Genetic Disruption of Adenosine Kinase in Mouse Pancreatic β-Cells Protects Against High Fat Diet-Induced Glucose Intolerance

Running Title: ADK negatively regulates β-cell expansion

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ABSTRACT

Islet β-cells adapt to insulin resistance through increased insulin secretion and expansion. Type 2 diabetes (T2D) typically occurs when prolonged insulin resistance exceeds the adaptive capacity of β-cells. Our prior screening efforts led to the discovery that Adenosine kinase (ADK) inhibitors stimulate β-cell replication. Here evaluated whether ADK disruption in mouse β-cells impacts β-cell mass and/or protects against high fat diet (HFD)-induced glucose dysregulation. Mice targeted at the Adk locus were bred to Rip-Cre and Ins1-Cre/ERT<sub>1Lphi</sub> mice to enable constitutive (βADKO) and conditional (iβADKO) disruption of ADK expression in β-cells, respectively. Weight gain, glucose tolerance, insulin sensitivity and glucose stimulated insulin secretion (GSIS) were longitudinally monitored in normal chow (NC)-fed and HFD-fed mice. Additionally, β-cell mass and replication were measured by immunofluorescence-based islet morphometry. NC-fed adult βADKO and iβADKO mice displayed glucose tolerance, insulin tolerance and β-cell mass comparable to control animals. By contrast, HFD-fed βADKO and iβADKO animals had improved glucose tolerance and increased in vivo glucose-stimulated insulin secretion. Improved glucose handling was associated with increased β-cell replication and mass. We conclude that ADK expression negatively regulates the adaptive β-cell response to HFD challenge. Therefore, modulation of ADK activity is a potential strategy for enhancing the adaptive β-cell response.
Diabetes is a pathologic state of disrupted glucose homeostasis characterized by an absolute or relative insulin deficiency and a loss of insulin-producing β-cells. In type 2 diabetes (T2D), β-cell failure results from a multi-factorial process initiated by insulin resistance, often in the setting of obesity (1-3). In T2D, a variety of insults contribute to progressive β-cell failure including endoplasmic reticulum stress, inflammatory cytokines, excess reactive oxygen species and glycolipid toxicity (2). β-cell loss occurs through a combination of increased apoptosis and de-differentiation, though the relative contribution of these outcomes remains unclear (3-6). Presently, a major research goal is to understand the molecular mechanisms of β-cell failure and devise strategies to reverse this process.

Although T2D is accompanied by reduced insulin secretion in late disease, increased insulin secretion is an early adaptation to insulin resistance (7; 8). Interestingly, non-diabetic individuals with high genetic risk for diabetes have a reduced glucose-stimulated insulin response (9). It is unclear whether this is a consequence of defective β-cell function or deficient β-cell mass; T2D-associated risk alleles implicate genes participating in both processes, e.g. CDKN2A and KCNQ1 (10-12). Murine studies demonstrate a central role for β-cell mass plasticity in the accommodation of obesity-associated insulin resistance (13). Although adaptive β-cell expansion is less evident in humans, nondiabetic obese humans have a 1.5-fold increase in β-cell mass and increased β-cell number (14). Hence, it is possible that human β-cell mass exhibits modest plasticity that influences an individual’s susceptibility to developing T2D (15).

In mature animals, numerous potential sources of new β-cells have been identified (16); however, the usual source of new β-cells is previously existing β-cells (17; 18). Consequently, understanding the signals that control self-duplication are critical to understanding how β-cell mass is controlled. To identify molecular mechanisms that regulate β-cell growth we developed a primary-islet-cell-based small
molecule screening platform (19; 20). Using this platform, we uncovered the β-cell replication-promoting activity of Adenosine Kinase inhibitors (ADKI’s). *ADK* is a broadly expressed metabolic enzyme that controls extracellular and intracellular adenosine pools via its enzymatic activity: conversion of adenosine to 5’-adenosine-monophosphate (AMP) (21). Several lines of evidence indicate that ADKI’s promote β-cell replication, in part, via ADK inhibition: multiple structurally dissimilar ADKI’s promote β-cell replication, ADK-directed RNAi triggered cell-autonomous β-cell replication and an independent screen for β-cell regeneration-promoting compounds identified distinct ADKI’s (19; 22). Notably, additional activities of some ADKI’s contribute to their β-cell replication promoting activity. For example, 5-IT was shown to promote human β-cell replication through inhibition of the dual-specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A) (23-25). To investigate the function of ADK in β-cells we generated mice conditionally targeted at the ADK locus. Using these mice we tested the hypothesis that ADK acts as a negative regulator of β-cell replication and limits the adaptive response of β-cells to diabetogenic challenge.
RESEARCH DESIGN AND METHODS

