tg⁺; $p < 0.0001$; Student's t-test; $n=3$ each genotype). The expression of human ZnT8 protein was also apparent by Western blotting using an antibody specific for the human protein (Fig. 6E).

Females from founder #31 displayed significant improvements in glucose tolerance at both 10- (Fig 7A, 12.2 ± 0.51 mmol/L vs 13.9 ± 1.1 mmol/L; ZnT8 Tg⁺ vs. ZnT8 Tg⁻; $p < 0.05$, 15 min time point; repeated measures two-way ANOVA; $n=6$ and 7 respectively) and 14 weeks of age (Fig. 7B, 8.84 ± 0.59 mmol/L vs 11.7 ± 1.01 mmol/L; ZnT8 Tg⁺ vs. ZnT8 Tg⁻; $p < 0.001$, 30 min. time point, repeated measures two-way ANOVA, $*p < 0.05$, AUC, Student's t-test, $n=8$ and 5 respectively), whereas changes were not apparent in males (Supp. Fig. 5). Insulin sensitivity was unchanged in 10 week old female ZnT8 transgene positive animals compared to wild-type littermates (Fig 7C; ns, repeated measures two-way ANOVA; $n=4$ ZnT8 Tg⁻ and 5 ZnT8 Tg⁺). Measured *in vivo* after intraperitoneal injection of 3g/kg glucose, insulin secretion was significantly enhanced by almost 2-fold compared to wild-type littermates, despite a tendency towards lowered blood glucose levels (Fig. 7D; $*p < 0.05$; 15 & 30 min time point; repeated measures two way ANOVA; $n=6$ ZnT8 Tg⁻ & 8 ZnT8 Tg⁺ animals).

Both male and female mice derived from founder #23 (copy number 13) displayed no apparent changes in glucose tolerance at either 10 or 14 weeks of age (Supp. Fig. 6A-D), consistent with the significantly lower levels of over-expression of the transgene and co-expressed reporter gene (luciferase) in this line versus line #31 (Supp. Fig. 4).

ZnT8 Tg⁺ islets secrete less insulin but more Zn^{2+} in response to glucose.

Assayed *in vitro*, insulin secretion from isolated islets derived from 10-14 week old female mice in response to high glucose (16.7 mM) was significantly reduced (Fig. 8A; 0.94 ± 0.21 ng/mL vs. 0.40 ± 0.05 ng/mL, ZnT8 Tg⁻ vs ZnT8 Tg⁺ respectively; $p < 0.05$; two-way ANOVA, $n=10-13$ replicates). No differences were apparent in the response of transgenic islets to stimulation with incretin or depolarisation with KCl (Fig. 8A).

Demonstrating enhanced glucose-stimulated Zn^{2+} secretion from transgenic mouse islets, both the amplitude (0.053 ± 0.002 vs. 0.791 ± 0.05 ; ZnT8 Tg⁻ vs ZnT8 Tg⁺ respectively; $p < 0.001$; two-way ANOVA, $n=11$ & 14 respectively) and AUC (496 ± 1.72 vs. 505 ± 2.11 ; ZnT8 Tg⁻ vs ZnT8 Tg⁺ respectively; $p < 0.01$; $n=11$ & 14 respectively) of glucose-evoked ZIMIR

responses (Fig. 8B) were increased by ZnT8 overexpression. Interestingly, there were no changes in cytosolic Zn^{2+} concentrations (Fig. 8C; 1079 ± 176 pM vs. 1020 ± 127 ; ZnT8 Tg⁻ vs. ZnT8 Tg⁺; ns; Student's t-test; $n= 46$ and 61 cells respectively from 2-6 animals per genotype).

Staining pancreatic slices for insulin and glucagon (Fig 8D) revealed no changes in β or α cell mass nor the ratio of β to α cells (Fig 8E-G).

Discussion

In this report, we describe new mouse models for ZnT8 which provide insights into the pathogenic mechanisms likely to be involved in the actions of human alleles associated with increased T2D risk.

Ins1CreZnT8 KO mice showed dramatic changes in secretory granule morphology and plasma insulin level under glucose stimulation, similar to findings previously reported in global ZnT8 KO mice (14), in mice with conditional ZnT8 alleles deleted with the more promiscuous RIP2 Cre (15), or with MIPCre which also expresses GH (24). The present results thus confirm that such morphological changes are likely to be a β cell-autonomous event, and to reflect impaired Zn^{2+} uptake into dense core granules in the absence of ZnT8.

Despite the exaggerated glucose excursions and smaller plasma insulin increases observed in response to intraperitoneal injection of the sugar in these animals (Fig. 2B & E, respectively), islets derived from Ins1CreZnT8KO mice displayed unaltered glucose-stimulated insulin secretion *in vitro*. By contrast, glucose-induced Zn^{2+} release from these islets was reduced by > 80%, in line with earlier results with global ZnT8 KO mice (36), presumably reflecting impaired Zn^{2+} accumulation by secretory granules. These findings reinforce the recent proposal (19,35) that impaired β -cell Zn^{2+} secretion and de-inhibition of insulin receptor endocytosis leads to exaggerated clearance of mature insulin by the liver.

We extend support for the above view by showing that glucose tolerance is *improved* in a new model in which ZnT8 is selectively over-expressed in the β cell. Remarkably, insulin secretion from islets isolated from these mice was barely stimulated by glucose, whereas Zn^{2+} release was increased by >50%. Nonetheless, fasting insulin levels tended to be increased in ZnT8Tg animals (Fig. 7D) and these levels were further strongly increased by intraperitoneal glucose injection (Fig. 7D). Thus, in ZnT8Tg animals, elevated Zn^{2+} secretion may act both to impair insulin clearance through the internalisation of insulin receptors (19), and possibly also to enhance insulin signalling. At the molecular level, possible actions of the released Zn^{2+} included inhibition of insulin receptor dephosphorylation by protein tyrosine phosphatase B1 (PTPB1) (39), or of phosphatidylinositol (3,4,5) phosphate (PIP₃) degradation by phosphatase and tensin homologue on chromosome ten (PTEN) (40).

These studies show that, by manipulating *Slc30a8* expression selectively in the mouse β cell using molecular genetics, a near-linear relationship exists in this species between ZnT8 levels

and glucose tolerance (Fig. 9A). Note that the study of mice deleted for just one conditional *Slc30a8* allele was not feasible with the breeding strategy used here though, in earlier studies with global *Slc30a8* null mice (14), we noted that heterozygous ($\text{ZnT8}^{+/-}$) mice displayed intermediate glucose tolerance between wild-type and homozygous null animals, consistent with the current findings. Importantly, the changes in peak glucose observed in the present study were best correlated to Zn^{2+} release from the islet (Fig. 9B): this was essentially eliminated by *Slc308* deletion (Fig. 4D) but enhanced when the transporter was over-expressed (Fig. 8B). By contrast, insulin release *in vitro* was inversely correlated with ZnT8 expression (Fig. 8C).

Interestingly, we observed differences between the impact of *Slc308* deletion on male and female mice, with only males showing defective glucose tolerance over the age range examined. In contrast, both male and female mice deleted globally for the transporter (31) displayed glucose intolerance at six weeks of age, whereas only males were intolerant at 12 weeks. The reasons for the differences between the impact of *Slc308* deletion between sexes is presently unknown, but may in part reflect the intrinsically greater inter-measurement variability in females resulting from the reproductive cycle, and/or the lower insulin sensitivity of male animals which imposes a greater metabolic stress on the β -cell. Surprisingly, this position was reversed in transgenic animals with the greater penetrance of ZnT8 over-expression observed in females. In this case, the underlying mechanisms are less clear but might reflect sex-specific differences in the handling of enhanced Zn^{2+} loads by the liver or other target tissues (41).

Whether control of hepatic insulin clearance and/or action via Zn^{2+} assumes the same importance in man, where the much larger diameter and volume of the portal vein may mean greater dilution of Zn^{2+} after release from the β cell - and hence lowered action on the liver - is unclear. Nonetheless, and arguing for this possibility, carriers of risk (R) *SLC30A8* alleles show lowered C-peptide:insulin levels, consistent with the more efficient uptake of the latter by hepatocytes when Zn^{2+} levels are lowered (42).

The present approach further demonstrates the feasibility of using mouse genetics to explore the mechanisms through which T2D risk genes, identified in GWAS studies (12), act. Intriguingly, we provide additional evidence that the actions of *SLC30A8* involve interactions between multiple tissues (β cells and liver), despite the tight restriction of the expression of this gene to the endocrine pancreas (13). Whether *SLC30A8* variants also influence the release

of glucagon may require further investigation: global inactivation of the gene exerted little effect on glucagon release from islets, though detailed *in vivo* analysis involving hypoglycemic clamps were not reported in these studies (15). Fadista et al (43) recently reported a strong positive correlation between glucagon and *SLC30A8* expression in human islets, consistent with a role for *SLC30A8* variants in controlling glucagon production.

In the light of the present results, the possibility that other GWAS genes expressed in multiple tissues, e.g. *TCF7L2* (44,45), might act via extrapancreatic sites to regulate insulin secretion, would seem worthy of careful investigation. Of note, *TCF7L2* is an upstream regulator of the mouse *Slc30a8* (46) and human *SLC30A* (47) genes, and as a “master” regulator of T2D susceptibility (47), might act in part via ZnT8 to modify β cell Zn^{2+} release and insulin clearance.

Materials and Methods

Ethical approval

All animal procedures were approved by the home office according to the Animals (Scientific Procedures) Act 1986 of the United Kingdom (PPL 70/7349).

Generation of β -cell selective knockout mice by Ins1Cre-driven recombination

ZnT8 floxed mice ($\text{ZnT8}^{\text{fl/fl}}$) were generated by GenOway, Lyon, France (15). This involved the insertion of a LoxP site together with a flippase recognition target flanked neomycin selection cassette within intron 1 and a single distal LoxP site within the upstream exon 1 containing the translational start codon. $\text{ZnT8}^{\text{fl/fl}}$ animals were then bred with the Ins1Cre deleter strain, to produce 50% β -cell specific knockout animals ($\text{Ins1Cre}^{+/-}\text{ZnT8}^{\text{fl/fl}}$) and 50% littermate controls ($\text{Ins1Cre}^{-/-}\text{ZnT8}^{\text{fl/fl}}$). Note that, in contrast to RIP2Cre (24) and MIP2Cre (ADA), Ins1Cre mice do not express a GH cassette and the transgene alone does not affect glucose tolerance (25,29). Animals were maintained in a pathogen-free facility under a 12 h light/12 h dark cycle with free access to water and food.

Generation of β -cell specific transgenic mice

Plasmid pCDNA3, containing the human *ZnT8* (W325 form) coding sequence with the addition of a single COOH-terminal c-Myc epitope tag (14), was digested with *Xho*I, blunt-

end filled and further digested with *NotI*. The digested *hZnT8-Myc* DNA fragment was gel purified and cloned into plasmid pBI-L Tet (Clontech) between *NotI* and *PvuII* sites. This generated a plasmid with a bidirectional tetracycline-regulated promoter driving expression of both *hZnT8-Myc* and firefly luciferase. The positive clone was further confirmed by DNA sequencing using a pBI-L internal primer GAAAGAACAATCAAGGGTCC and a *hZnT8* primer ACACTAGCACGCCAGTCACC.

The expression cassette was excised from the plasmid backbone by *AatII* and *AseI* digestion and transferred by pronuclear microinjection into C57Bl/6 mouse oocytes (MRC Clinical Sciences Centre transgenic facility, Hammersmith Hospital, Imperial College London). Successful integration was identified by PCR screening of DNA extracted from ear biopsies by the HotSHOT method (48) using two sets of primers: 1. *hZnT8* gene, forward: CTGTCATCGAAGCCTCCCTC and reverse: AAGGGCATGCACAAAAGCAG; 2. Luciferase gene, forward: CATTAAAACCGGGAGGTAGATGA and reverse: CATGGATTCTAAAACGGATTACCA. The relative transgene copy number was determined by SYBR green quantitative PCR (qPCR) method (life Technology) using a set of luciferase gene primers: forward CAACTGCATAAGGCTATGAAGAGA and reverse ATTTGTATTTCAGCCCATATCGTTT and, as an internal control, a set of mouse *Cxcl12* gene primers: forward GGACGAGCTCCACTTAGACG and reverse: CAACATGTCCAGATC GAAATC. Two founders were crossed twice with C57Bl/6 mice to generate the *hZnT8-Luc* strain. *RIP7-rtTA* mice on a C57Bl/6 background (28), expressing the reverse tetracycline transactivator under the control of the rat insulin promoter, were crossed with *hZnT8-Luc* mice to permit β -cell-specific, tetracycline-inducible expression of *hZnT8-Myc* and luciferase. *hZnT8-Luc* mice were crossed with homozygous *RIP7-rtTA* mice to produce littermates of two genotypes as follows: *hZnT8-Luc*⁺/*RIP7-rtTA*⁺ (ZnT8 Tg⁺) and *hZnT8-Luc*⁻/*RIP7-rtTA*⁺ (ZnT8 Tg⁻). All offspring were genotyped for both the *hZnT8* and *RIP7-rtTA* genes (49). Mice were treated with 0.5 g/L doxycycline from 5 weeks of age.

Islet Isolation

Mice were euthanised by cervical dislocation and pancreatic islets isolated by collagenase digestion as previously described (50). Given the sex- and age-dependent differences between mouse lines, islets used for ex vivo analysis were obtained from mice of the appropriate sex and, importantly, at an age where an in vivo phenotype was apparent i.e. 10 week old male *Ins1Cre*^{+/-}::*ZnT8*^{fl/fl} mice and 10 – 14 week old female *Rip7rTta*^{+/-}::*ZnT8Tg*^{+/-} mice.

Quantitative real-time PCR

Total islet RNA was extracted using Trizol reagent (Invitrogen, Paisley). After reverse transcription, relative expression was assessed using SYBR Green (Invitrogen, Paisley). Primers were designed using PerlPrimer and gene expression was normalised to β -actin (*Actb*).

Immunofluorescence

Isolated islets were fixed overnight at 4°C in 4% paraformaldehyde (vol./vol.) before the addition of primary antibodies against murine ZnT8 (Mellitech, 1:200, raised in rabbit) insulin (DAKO, 1:200, raised in guinea pig) and glucagon (Sigma Aldrich, 1:1000 raised in mouse). Detection was performed using goat anti-rabbit Alexa-Fluor 488, goat anti guinea-pig Alexa-Fluor 568 and goat anti-mouse 568 (all Invitrogen, 1:500). Islets were mounted on Superfrost slides (Fisher Scientific) using Vectashield DAPI-containing hardset mounting medium (Vector Labs). Data capture was performed using a Zeiss LSM780 confocal microscope equipped with GaAsP spectral detectors and a 64x/1.4NA oil-immersion objective. The proportion of ZnT8-immunopositive α and β cells was quantified according to co-localisation of ZnT8 with either insulin or glucagon, above a background threshold (i.e. twice the signal-to-noise ratio). In all cases, uniform linear adjustments were applied to contrast/brightness to improve image quality for presentation, while preserving the pixel dynamic range. Background fluorescence after insulin staining is likely to correspond to autofluorescence and was left uncorrected to preserve image integrity for comparisons. Likewise, stippled background fluorescence after glucagon staining can be attributed to non-specific staining apparent when the ZnT8 antibody was used in the presence of the anti-glucagon antibody.