Generation, Genotyping and Feeding of ADK-Targeted Mice

All animal work was approved and carried out in accordance with our institutional animal care and use committee and the Guide for the care and use of laboratory animals (2011). Adk-targeted mice were generated from IKMC clone EUCE0154a03 (26). Genotyping of ADK-targeted mice was performed by PCR: wild-type allele (ADK_F (5'-AGCCTAGACTACACAACAAG-3') and ADK_R (5'-GCTCAATCACCTATGGCC-3')); Adk-targeted allele (ADK1) (ADK_F and B32 (5'-CAAGGCGATTAAGTGGTACAG-3')); non-mutagenic orientation (ADK2; Fig. la) (B32 and ADK_6898 (5'-TCAAGCCCTTGTACACCTAAG-3')). The FLP deleter strain (FLPo-10, Jax 011065) was used to convert Adk1 to ADK2. Two Cre-expressing strains were used for constitutive (Tg(Ins2-Cre)25Mgn; JAX 0035731) and conditional (Ins1-cre/ERT1Lphi; JAX 024709) recombination. For Cre/ERT activation, tamoxifen (Cayman Chemical, #13258, 100mg/kg) was dissolved in 100% ethanol, solubilized in sterile warm corn oil ([10 mg/mL], Sigma C8267) and administered by i.p. injection on four consecutive days. β-galactosidase activity was used to confirm efficient tamoxifen-induced recombination. One week after tamoxifen injection, pancreata were excised and fixed (4% PFA/PBS at 4 °C for 30 minutes), washed with PBS and placed in 30% sucrose/PBS for 48h at 4 °C before freezing in O.C.T (Tissue Tek). Animals were fed normal chow (NC; Harlan Teklad 2018) or high fat diet (HFD; Research Diets D12492) as indicated. Experiments were conducted with female mice unless stated otherwise. Mice are mixed background (129P2/OlaHsd and C57BL/6J).

Western Blotting
Isolated islets and homogenized liver tissue lysates were used for SDS-PAGE (RIPA Lysis System, sc-24948). Islet-restricted ADK disruption was assessed using liver and islet lysates from adult βADKO and control animals. Islets were isolated as previously described (20). Anti-ADK (1:200, sc-32908) and anti-enolase (1:500, sc-15343) were used for protein detection.

**Glucose Physiology Experiments**

All glucose physiology experiments were performed on age- and gender-matched cohorts. Glucose measurements were made at the indicated times with a TRUEresult glucometer and TRUEtest strips. For fasting glucose measurement and i.p. glucose tolerance testing (ipGTT), mice were weighed and fasted overnight (14 hours, 6pm-8am). Glucose levels were obtained at 0 minutes, prior to i.p. glucose (1.5 to 2.0 grams/kg, 10 µl/gram) injection, and at the subsequent indicated time points. Random glucose levels were obtained on fed mice at 10 am. IpITT was performed using 0.5-1.5 U/kg Humulin (Eli Lilly, R-100) as indicated after four hour fast starting at 8 am. *In vivo* GSIS was measured in blood collected from overnight fasted mice at time 0 and after i.p. injection of glucose (3 g/kg). Insulin was measured with Alpco Mouse Ultrasensitive Insulin ELISA kits (80-INSMSU-E01). ADK^2/+^ Rip-Cre animals displayed no detectable phenotype and were included with ADK^+/+^ Rip-Cre as control animals.

**Histology**

Tissue β-galactosidase staining was performed on fresh frozen 25 µm frozen sections that were fixed for 5 minutes with cold 4% paraformaldehyde in PBS and washed in cold PBS. Slides were incubated in X-gal staining solution (PBS with potassium ferricyanide [5mM] (Sigma, P8131), potassium ferricyanide trihydrate [5mM] (Sigma, P3289), magnesium chloride [2mM] and X-gal 20 mg/100 mL (Promega)) at 37°C Celsius. Slides were counterstained as indicated. Antigen retrieval was performed by heating slides to
90° Celsius for 10 minutes in 10mM sodium citrate (pH6.0) solution. Primary antibodies were incubated overnight at 4 degrees Celsius. To measure islet size distribution, pancreatic sections were insulin (Dako A0564, 1:300) or glucagon (Dako A0565, 1:1000) stained along with DAPI. Affinity purified secondary antibodies (donkey) were obtained from Jackson ImmunoResearch (1:300). Entire sections (8 per mouse) were scanned using an Inverted Nikon Spinning Disk confocal microscope. Images were stitched together and analyzed using Velocity 6 (PerkinElmer). Cellular clusters of >900 µm² were counted. Total pancreatic area was determined by the DAPI⁺ area. The β-cell mass was calculated by multiplying pancreatic mass by the percent of pancreatic area that is insulin-positive. β-cell replication indices were determined by 5-Bromo-2’-deoxyuridine (BrdU) incorporation (Dako M074401-8, 1:50) and/or ki67 expression (BD Bioscience 556003, 1:300); ki67 detection was performed using biotin amplification (Jackson ImmunoResearch, 1:200). β-cells were identified by the expression of insulin or PDX1 (R&D AF2419; 1:200); use of insulin staining to confirm findings obtained with PDX-1 is necessary given that δ-cells also express PDX-1 (27). A minimum of 2,000 β-cells from non-consecutive sections (>50 µM apart) were used to determine the β-cell replication rate for each animal. Replication events were adjudicated in a blinded fashion. Mice were provided BrdU-containing water (Acros 228590100, 0.8mg/mL) for one week in opaque bottles that were changed every two days.