Histology and Immunohistochemistry.

Mouse pancreata were extracted and fixed in 10% neutral balanced formalin (Sigma, Dorset, U.K.) at 4°C for 18 h before dehydration and wax embedding and processing to obtain 5- μ m slices (Carleton's Histological Technique, Drury and Wallington). Sections were labelled with anti-insulin (1:200 dilution, Secondary-Alexa Fluor 488 1:1000) and anti-glucagon (1:100 dilution, secondary-Alexa Fluor 568 1:500) and sealed using Vector Shield Antifade Hard Set reagent (Vector Laboratories). β cell mass was determined as described (51). Data capture was performed using a Zeiss AxioObserver and a 40x/0.75NA objective. β/α cell mass was calculated using the threshold plugin for ImageJ (NIH), as previously detailed (52).

Intraperitoneal Glucose and Insulin Tolerance Tests

Glucose (1 g /Kg body weight) was injected into the abdomen of mice that had been fasted overnight. Blood glucose measurements were taken at 0, 15, 30, 60, 90 and 120 min. using an automatic glucometer (Accucheck). Insulin tolerance tests are performed as per glucose tolerance test but animals were fasted for 5 h prior to 0.75 U insulin/Kg bodyweight insulin injection.

Plasma Insulin Measurements

Mice fasted overnight were injected with glucose (3 g glucose/Kg body weight) and blood from the tail vein was collected into heparin coated tubes (Sarstedt, Beaumont Leys, UK) at 0, 15 and 30 min. Plasma was separated by centrifugation at 2000g for 10 min, 5 μ L of blood plasma was used to measure insulin levels using an ultrasensitive mouse insulin ELISA kit (CrystalChem, IL, USA).

Insulin Secretion Assay

Five size matched islets were pre incubated for 1 h at 37 °C in a Krebs-HEPES-bicarbonate (KHB) buffer (130 mM NaCl, 3.6 mM KCl, 1.5 mM CaCl_2 , 0.5 mM MgSO_4 , 0.5 mM NaH_2PO_4 , 2 mM NaHCO_3 , 10 mM HEPES, and 0.1 % [wt/vol] BSA, pH 7.4) containing 3 mmol/L glucose with gentle shaking (120 rev/min). Islets were further incubated for 30 min at 37 °C in either: 3 mmol/L glucose, 8 mmol/L glucose, 16.7 mmol/L glucose, 16.7 mmol/L glucose plus 20 nmol/L GLP1 or 3 mmol/L glucose plus 30 mmol/L KCl, before collection of supernatant fractions for insulin secretion analysis. Total insulin was collected by lysing islets in 500 μ L acidified ethanol solution (1.5% [vol./vol.] HCl, 75% [vol./vol.] ethanol, 0.1% [vol./vol.] Triton X-100) followed by sonication. Secreted and total insulin was measured using HTRF assay kit (Cisbio, MA, USA). Insulin release during perfusion was monitored using a custom-built device. Experiments were performed in triplicate and 50 islets were perfused at a rate of 500 μ L min^{-1} at 37 °C.

Intracellular free Ca^{2+} ($[\text{Ca}^{2+}]_i$) imaging & connectivity analysis

Isolated islets were incubated (37°C, 95% O_2 /5% CO_2) for 1 h in fluo2-AM (10 μ M; Teflabs, Austin, USA) diluted in a HEPES-bicarbonate buffer solution (120 mM NaCl, 4.8 mM KCl, 1.25 mM NaH_2PO_4 , 24 mM NaHCO_3 , 2.5 mM CaCl_2 , 1.2 mM MgCl_2 , 10 mM HEPES and 3 mM D-glucose; all Sigma). Functional multicellular Ca^{2+} imaging was achieved using a

Nipkow spinning disk head allowing rapid scanning of islet areas for long periods of time with minimal phototoxicity. A solid-state laser (CrystaLaser) controlled by a laser-merge module (Spectral Applied Physics) provided wavelengths of 491 nm to excite fluo-2 (rate = 0.5Hz; exposure time = 600 ms). Emitted light was filtered at 525/50 nm, and images captured by a highly sensitive 16-bit, 512 × 512 pixel back-illuminated EM-CCD camera (ImageEM 9100-13; Hamamatsu). Volocity software (PerkinElmer) provided the user interface. During recordings, islets were maintained at 35°C to 36°C and continuously irrigated with bicarbonate buffer aerated with 95% O₂/5% CO₂. Connectivity analysis was performed as previously described (32).

ZIMIR imaging

ZIMIR imaging was performed as previously described (36). Briefly, isolated islets were incubated (37°C, 95% O₂/5% CO₂) in ZIMIR (1 μM) for 30 min and imaged in bicarbonate buffer solution supplemented with 1 μM EDTA to improve the signal-to-noise ratio. ZIMIR was excited at 491 nm and emitted signals captured at 525 nm. After acquisition, islets were divided into sub-regions before extraction of intensity over time to allow analysis of amplitude and area under the curve (AUC) of glucose-stimulated ZIMIR responses.

Cytosolic free Zn²⁺ measurements

Zn²⁺ measurements were acquired as previously described (32). Briefly, islets were dispersed onto coverslips before infection with adenovirus containing the FRET-based Zn²⁺ sensor eCALWY4. Steady-state fluorescence intensity ratio citrine/cerulean (R) was measured, then maximum and minimum ratios were determined to calculate free Zn²⁺ concentration using the following formula: $[Zn^{2+}] = K_d(R_{max} - R) / (R - R_{min})$. The maximum ratio (R_{max}) was obtained upon intracellular zinc chelation with 50 μM TPEN and the minimum ratio (R_{min}) was obtained upon Zn²⁺ saturation with 100 μM ZnCl₂ in the presence of the Zn²⁺ ionophore, pyrithione (5 μM).

Protein Extraction and Western (Immuno-) blotting Analysis

For protein analysis, roughly 100 islets were washed twice in ice-cold PBS and lysed in ice-cold radioimmuno-precipitation assay (RIPA) buffer [50 mM Tris HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS]. Protein was assayed with a BCA kit (Pierce). Total protein extracts (30 μg) were resolved by SDS-PAGE (8% v/v acrylamide) and transferred to PVDF membranes, followed by immunoblotting with either rabbit polyclonal anti-mouse or anti-human ZnT8 (both 1:200, Millitech clone R/PZ8) and mouse monoclonal anti-tubulin

(1:5000, Sigma clone B-5-1-2) antibodies. Secondary HRP-linked anti-rabbit or anti-mouse antibodies (1:3000, GE Healthcare) were revealed by using ECL detection reagent (GE Healthcare).

Transmission Electron Microscopy

Isolated islets were fixed in Vincenzo's fixative (2 % PFA, 2.5 % glutaraldehyde, 3 mM CaCl_2 , 0.1 M sodium cacodylate buffer (pH 7.4)) for 20 min at 37°C initially followed by a further 2 h at room temperature and finally overnight at 4°C. Electron microscopy was performed as previously described (53).

Statistical analysis.

Values represented are the mean $\pm$ SEM. Statistical significance was assessed using either Student's t-test or the Mann-Whitney U test depending on data distributions. Two-way ANOVA (with Bonferroni or Sidak multiple comparison test) was used to examine the effect of multiple variables. Statistical analyses were performed using Graph Pad Prism 6.0, ImageJ and IgorPro.

Acknowledgements.

Funded by grants to GAR from the Wellcome Trust (Programme 081958/Z/07/Z; Senior Investigator Award WT098424AIA), the Medical Research Council (Programme MR/J0003042/1), European Foundation for the Study of Diabetes (EFSD), Diabetes UK (BDA 11/0004210) and the Royal Society (Wolfson Research Merit Award). DJH was supported by Diabetes UK R.D. Lawrence (12/0004431) [and EFSD/Novo Nordisk Rising Star Fellowships, and an MRC Project Grant \(MR/N00275X/1\) with GAR.](#) WHL was supported by an award from the NIH (R01 GM077593). LC and RC would like to acknowledge funding from Cancer Research UK, and from the MRC, BBSRC and EPSRC under grant award MR/K01580X/1 to LC and Peter O'Toole (York University). The work leading to this publication also received support from the Innovative Medicines Initiative Joint Undertaking under grant agreement n° 155005 (IMIDIA), resources of which are composed of a financial contribution from the European Union's Seventh Framework Programme (FP7/2007-2013) and EFPIA companies' in kind contribution (GAR). We thank J. Ferrer (Imperial College London, U.K.) and Bernard Thorens (University of Lausanne, Switzerland) for providing *Ins1Cre* mice and Gerhard Christofori (University of Basel, Basel, Switzerland) for supplying *RIP7-rtTA* mice.

Reference List

1. Matschinsky, F.M., Meglasson, M., Ghosh, A., Appel, M., Bedoya, F., Prentki, M., Corkey, B., Shimizu, T., Berner, D., Najafi, H., et al. (1986) Biochemical design features of the pancreatic islet cell glucose- sensory system. *Adv. Exp. Med. Biol.*, **211**, 459-469.
2. Rutter, G.A., Pralong, W.-F., Wollheim, C.B. (1992) Regulation of mitochondrial glycerol phosphate dehydrogenase by Ca^{2+} within electroporated insulin secreting cells (INS-1). *Biochim. Biophys. Acta*, **1175**, 107-113.
3. Rutter, G.A., Theler, J.-M., Li, G., Wollheim, C.B. (1994) Ca^{2+} stores in insulin-secreting cells: lack of effect of cADP ribose. *Cell Calcium*, **16**, 71-80.
4. Tsuboi, T., Kikuta, T., Warashina, A., Terakawa, S. (2001) Protein kinase C-dependent supply of secretory granules to the plasma membrane. *Biochem Biophys. Res. Commun.*, **282**, 621-628.
5. Rutter, G.A. (2004) Visualising Insulin Secretion. The Minkowski lecture 2004. *Diabetologia*, **47**, 1861-1872.
6. Dodson, G., Steiner, D. (1998) The role of assembly in insulin's biosynthesis. *Curr. Opin. Struct. Biol.*, **8**, 189-194.
7. Kahn, S.E., Zraika, S., Utzschneider, K.M., Hull, R.L. (2009) The beta cell lesion in type 2 diabetes: there has to be a primary functional abnormality. *Diabetologia*, **52**, 1003-1012.
8. Scully, T. (2012) Diabetes in numbers. *Nature*, **485**, S2-S3.
9. Rutter, G.A., Pullen, T.J., Hodson, D.J., Martinez-Sanchez, A. (2015) Pancreatic beta cell identity, glucose sensing and the control of insulin secretion. *Biochem. J.*, **466**, 202-218.
10. Pal, A., McCarthy, M.I. (2013) The genetics of type 2 diabetes and its clinical relevance. *Clin. Genet.*, **83**, 297-306.
11. Rutter, G.A., Parton, L.E. (2008) The beta-cell in type 2 diabetes and in obesity. *Front Horm Res.*, **36**, 118-134.
12. Sladek, R., Rocheleau, G., Rung, J., Dina, C., Shen, L., Serre, D., Boutin, P., Vincent, D., Delisle, A., Hadjadj, S., et al. (2007) A genome-wide association study identifies novel risk loci for type 2 diabetes. *Nature*, **445**, 881-885.
13. Chimienti, F., Devergnas, S., Favier, A., Seve, M. (2004) Identification and cloning of a beta-cell-specific zinc transporter, ZnT-8, localized into insulin secretory granules. *Diabetes*, **53**, 2330-7.
14. Nicolson, T.J., Bellomo, E.A., Wijesekara, N., Loder, M.K., Baldwin, J.M., Gyulkhandanyan, A.V., Koshkin, V., Tarasov, A.I., Carzaniga, R., Kronenberg, K., et al. (2009) Insulin storage and glucose homeostasis in mice null for the granule zinc

- transporter ZnT8 and studies of the type 2 diabetes-associated variants. *Diabetes*, **58**, 2070-2083.
15. Wijesekara,N., Dai,F.F., Hardy,A.B., Giglou,P.R., Bhattacharjee,A., Koshkin,V., Chimienti,F., Gaisano,H., Rutter,G.A., Wheeler,M.B. (2010) Beta cell specific ZnT8 deletion in mice causes marked defects in insulin processing, crystallisation and secretion. *Diabetologia*, **53**, 1656-1668.
 16. Carroll,R.J., Hammer,R.E., Chan,S.J., Swift,H.H., Rubenstein,A.H., Steiner,D.F. (1988) A mutant human proinsulin is secreted from islets of Langerhans in increased amounts via an unregulated pathway. *Proc. Natl. Acad. Sci. U. S. A.*, **85**, 8943-8947.
 17. Kim,I., Kang,E.S., Yim,Y.S., Ko,S.J., Jeong,S.H., Rim,J.H., Kim,Y.S., Ahn,C.W., Cha,B.S., Lee,H.C., Kim,C.H. (2010) A low-risk ZnT-8 allele (W325) for post-transplantation diabetes mellitus is protective against cyclosporin A-induced impairment of insulin secretion. *Pharmacogenomics. J.*, **11**, 191-198.
 18. Lemaire,K., Ravier,M.A., Schraenen,A., Creemers,J.W., Van de,P.R., Granvik,M., Van,L.L., Waelkens,E., Chimienti,F., Rutter,G.A., *et al.* (2009) Insulin crystallization depends on zinc transporter ZnT8 expression, but is not required for normal glucose homeostasis in mice. *Proc. Natl. Acad. Sci. U. S. A.*, **106**, 14872-14877.
 19. Tamaki,M., Fujitani,Y., Hara,A., Uchida,T., Tamura,Y., Takeno,K., Kawaguchi,M., Watanabe,T., Ogihara,T., Fukunaka,A., *et al.* (2013) The diabetes-susceptible gene SLC30A8/ZnT8 regulates hepatic insulin clearance. *J. Clin. Invest.*, **123**, 4513-4524.
 20. Rutter,G.A., Chimienti,F. (2015) SLC30A8 mutations in type 2 diabetes. *Diabetologia*, **58**, 31-36.
 21. Pound,L.D., Sarkar,S.A., Ustione,A., Dadi,P.K., Shadoan,M.K., Lee,C.E., Walters,J.A., Shiota,M., McGuinness,O.P., Jacobson,D.A., *et al.* (2012) The physiological effects of deleting the mouse slc30a8 gene encoding zinc transporter-8 are influenced by gender and genetic background. *PLoS One.*, **7**, e40972.
 22. Wicksteed,B., Brissova,M., Yan,W., Opland,D.M., Plank,J.L., Reinert,R.B., Dickson,L.M., Tamarina,N.A., Philipson,L.H., Shostak,A., *et al.* (2010) Conditional gene targeting in mouse pancreatic {beta}-cells: Analysis of ectopic Cre transgene expression in the brain. *Diabetes.*, **59**, 3090-3098.
 23. Hardy,A.B., Wijesekara,N., Genkin,I., Prentice,K.J., Bhattacharjee,A., Kong,D., Chimienti,F., Wheeler,M.B. (2012) Effects of high-fat diet feeding on Znt8-null mice: differences between beta-cell and global knockout of Znt8. *Am. J. Physiol Endocrinol. Metab.*, **302**, E1084-E1096.
 24. Brouwers,B., de,F.G., Osipovich,A.B., Goyvaerts,L., Lemaire,K., Boesmans,L., Cauwelier,E.J., Granvik,M., Pruniau,V.P., Van,L.L., *et al.* (2014) Impaired islet function in commonly used transgenic mouse lines due to human growth hormone minigene expression. *Cell Metab.*, **20**, 979-990.
 25. Kone,M., Pullen,T.J., Sun,G., Ibberson,M., Martinez-Sanchez,A., Sayers,S., Nguyen-Tu,M.S., Kantor,C., Swisa,A., Dor,Y., *et al.* (2014) LKB1 and AMPK differentially regulate pancreatic beta-cell identity. *FASEB J.*, **28**, 4972-4985.

26. Patel,D., Ythier,D., Brozzi,F., Eizirik,D.L., Thorens,B. (2015) Clic4, a novel protein that sensitizes beta cells to apoptosis. *Mol Metab*, **4**, 253-264.
27. Oropeza,D., Jouvet,N., Budry,L., Bouyakdan,K., Sczelecki,S., Perron,G., Bergeron,V., Neuman,J.C., Brar,H., Fenske,R., *et al.* (2015).
28. Milo-Landesman,D., Surana,M., Berkovich,I., Compagni,A., Christofori,G., Fleischer,N., Efrat,S. (2001) Correction of hyperglycemia in diabetic mice transplanted with reversibly immortalized pancreatic beta cells controlled by the tet-on regulatory system. *Cell Transplant.*, **10**, 645-650.
29. Thorens,B., Tarussio,D., Maestro,M.A., Rovira,M., Heikkila,E., Ferrer,J. (2015) Ins1 knock-in mice for beta cell-specific gene recombination. *Diabetologia*, **58**, 558-656.
30. Elayat,A.A., el-Naggar,M.M., Tahir,M. (1995) An immunocytochemical and morphometric study of the rat pancreatic islets. *J. Anat.*, **186**, 629-637.
31. Nicolson,T.J., Bellomo,E.A., Wijesekara,N., Loder,M.K., Baldwin,J.M., Gyulkhandanyan,A.V., Koshkin,V., Tarasov,A.I., Carzaniga,R., Kronenberger,K., *et al.* (2009) Insulin storage and glucose homeostasis in mice null for the granule zinc transporter ZnT8 and studies of the type 2 diabetes-associated variants. *Diabetes*, **58**, 2070-2083.
32. Hodson,D.J., Mitchell,R.K., Bellomo,E.A., Sun,G., Vinet,L., Meda,P., Li,D., Li,W.H., Bugliani,M., Marchetti,P., *et al.* (2013) Lipotoxicity disrupts incretin-regulated human beta cell connectivity. *J. Clin. Invest.*, **123**, 4182-4194.
33. Vinkenborg,J.L., Nicolson,T.J., Bellomo,E.A., Koay,M.S., Rutter,G.A., Merckx,M. (2009) Genetically encoded FRET sensors to monitor intracellular Zn²⁺ homeostasis. *Nat. Methods*, **6**, 737-740.
34. Chabosseau,P., Tuncay,E., Meur,G., Bellomo,E.A., Hessels,A., Hughes,S., Johnson,P.R., Bugliani,M., Marchetti,P., Turan,B., *et al.* (2014) Mitochondrial and ER-Targeted eCALWY Probes Reveal High Levels of Free Zn. *ACS Chem. Biol.*
35. Gerber,P.A., Bellomo,E.A., Hodson,D.J., Meur,G., Solomou,A., Mitchell,R.K., Hollinshead,M., Chimienti,F., Bosco,D., Hughes,S.J., *et al.* (2014) Hypoxia lowers SLC30A8/ZnT8 expression and free cytosolic Zn²⁺ in pancreatic beta cells. *Diabetologia*, **57**, 1635-1644.
36. Li,D., Chen,S., Bellomo,E.A., Tarasov,A.I., Kaut,C., Rutter,G.A., Li,W.H. (2011) Imaging dynamic insulin release using a fluorescent zinc indicator for monitoring induced exocytotic release (ZIMIR). *Proc Natl Acad Sci U S A*, **108**.
37. Tamaki,M., Fujitani,Y., Uchida,T., Hirose,T., Kawamori,R., Watada,H. (2009) Downregulation of ZnT8 expression in pancreatic beta-cells of diabetic mice. *Islets.*, **1**, 124-128.
38. Eldor,R., Yeffet,A., Baum,K., Doviner,V., Amar,D., Ben-Neriah,Y., Christofori,G., Peled,A., Carel,J.C., Boitard,C., *et al.* (2006) Conditional and specific NF-kappaB blockade protects pancreatic beta cells from diabetogenic agents. *Proc. Natl. Acad. Sci. U. S. A.*, **103**, 5072-5077.

39. Bellomo,E., Massarotti,A., Hogstrand,C., Maret,W. (2014) Zinc ions modulate protein tyrosine phosphatase 1B activity. *Metallomics.*, **6**, 1229-1239.
40. [Plum,L.M., Brieger,A., Engelhardt,G., Hebel,S., Nessel,A., Arlt,M., Kaltenberg,J., Schwaneberg,U., Huber,M., Rink,L., Haase,H. \(2014\) PTEN-inhibition by zinc ions augments interleukin-2-mediated Akt phosphorylation. *Metallomics.*, **6**, 1277-1287.](#)
41. Tamaki,M., Fujitani,Y., Hara,A., Uchida,T., Tamura,Y., Takeno,K., Kawaguchi,M., Watanabe,T., Ogihara,T., Fukunaka,A., *et al.* (2013) The diabetes-susceptible gene SLC30A8/ZnT8 regulates hepatic insulin clearance. *J. Clin. Invest.*, **123**, 4513-4524.
42. Kirchhoff,K., Machicao,F., Haupt,A., Schafer,S.A., Tschritter,O., Staiger,H., Stefan,N., Haring,H.U., Fritsche,A. (2008) Polymorphisms in the TCF7L2, CDKAL1 and SLC30A8 genes are associated with impaired proinsulin conversion. *Diabetologia.*, **51.**, 597-601.
43. Fadista,J., Vikman,P., Laakso,E.O., Mollet,I.G., Esguerra,J.L., Taneera,J., Storm,P., Osmark,P., Ladenvall,C., Prasad,R.B., *et al.* (2014) Global genomic and transcriptomic analysis of human pancreatic islets reveals novel genes influencing glucose metabolism. *Proc. Natl. Acad. Sci. U. S. A.*, **111**, 13924-13929.
44. Grant,S.F., Thorleifsson,G., Reynisdottir,I., Benediktsson,R., Manolescu,A., Sainz,J., Helgason,A., Stefansson,H., Emilsson,V., Helgadottir,A., *et al.* (2006) Variant of transcription factor 7-like 2 (TCF7L2) gene confers risk of type 2 diabetes. *Nat. Genet.*, **38**, 320-323.
45. Rutter,G.A. (2014) Understanding GWAS genes for Type 2 diabetes. *Diabet. Med.*, **31**, 1480-1487.
46. da Silva Xavier,G., Loder,M.K., McDonald,A., Tarasov,A.I., Carzaniga,R., Kronenberger,K., Barg,S., Rutter,G.A. (2009) TCF7L2 regulates late events in insulin secretion from pancreatic islet beta-cells. *Diabetes*, **58**, 894-905.
47. Zhou,Y., Park,S.Y., Su,J., Bailey,K., Ottosson-Laakso,E., Shcherbina,L., Oskolkov,N., Zhang,E., Thevenin,T., Fadista,J., *et al.* (2014) TCF7L2 is a master regulator of insulin production and processing. *Hum. Mol. Genet.*, **23**, 6419-6431.
48. Truett,G.E., Heeger,P., Mynatt,R.L., Truett,A.A., Walker,J.A., Warman,M.L. (2000) Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). *Biotechniques.*, **29**, 52, 54.
49. Pullen,T.J., Sylow,L., Sun,G., Halestrap,A.P., Richter,E.A., Rutter,G.A. (2012) Over-expression of Monocarboxylate Transporter-1 (*Slc16a1*) in the pancreatic α -cell leads to relative hyperinsulinism during exercise. *Diabetes*, **61**, 1719-1725.
50. Ravier,M.A., Rutter,G.A. (2010) Isolation and culture of mouse pancreatic islets for ex vivo imaging studies with trappable or recombinant fluorescent probes. *Methods Mol. Biol.*, **633**, 171-184.
51. Sun,G., da Silva Xavier,G., Gorman,T., Priest,C., Solomou,A., Hodson,D.J., Foretz,M., Viollet,B., Herrera,P.L., Parker,H.E., *et al.* (2015) LKB1 and AMPK α 1

are required in pancreatic alpha cells for the normal regulation of glucagon secretion and responses to hypoglycemia. *Mol. Metab.*, **4**, 277-286.

52. [Martinez-Sanchez,A., Nguyen-Tu,M.S., Rutter,G.A. \(2015\) DICER Inactivation Identifies Pancreatic beta-Cell "Disallowed" Genes Targeted by MicroRNAs. *Mol Endocrinol.*, **29**, 1067-1079.](#)
53. Deerinck,T., Bushong,E., Thor,A., Ellisman,M. (2010) NCMIR methods for 3D EM: A new protocol for preparation of biological specimens for serial block face scanning electron microscopy. *Microscopy*, 6-8.
54. Hodson,D.J., Mitchell,R.K., Bellomo,E.A., Sun,G., Vinet,L., Meda,P., Li,D., Li,W.H., Bugliani,M., Marchetti,P., *et al.* (2013) Lipotoxicity disrupts incretin-regulated human beta cell connectivity. *J. Clin. Invest.*, **123**, 4182-4194.

Figure Legends

Figure 1 – Ins1Cre-mediated deletion of ZnT8 in pancreatic β -cells.

Mice carrying a LoxP site together with a flippase recognition target-flanked neomycin selection cassette within intron 1, and a single distal LoxP site within the upstream exon 1 containing the translational start codon, were bred with the Ins1Cre deleter strain, leading to the removal of exon 1 of *Slc30A8*/ZnT8 (A). Ins1Cre-mediated deletion of ZnT8 resulted in an approximate 80-90% reduction in *Slc30a8* expression (B; ** $p < 0.001$ vs. 3 Cre⁻, 4 Cre⁺), with no significant changes in the expression of other ZnT family members ($p > 0.05$ by two-way ANOVA). Gene expression was normalised to β -actin and fold change gene expression was determined using $2^{-\Delta\Delta CT}$. (B). ZnT8 protein expression is reduced in islets from Cre⁺ animals, as shown by immunofluorescence staining, which demonstrates deletion specifically in β ($n = 90$) but not α cells ($n = 24$) (C-D). Deletion of ZnT8 revealed by Western (immuno-) blotting of isolated islets from Ins1Cre^{+/-}:ZnT8^{fl/fl} mice and controls (E). Values represent mean $\pm$ SEM. Scale bar in C, 12.5 μ m.

Figure 2 – *In vivo* assessment of glucose homeostasis in Ins1CreZnT8KO mice.

Intraperitoneal glucose tolerance test (IPGTT) and area under the curve (AUC) of 6 week old (A), 10 week old (B; * $p < 0.05$, 30 min time point) and 14-week old (C) male Ins1Cre^{+/-} ZnT8^{fl/fl} (Cre⁺) and littermate control (Ins1CreZnT8^{-/-} ZnT8^{fl/fl}, Cre⁻) mice. Animals were injected with 1g/kg body weight glucose and blood glucose measured at time point 0, 15, 30, 60, 90 and 120 min. post glucose injection ($n=7-14$ animals). Glucose (D, *** $p < 0.01$, 15 min. time, point, **** $p < 0.001$, 30 min. time point) and insulin (E, , * $p < 0.05$, 30 min. time point) responses of 10 week old male Ins1CreZnT8 mice after 3 g/kg bodyweight glucose injection ($n=13-14$ animals per genotype). (F) Insulin tolerance test of 10 week-old Ins1CreZnT8 and littermate control male mice. Animals were injected with 0.75 U/kg body weight insulin and blood glucose measured as per IPGTT. Numbers in solid bars in the histograms indicate the number of animals studied. Values are mean $\pm$ SEM.

Figure 3 – *In vitro* assessment of islets isolated from Ins1CreZnT8KO mice.

Insulin secretion from 5 size-matched islets was assessed using a HTRF-based assay (Methods). Briefly, islets were pre-treated at 3 mM glucose for 1 h at 37 °C before being exposed to either 3 mM glucose (G3), 16.7 mM glucose (G16.7), 16.7 mM glucose plus 20 nM GLP-1 (GLP-1) or 3mM glucose + 30mM KCl (KCl) for 30 min. at 37 °C. Secreted

insulin was determined and, after normalisation to total insulin, expressed as fold change vs. the 3 mM glucose condition (A). Intracellular free Ca^{2+} ($[\text{Ca}^{2+}]_i$) dynamics were assessed using Nipkow spinning disk microscopy. No significant differences were seen in either the amplitude or the AUC of glucose-evoked (17 mM; G17) whole islet Ca^{2+} rises (B; ns vs. Cre^- ; Student's t test). The number of glucose-responsive cells was unchanged between Cre^- and Cre^+ mice (data not shown). Likewise, there were no differences seen in KCl-induced (30mM KCl) intracellular free Ca^{2+} rises (C; ns vs. Cre^- ; Student's t-test). Correlation analyses of glucose-evoked Ca^{2+} traces (54) showed no difference in β cell- β cell connectivity in Cre^+ islets vs. Cre^- islets at both low (3 mM) and high (11 mM) glucose, indicating maintained cell quiescence and synchronicity respectively. (D) Connectivity map depicting location, number and strength (colour coded; 0 [blue] = lowest, 1[red] = highest) of significantly correlated cell pairs (E). Values represent mean $\pm$ SEM.

Figure 4. Intracellular zinc dynamics and secretion in control in *Ins1CreZnT8KO* mouse β cells.

To measure cytosolic free Zn^{2+} levels, isolated islets were dispersed and infected with an adenovirus expressing the Zn^{2+} -sensitive FRET probe eCALWY4 (34) (A). Steady-state fluorescence intensity ratio (citrine/cerulean) was first measured (1) before obtaining the maximum ratio (2) under perfusion with KHB buffer containing the zinc chelator TPEN (50 μM ; zinc-free condition). Finally, the minimum ratio (3) was obtained under perfusion with KHB buffer containing 5 μM pyrithione and 100 μM Zn^{2+} (zinc-saturated condition), providing saturating intracellular Zn^{2+} concentrations (B). The free cytosolic concentration of Zn^{2+} (C) was calculated using the following formula: $[\text{Zn}^{2+}] = K_d(R_{\text{max}} - R)/(R - R_{\text{min}})$, revealing significant decreases in cytosolic free zinc levels in *Ins1Cre:ZnT8^{fl/fl}* animals compared to littermate controls (** $p < 0.01$, Cre^+ vs. Cre^- respectively, $n = 20$ Cre^+ & 11 Cre^- islets). Zinc secretion from isolated islets, using the zinc binding probe ZIMIR, was decreased in Cre^+ islets as shown by significant decreases in both the amplitude (**** $p < 0.0001$, Student's t-test) and AUC (* $p < 0.05$, Student's t test) of glucose-evoked ZIMIR responses. $n = 22$ Cre^- islets & 17 Cre^+ islets (D).

Figure 5 – *Ins1CreZnT8KO* mice exhibit abnormal granule morphology but unchanged β cell mass

TEM images of β -cells from islets isolated from Cre^- (upper) or Cre^+ (lower) animals show altered dense core granule structure. Scale bar, 1 μm . (A). Insulin granules were grouped into

3 categories according to morphological structure and counted. Cre⁺ animals showed a significant reduction in granules containing a dense core coupled with significant increases in granules showing a rod like structure or a grey interior (B; *** $p < 0.001$ vs. Cre⁻). No changes in total granule number were seen between genotypes (C; ns vs. Cre⁻). Granule diameter was increased in Cre⁺ β cells (D, **** $p < 0.0001$ vs. Cre⁻, Student's t-test). Staining pancreatic slices from Cre⁻ (E, left) and Cre⁺ (E, right scale bar 50 μ m) for insulin and glucagon revealed no significant differences in α : β cell ratio (F), β cell mass (G) or α cell mass (H) ($n=3$ animals per genotype). Values represent mean $\pm$ SEM.

Figure 6 – Selective overexpression of ZnT8 in the mouse β cell.

Mice expressing human ZnT8 (hZnT8) and luciferase under the control of a bidirectional, tetracycline-regulated promoter were crossed with *RIP7-rtTA* mice (28) and bred to give ZnT8 Tg⁺ mice (*Rip7-rtTA*⁺*ZnT8Tg*⁺) and littermate control animals (*Rip7-rtTA*⁺*ZnT8*⁻). Administration of doxycycline in the drinking water was used to induce ZnT8 overexpression specifically in the β -cell (A). qPCR revealed increased hZnT8 (B, **** $p < 0.0001$ vs. ZnT8 Tg⁻, $n=3$ animals per genotype) and luciferase (C, **** $p < 0.0001$ vs. ZnT8 Tg⁻, $n=3$ animals per genotype) gene expression in isolated mouse islets. Luciferase activity was also increased in islets isolated from ZnT8 Tg⁺ animals (D, *** $p < 0.001$ vs. ZnT8 Tg⁻, $n=4$). Western (immuno-) blotting demonstrated increased hZnT8 protein expression in isolated islets from transgenic mice (E). Values represent mean $\pm$ SEM.

Figure 7 – Female ZnT8 Tg⁺ mice show improvements in glucose tolerance.

Glucose tolerance of *Rip7-rtTA*⁺*ZnT8Tg*⁺ animals was assessed by IPGTT. A significant improvement in glucose tolerance was seen in 10 week- (A, * $p < 0.05$, 15 min. time point) and 14-week old female (B, ** $p < 0.01$, 30 min time point; * $p < 0.05$ AUC) animals. Insulin sensitivity was unchanged by ZnT8 overexpression (C). Plasma glucose (D, left) tended to be reduced in ZnT8 Tg⁺ mice, but insulin (D, right) levels were significantly increased in response to a 3g/kg bodyweight IP injection of glucose (* $p < 0.05$, 15 and 30 min. time points). Values represent mean $\pm$ SEM.

Figure 8 – Insulin secretion and endocrine cell mass in ZnT8 Tg⁺ islets.

Glucose-stimulated insulin secretion was significantly reduced in isolated islets (A, * $p < 0.05$), whereas Zn²⁺ secretion was enhanced as shown by significant increases in both the amplitude (B, top, *** $p < 0.001$) and AUC (B, bottom, ** $p < 0.01$) of glucose-stimulated ZIMIR

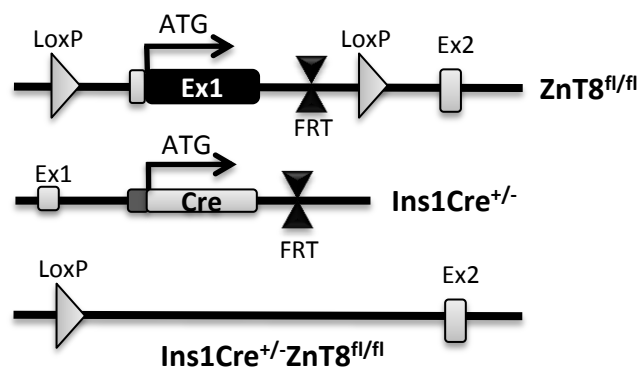
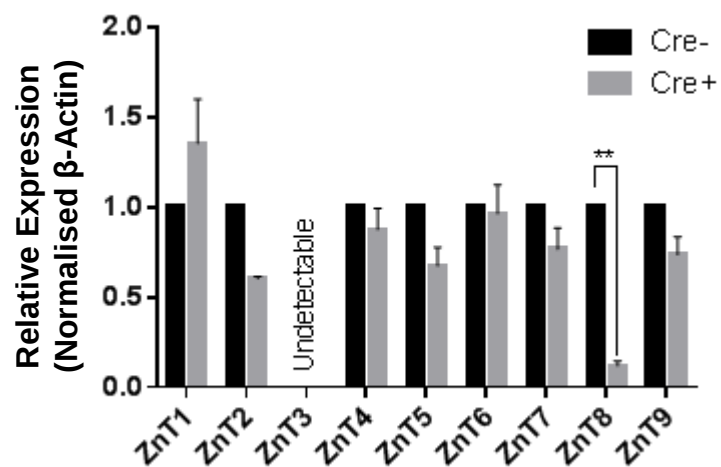
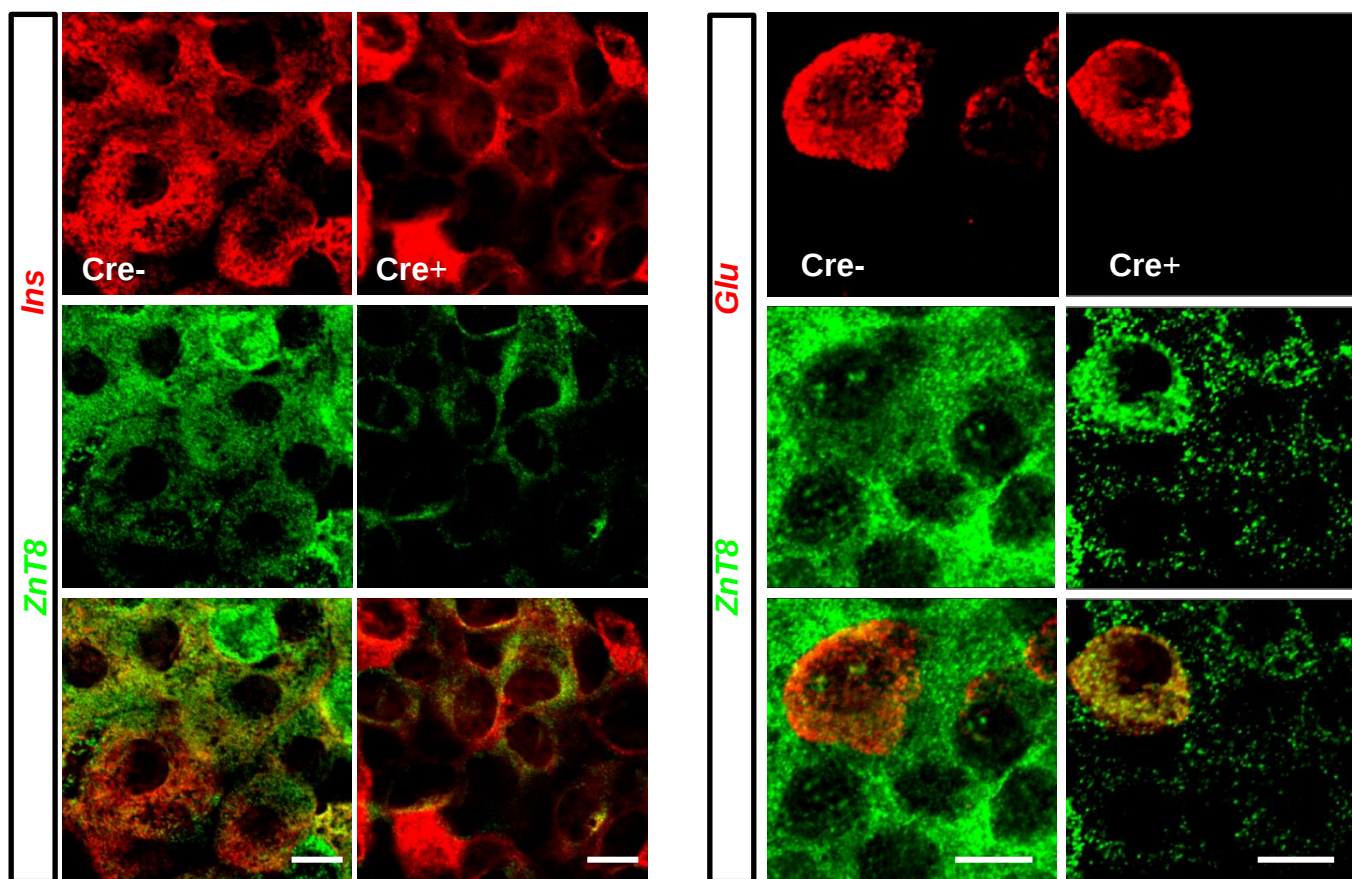
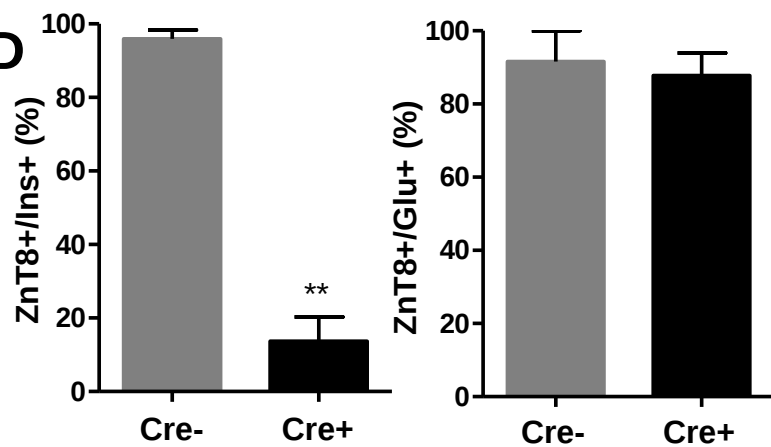
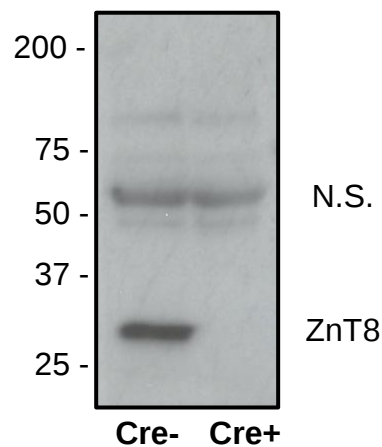
responses. Cytosolic Zn^{2+} concentrations, measured using eCALWY4 (Fig. 4), were unchanged by ZnT8 overexpression (C). Staining pancreatic slices for insulin and glucagon (D, scale bar 50 μm) revealed no changes in β (E) or α cell (F) mass, nor in $\alpha:\beta$ cell ratio (G). Values represent mean $\pm$ SEM.

Figure 9 – Impact of ZnT8 manipulation in pancreatic β cells on murine glucose homeostasis

Whereas glucose tolerance (A) and Zn^{2+} secretion (B) are both increased with increasing ZnT8 levels, glucose-stimulated insulin secretion is impaired (C). Data are taken from Figs 2-4 and 7,8. Schematic: deletion of ZnT8 specifically in pancreatic β cells leads to impaired glucose tolerance and abnormal insulin granule morphology. Conversely, overexpressing ZnT8 in the β cell causes improvements in glucose tolerance but reduced glucose-stimulated insulin secretion (D).

Abbreviations

K_{ATP} , ATP-sensitive K^+ channel; GH, growth hormone; T1D, T2D, Type 1 and Type 2 diabetes, respectively; ZnT8, zinc transporter 8.

A**B****C****D****E**

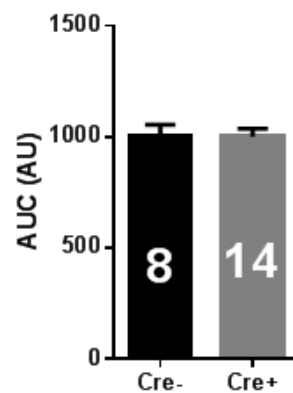
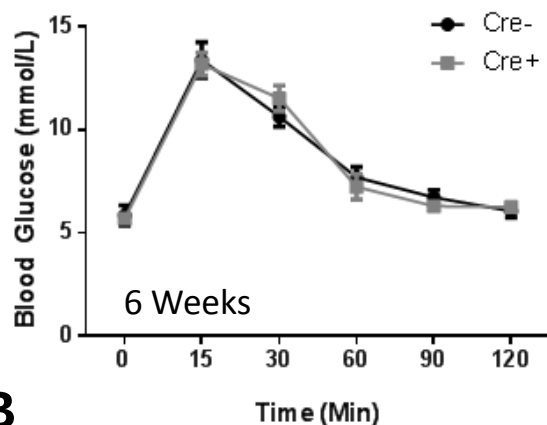
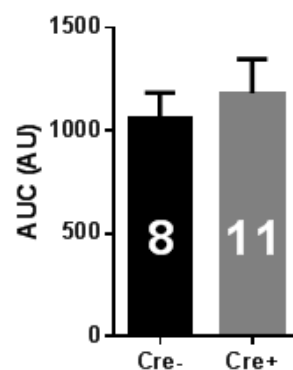
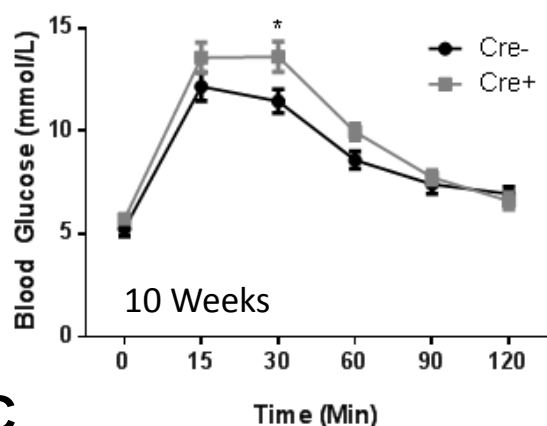
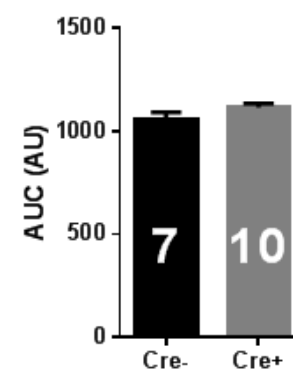
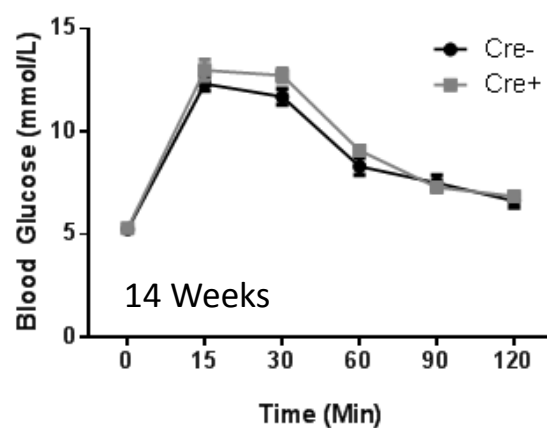
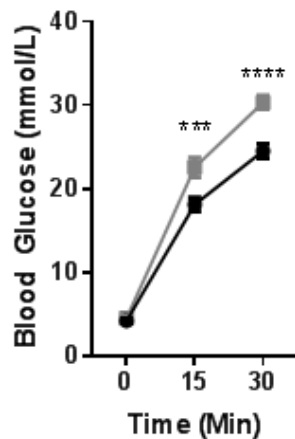
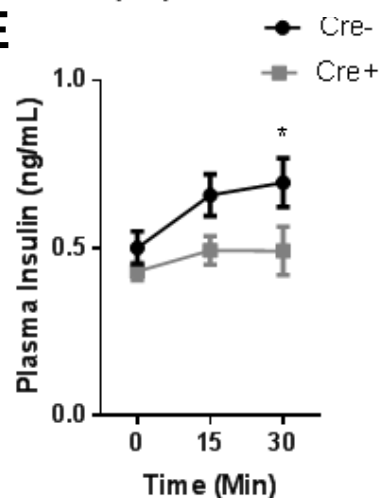
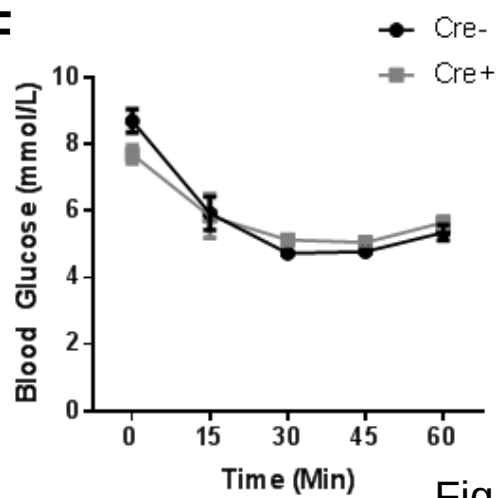
A**B****C****D****E****F**

Fig. 2

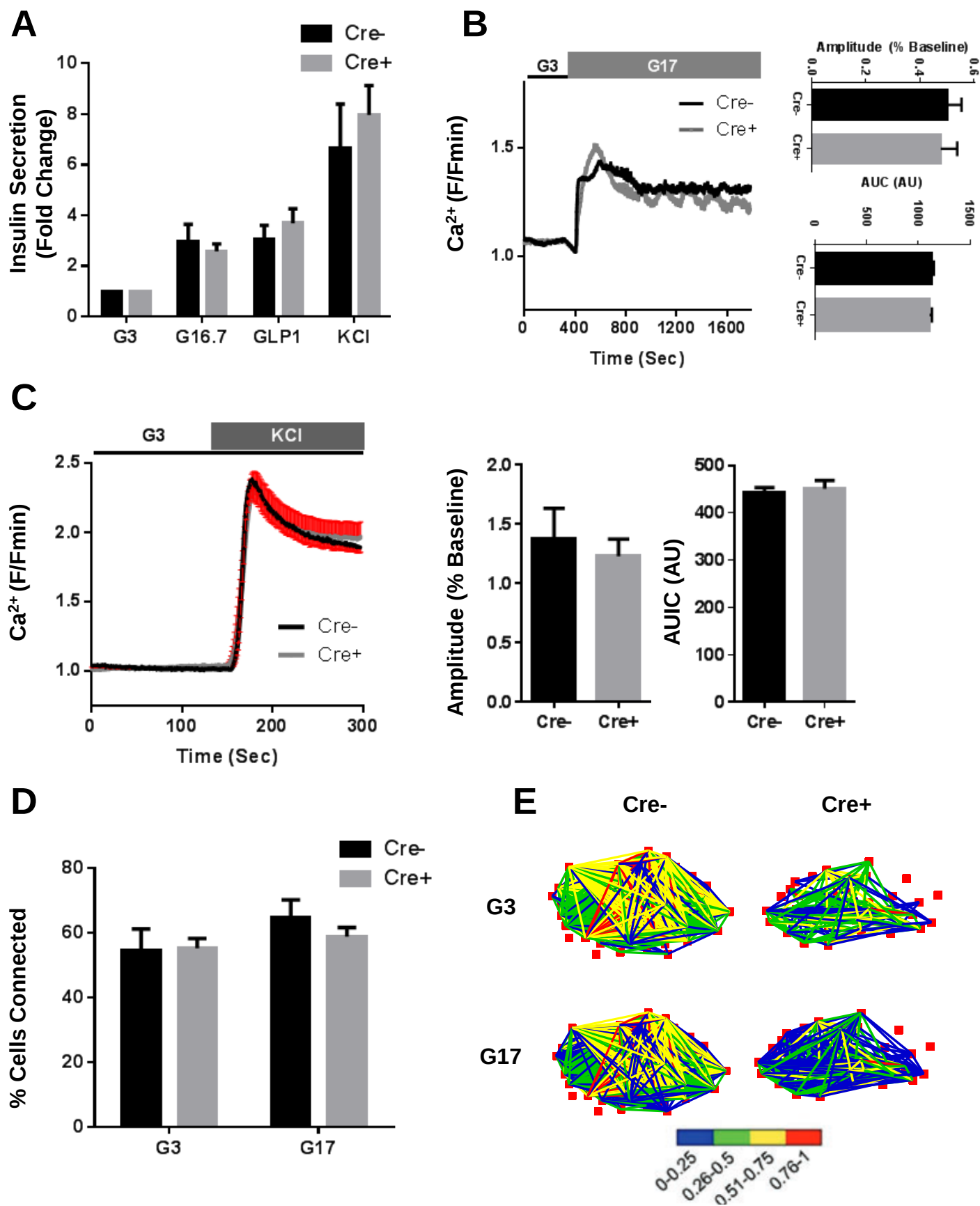


Fig. 3

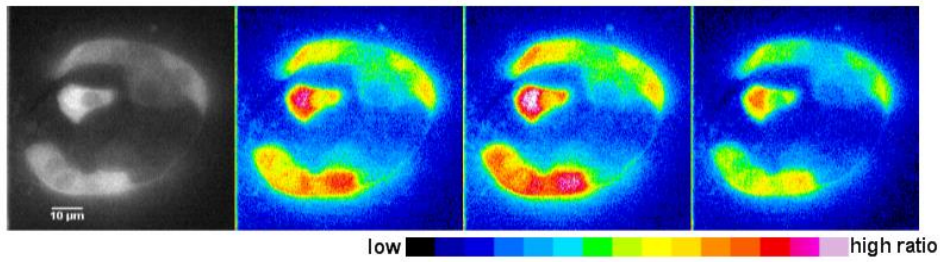
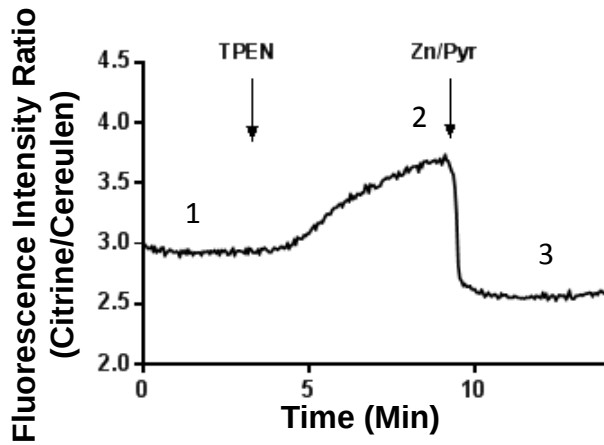
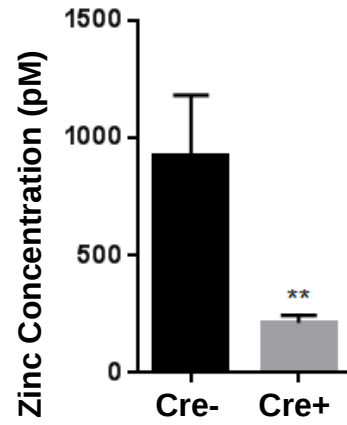
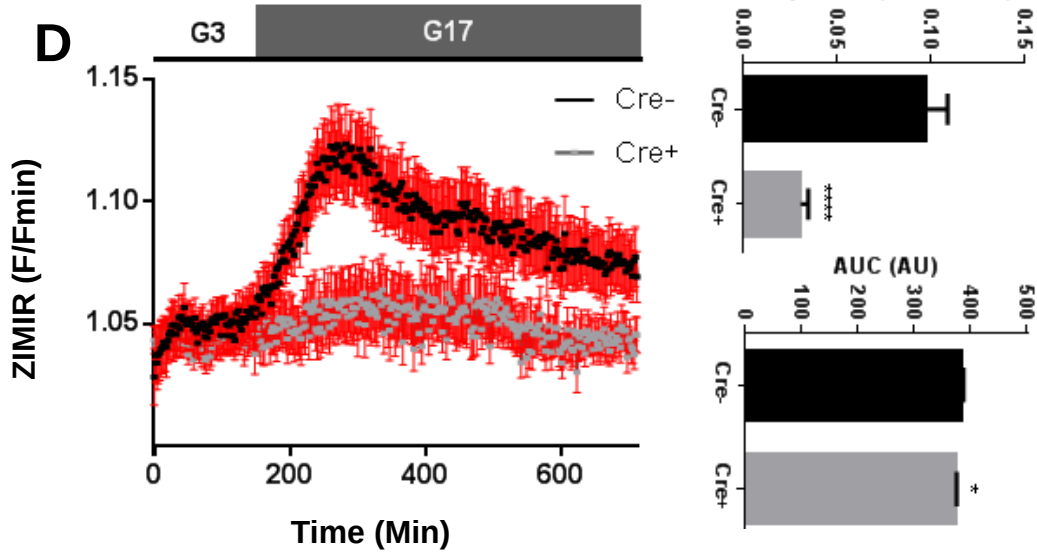
A**B****C****D**

Fig. 4

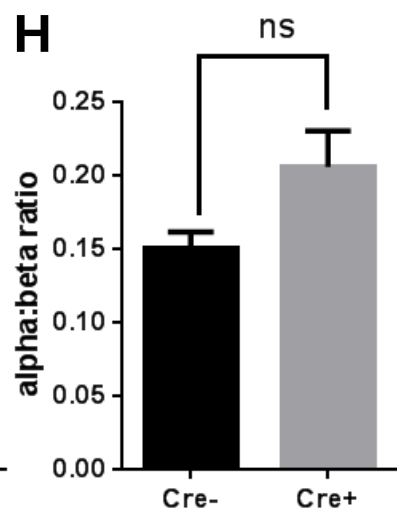
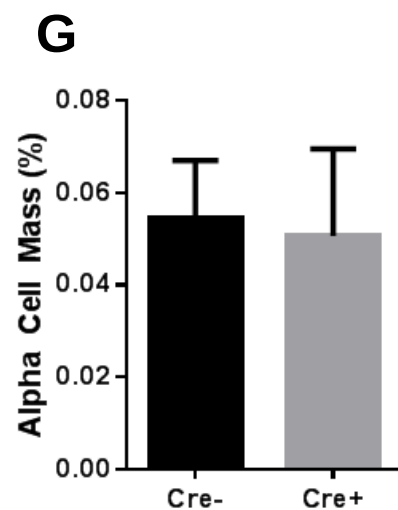
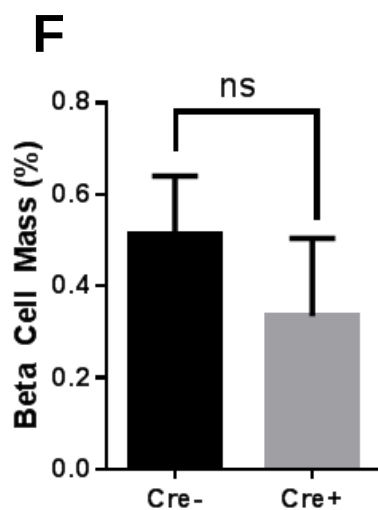
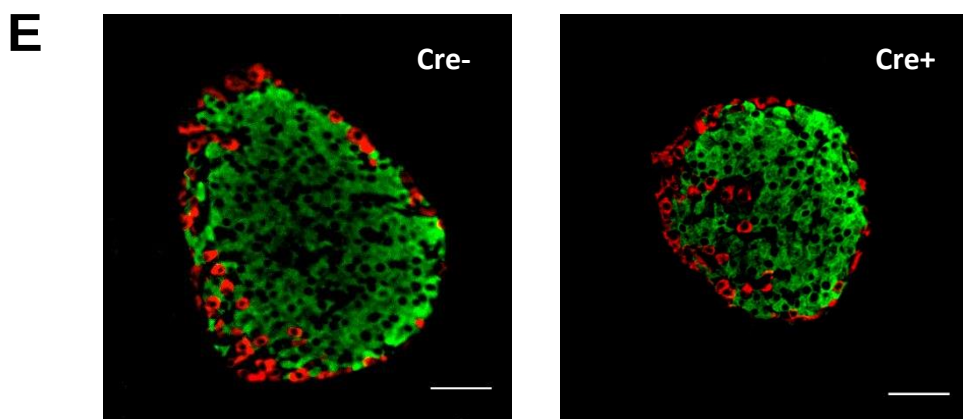
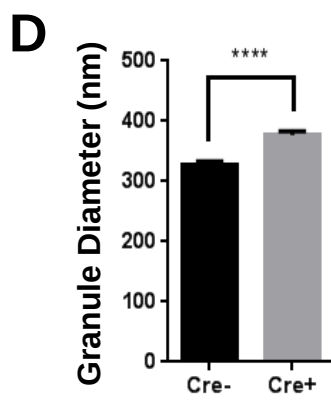
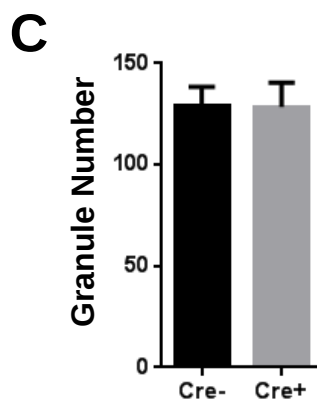
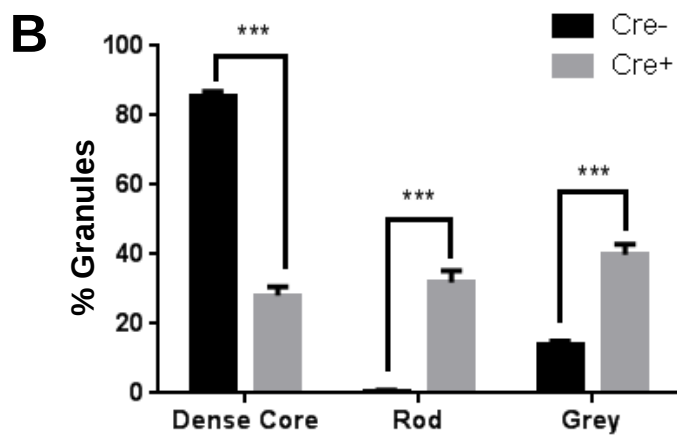
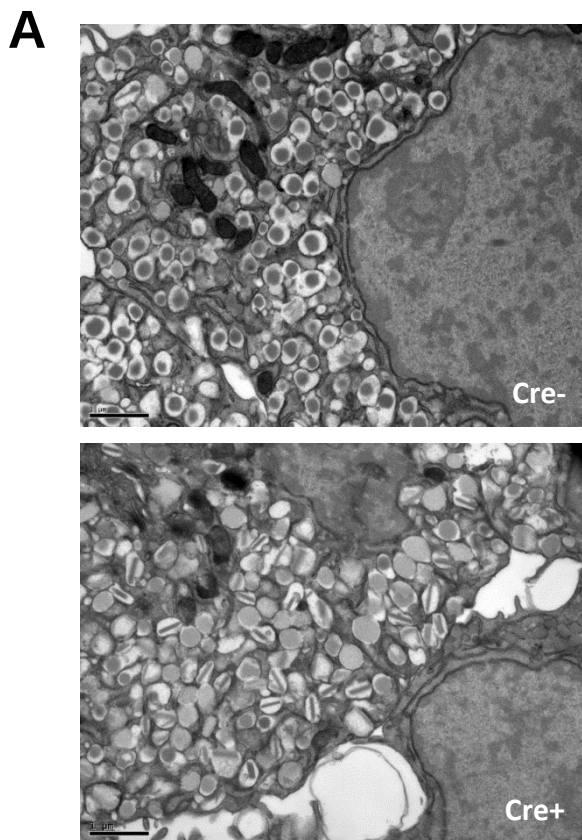


Fig. 5

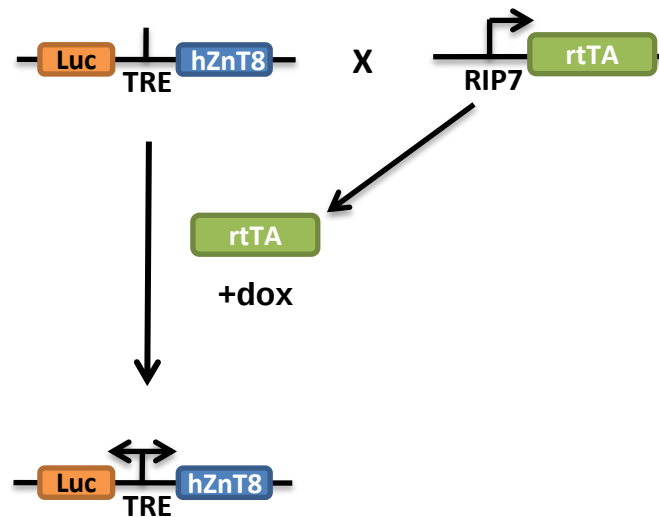
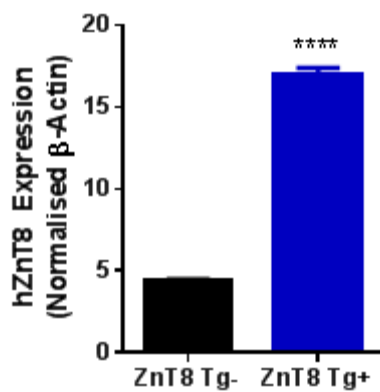
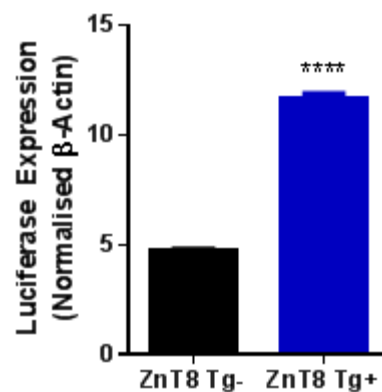
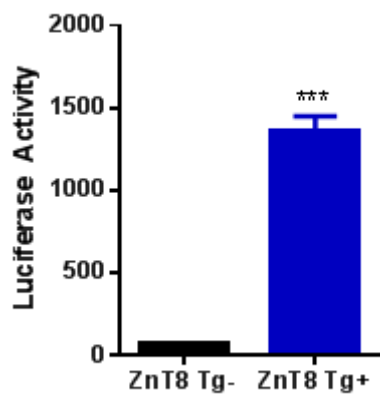
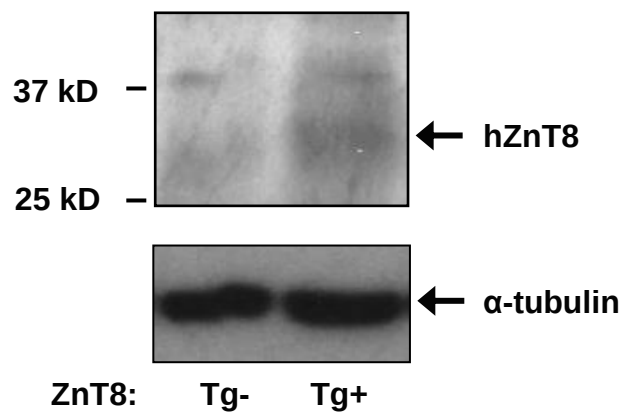
A**B****C****D****E**

Fig. 6

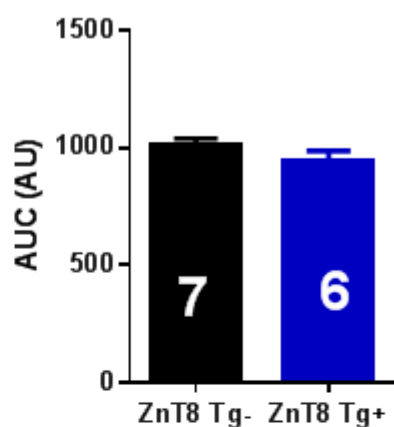
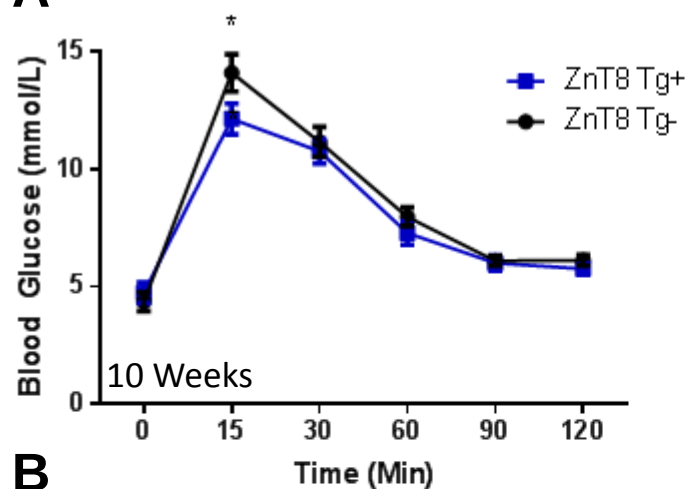
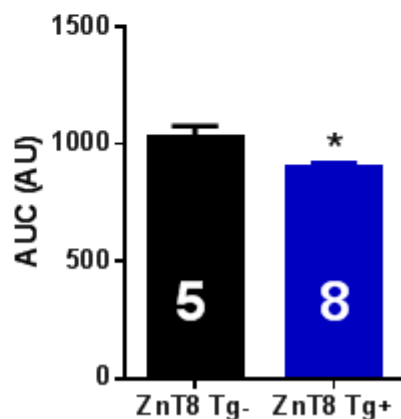
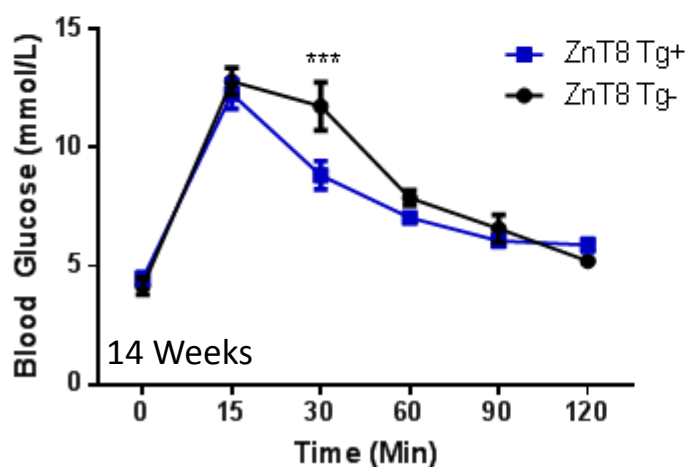
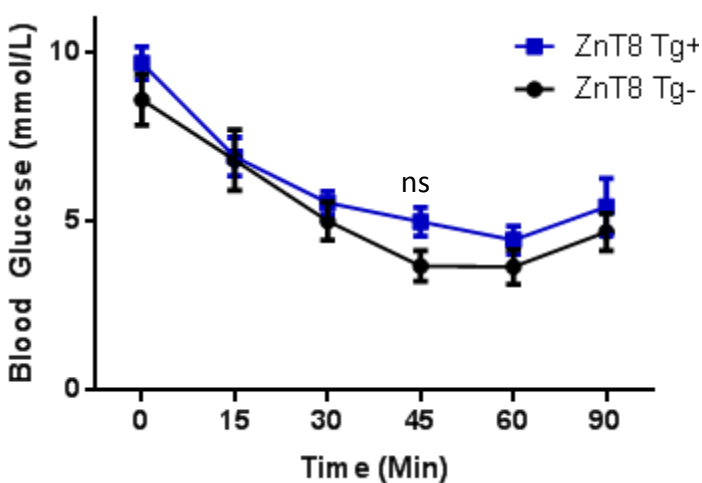
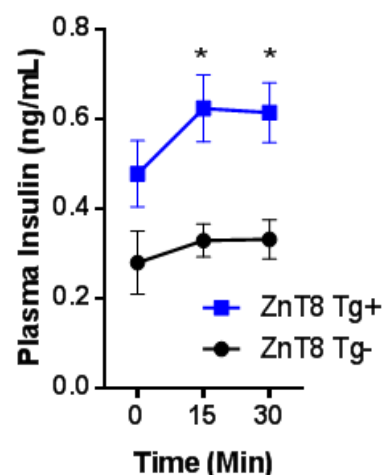
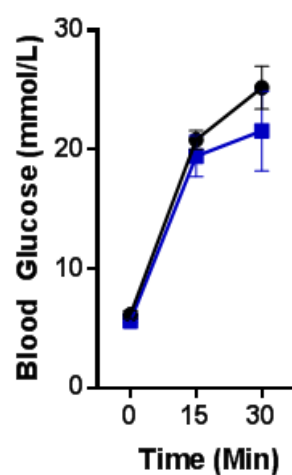
A**B****C****D**

Fig. 7

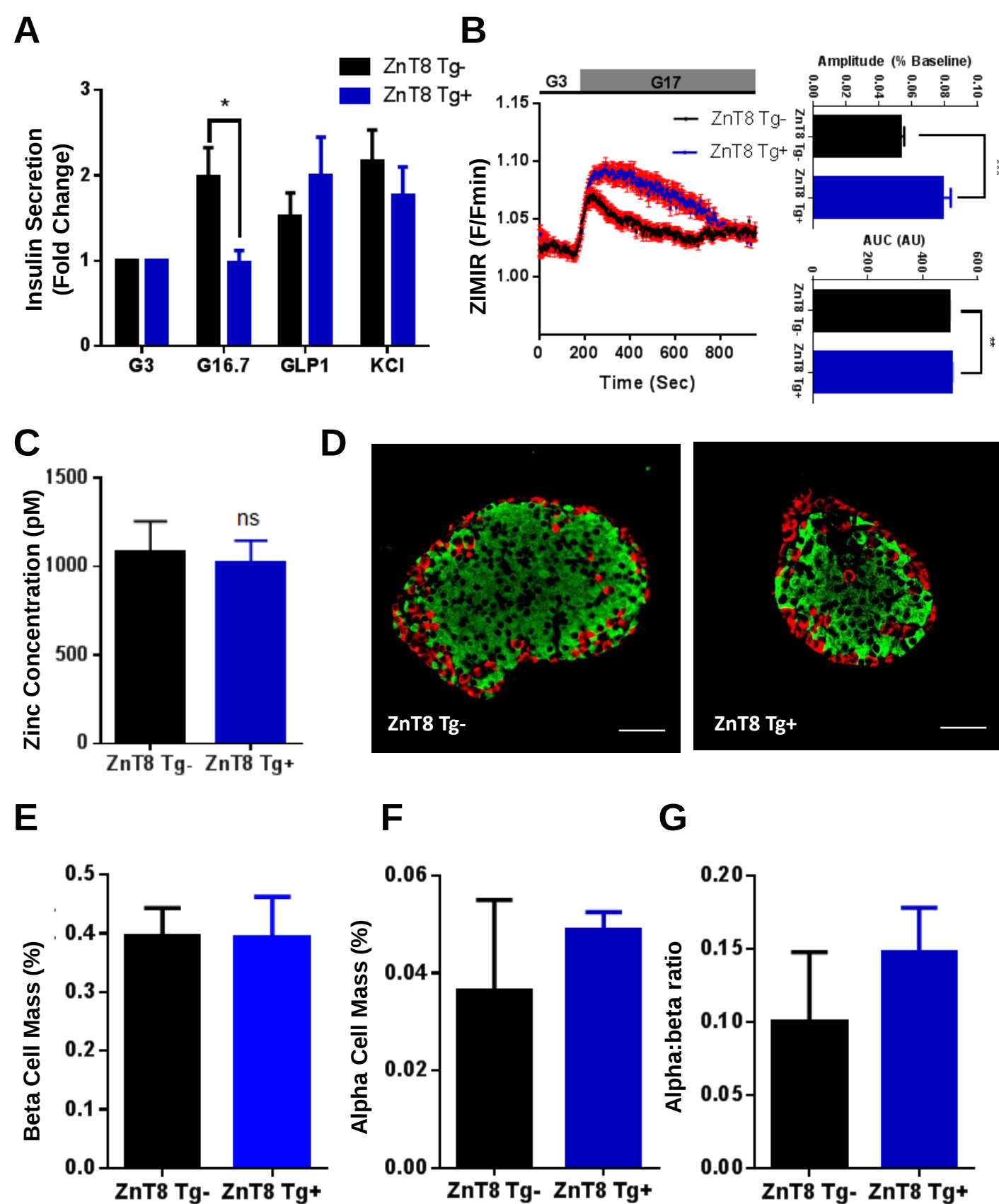
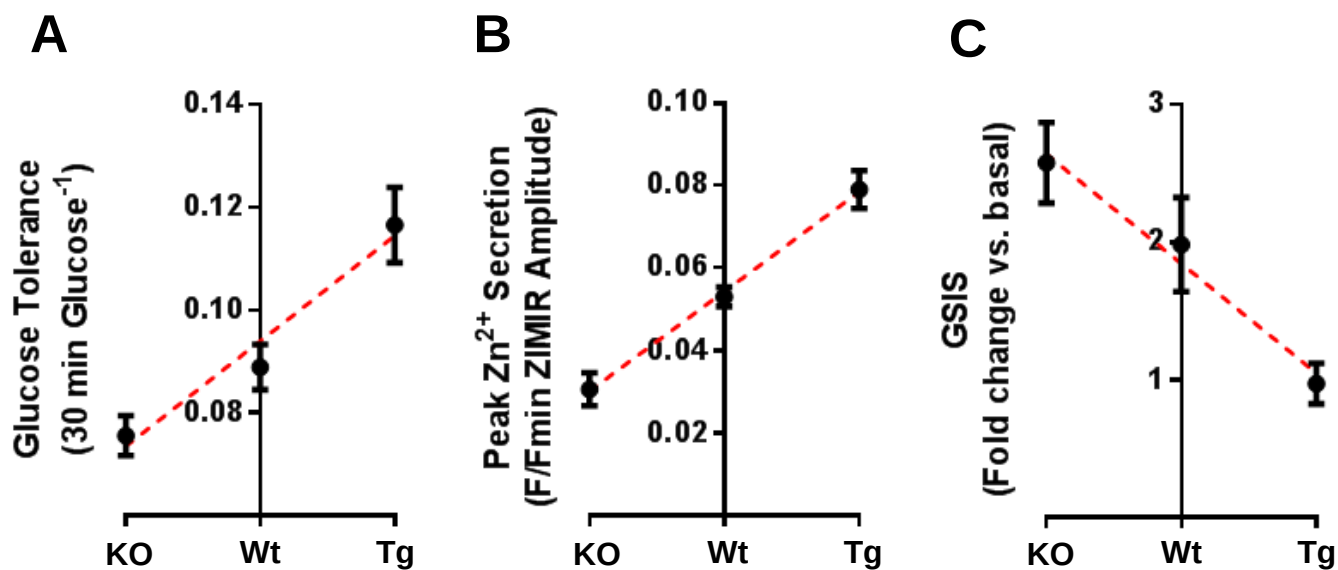


Fig. 8



D

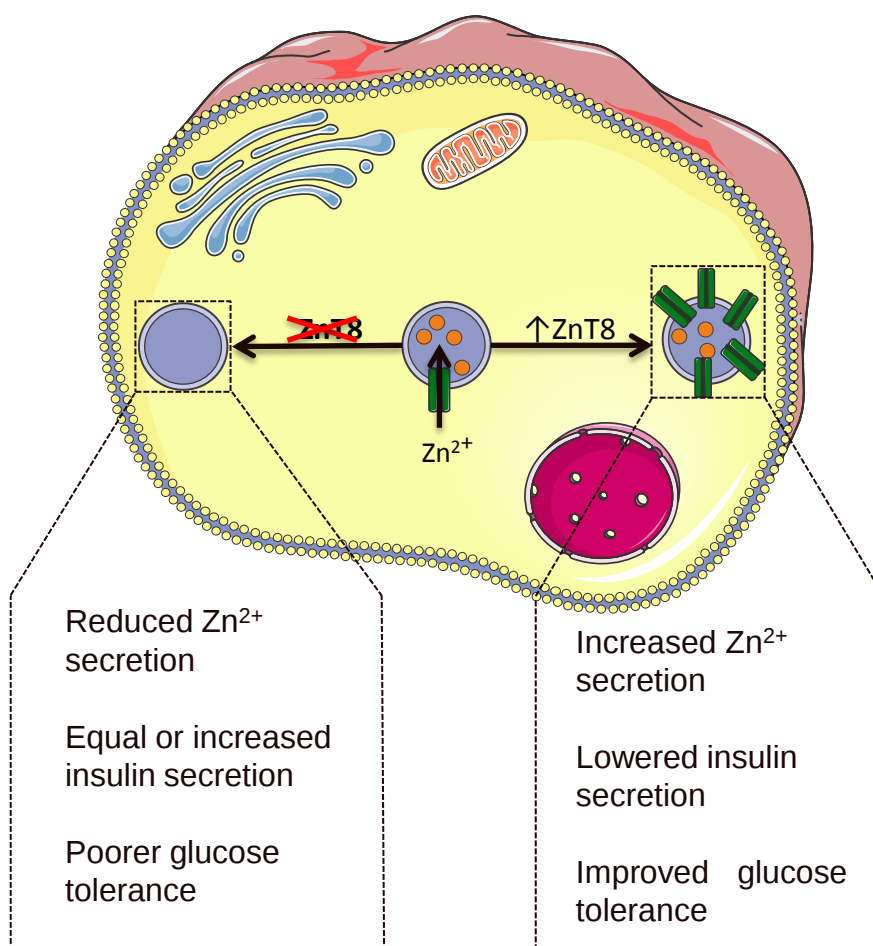
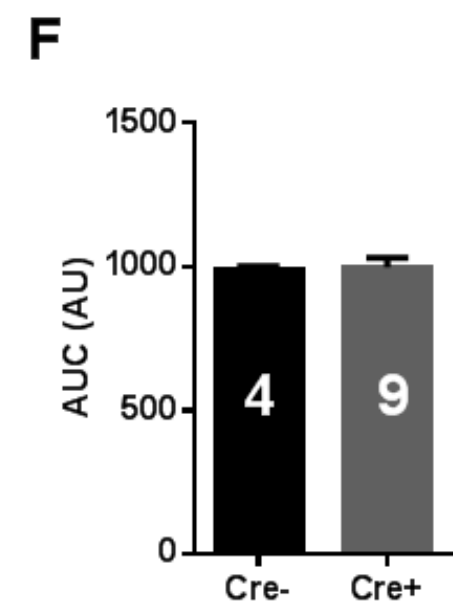
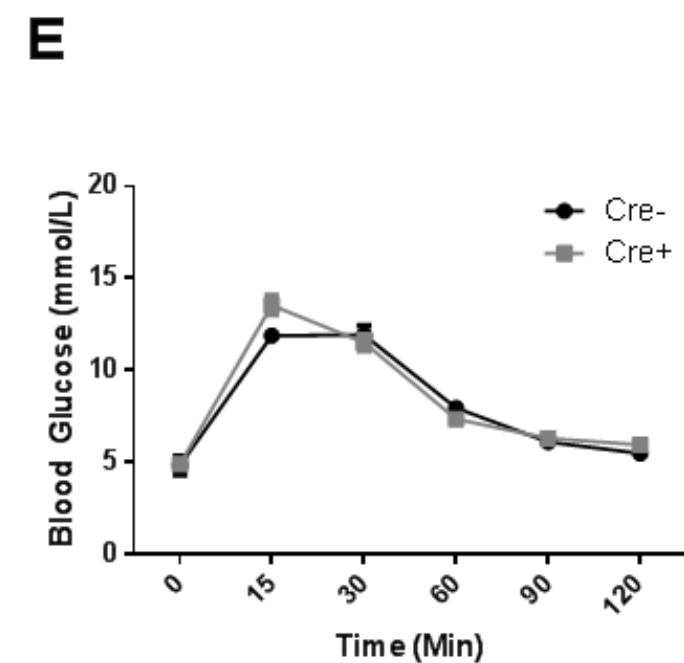
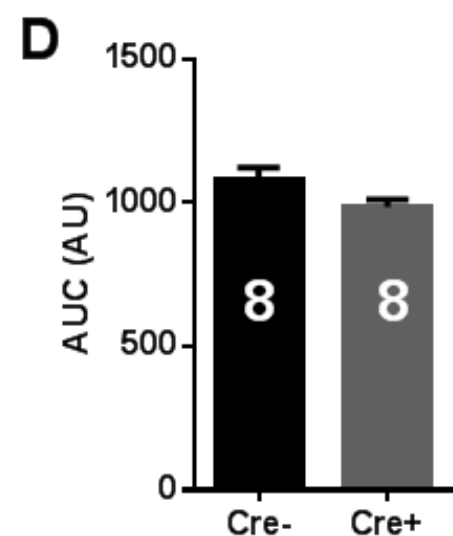
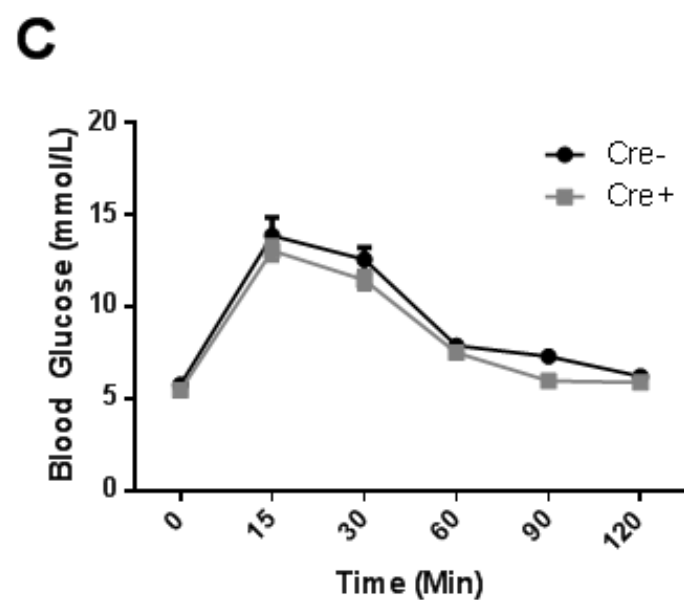
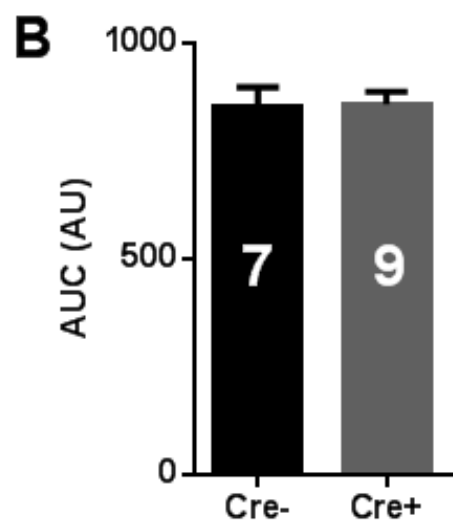
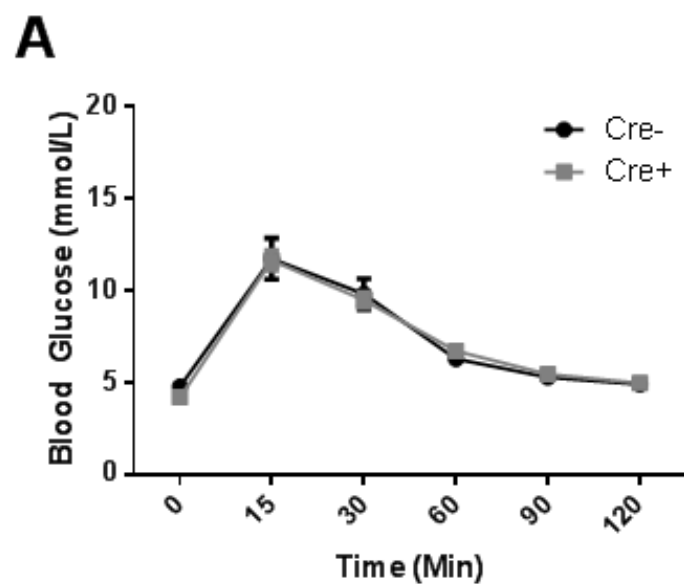
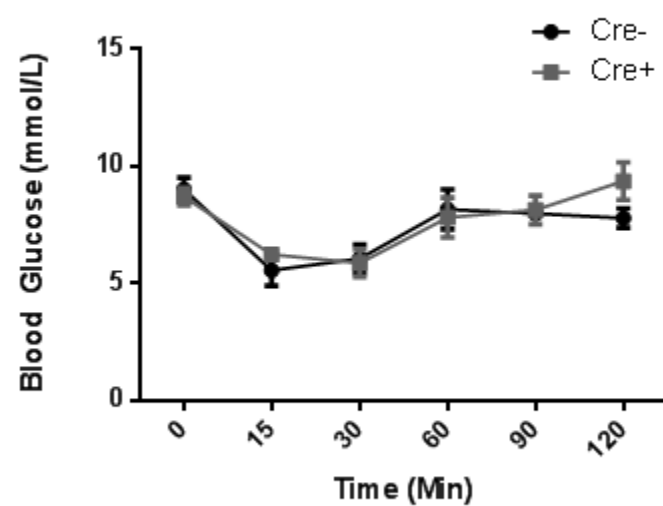
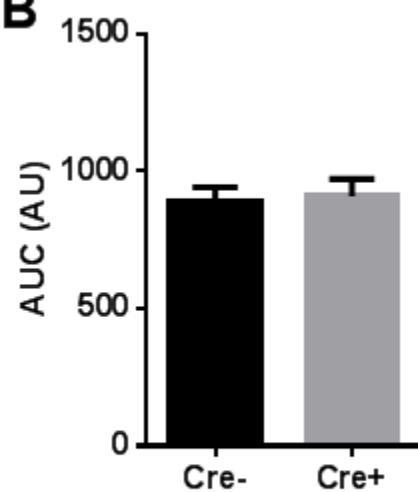
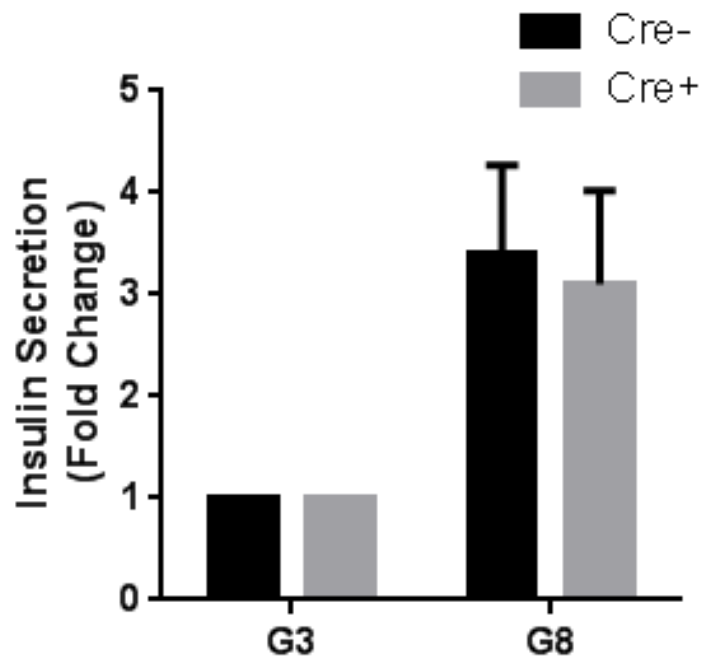
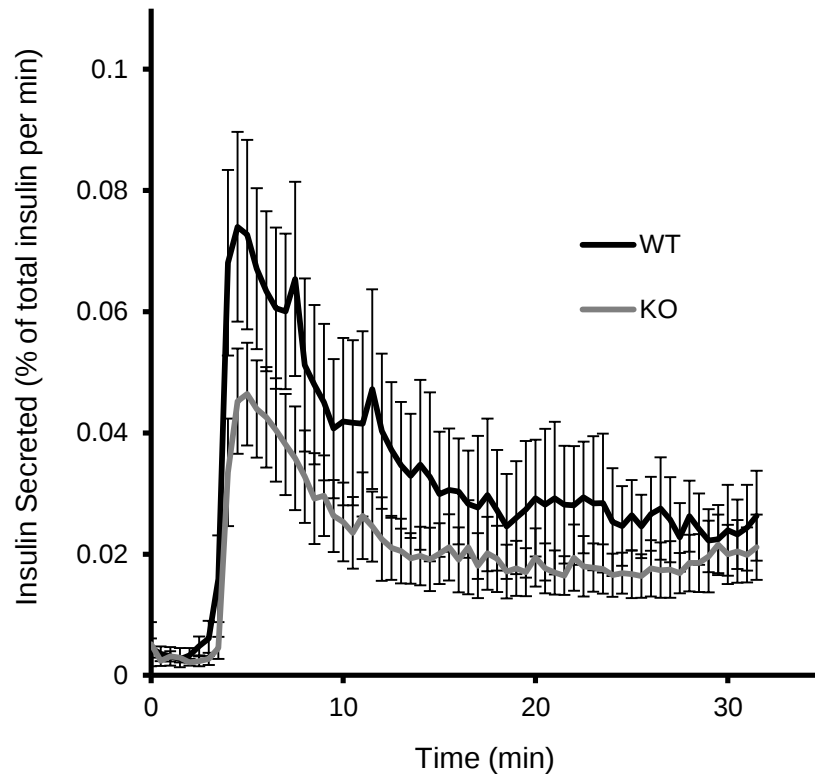


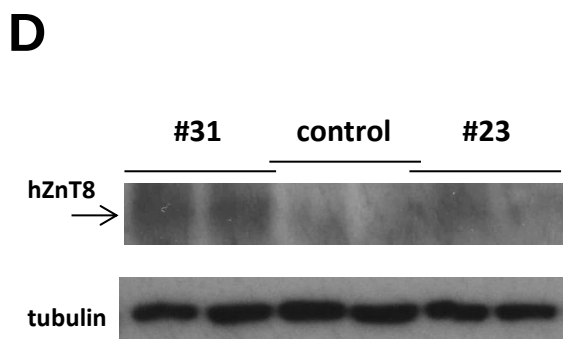
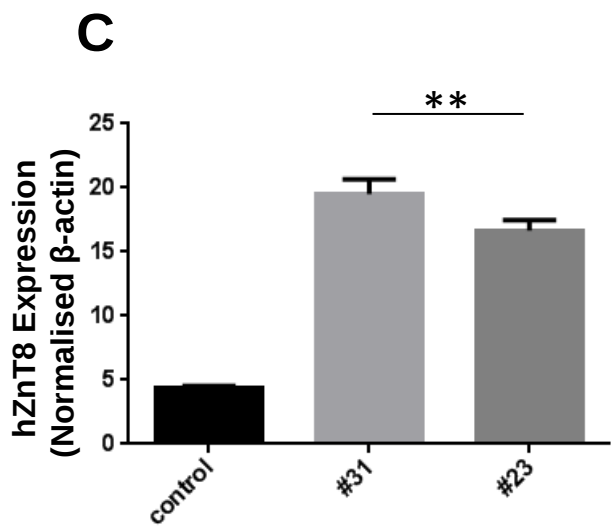
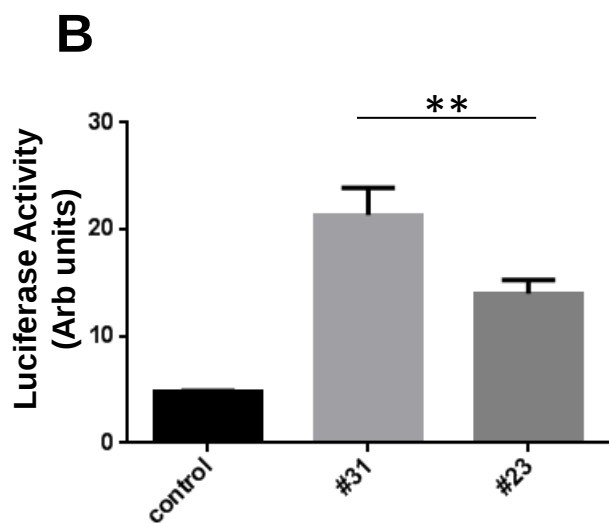
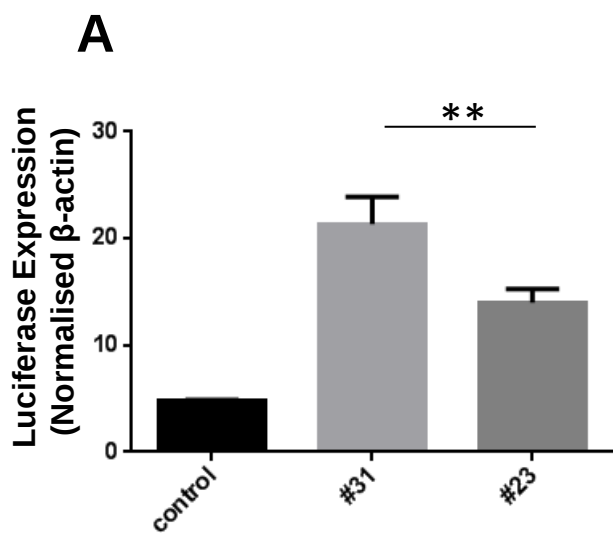
Fig. 9

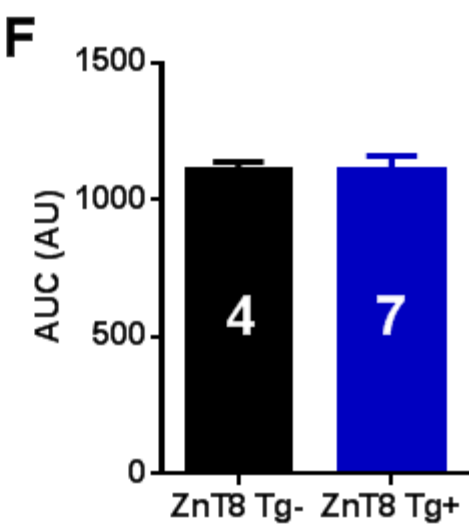
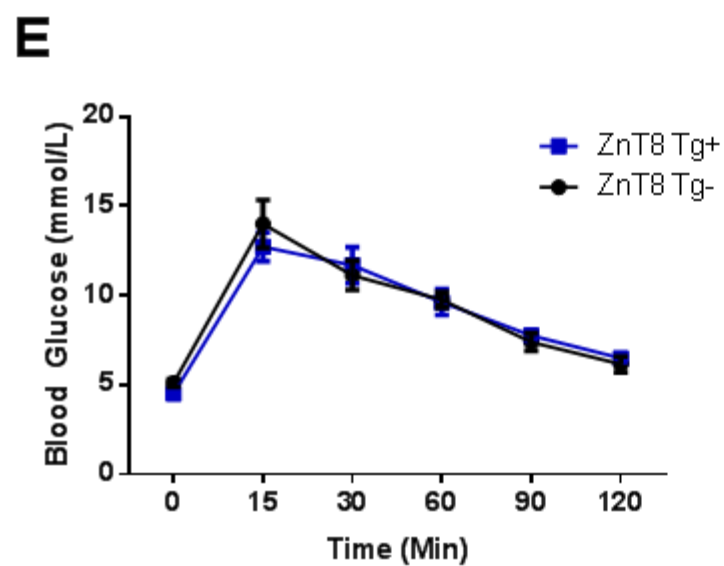
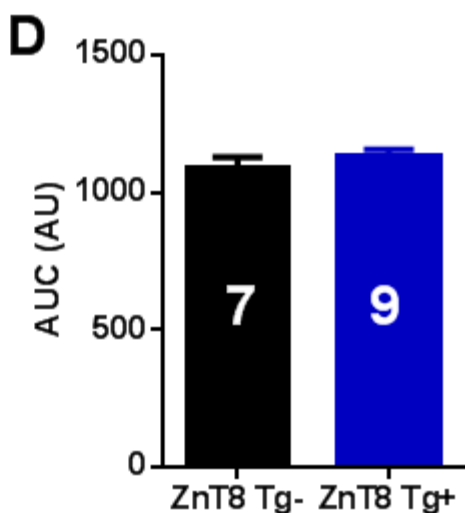
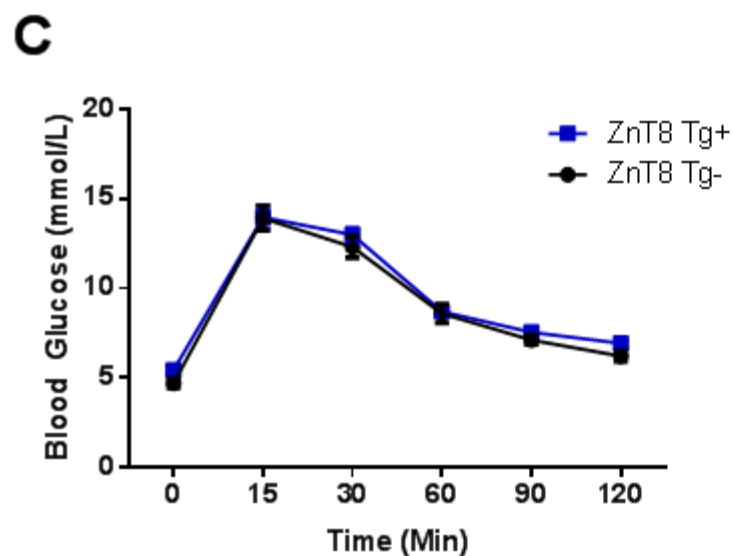
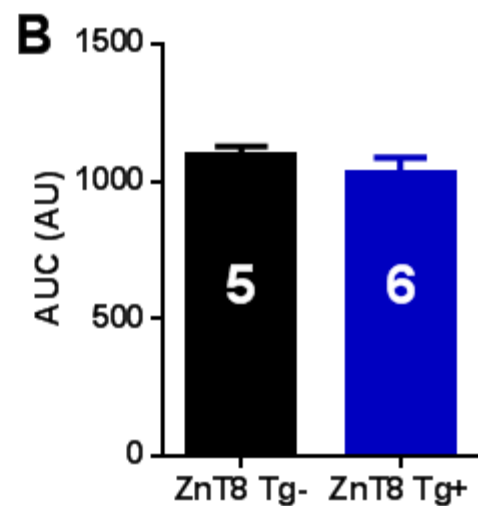
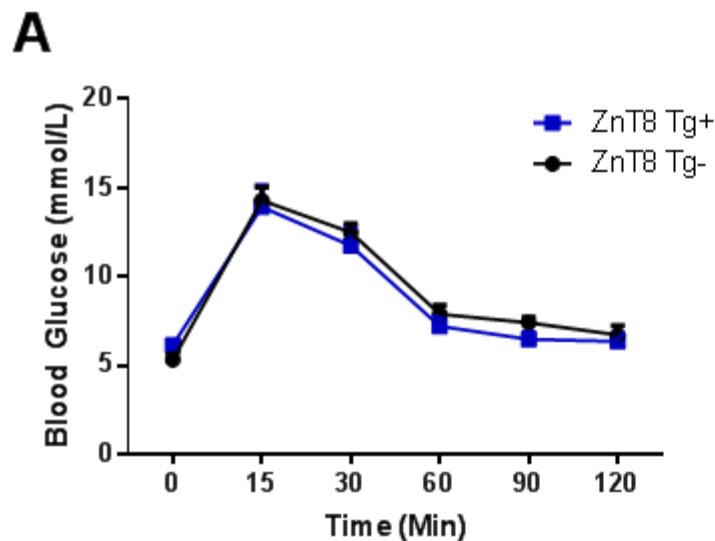


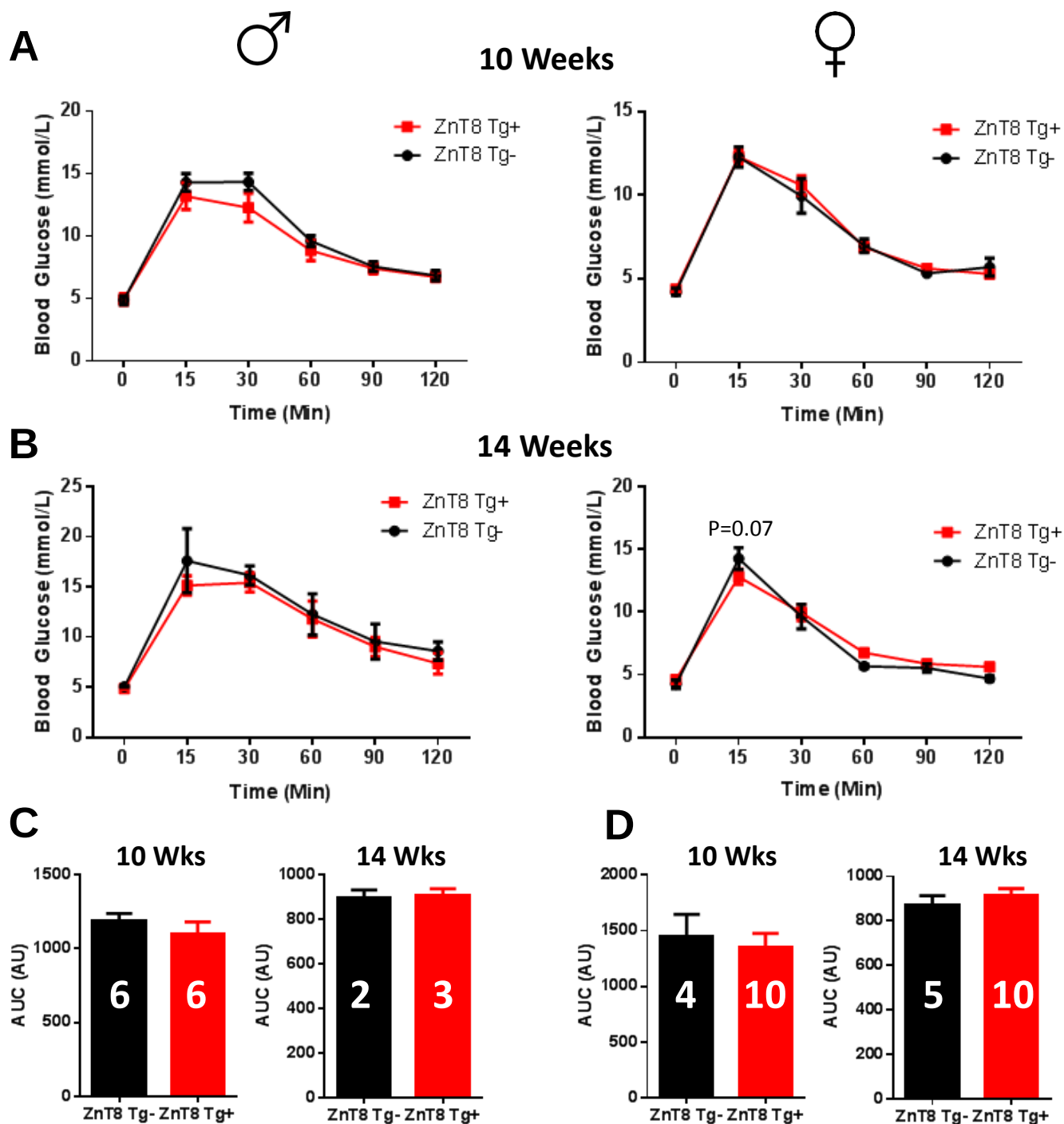
Supp. Fig. 1

A**B**

A**B**







Supp. Fig. 6