**In Vitro β-cell replication**

Islets were isolated from 36 week vehicle- (n=4) and tamoxifen-injected (n=4) iβADK mice. Injections were performed at age 18 weeks to avoid any developmental impact and islets were harvested after an 18 week delay to avoid potential short-term tamoxifen and recombination effects. Islets were dispersed, plated (allowed 60h to attach), treated (60 h duration), fixed and assayed (PDX1 and ki67 staining) as previously described (19; 20).
Statistics

Experimental data are plotted as scatter plots with an adjacent representation of the statistical mean that includes an error bar representing the standard deviation. Statistically significant differences were determined using the student’s two-tailed $t$ test where $p \leq 0.5$ was taken to be significant. Experimental results were confirmed in independent experimentation in all cases except for the \textit{in vivo} $\beta$-cell replication experiments.
RESULTS

Adk-Targeted Mouse Model.

To study the in vivo function of ADK in β-cells, we generated mice conditionally targeted at the Adk locus (Fig. 1A). The integrated mutagenic orientation (ADK₁) placed a highly efficient splice-acceptor sequence (SA) / βgeo expression cassette (β-galactosidase-neomycin resistance fusion gene) in frame with exons 1a and 1b, the ADK transcriptional start sites, which encode ADK-short (cytoplasmic) and ADK-long (nuclear) isoforms, respectively (28). β-galactosidase activity was used to assess ADK-expression in the pancreas of Adk₁⁺ mice (Fig. 1B, left panels). ADK was highly expressed in the exocrine pancreas and modestly expressed in the endocrine pancreas where the nuclear-located isoform (ADK-long) predominates. Newborn litters from heterozygous (Adk₁⁺ x Adk₁⁺) breeding pairs contained Adk₁⁺ pups at Mendelian frequency. However, consistent with the published phenotype of ADK-null animals, no Adk₁⁻ mice were identified at weaning (>150 mice screened) (29). Furthermore, ADK protein was nearly undetectable in hepatocyte lysates from Adk₁⁻ newborn pups (Fig. 1C). These results indicated efficient disruption of ADK expression by the targeting strategy.

We disrupted ADK expression in β-cells (βADKO mice) using the Rip-Cre driver strain (30). We confirmed β-cell-restricted disruption by measuring β-galactosidase activity in pancreatic tissue sections from (1) mice harboring the gene-trap vector in the non-mutagenic orientation (Adk²⁺ mice), (2) Rip-Cre mice and (3) Rip-Cre Adk²⁺ mice (βADKO-Het) (Fig. 1B). As anticipated, pancreatic sections from Adk²⁺ and Rip-Cre animals lacked β-galactosidase activity whereas βADKO-Het sections displayed β-cell restricted β-galactosidase activity. Notably, Rip-Cre mice have recombination activity in the brain and exhibit glucose intolerance (31; 32). Consequently, all studies included Rip-Cre control animals. Next, we assessed ADK disruption by Western blot of liver and islet lysates from Rip-Cre, βADKO-Het and
βADKO mice (Fig. 1D). Whereas hepatic ADK expression was similar among genotypes, islet ADK expression was reduced in βADKO animals. Residual ADK expression in islet samples might reflect exocrine and/or insulin-negative islet-cell contamination. Taken together, these data indicate successful disruption of ADK expression in pancreatic β-cells.

**βADKO Mice Are Protected Against HFD-Induced Glucose Intolerance.**

Young βADKO animals were heavier than Rip-Cre control littermates (Fig. 2A). At eight weeks of age, female βADKO mice weighed ~2g more than Rip-Cre control animals (18.3±0.3 g vs.16.1±0.6 g, p<0.01). As mice aged the bodyweights of βADKO and Rip-Cre mice converged; no statistical difference was present beyond 14 weeks (p>0.05). By 52 weeks, the body weights of Rip-Cre (28.9+/−1.4 g) and βADKO (29.2+/−1.5 g) were indistinguishable. HFD-fed 13-week βADKO and Rip-Cre mice demonstrated increased weight gain compared to genotype-matched NC-fed mice beginning two weeks after HFD initiation (p<0.05 for weeks 15-21; filled vs. open symbols). HFD-induced weight gain in Rip-Cre and βADKO mice was similar (Fig. 2B, p>0.05 at every time point). Thus, βADKO animals are transiently heavier than control animals but not predisposed to increased HFD-induced weight gain.

Next, we assessed the impact of β-cell-targeted ADK disruption on glucose tolerance by performing ipGTTs every 4-8 weeks on NC-fed Rip-Cre and βADKO mice. No difference was observed until 52-weeks when subtle improvement in the ipGTT of βADKO mice emerged (Fig. 2C,D). To assess a potential protective role of ADK disruption against diabetogenic challenge, ipGTTs were performed on Rip-Cre and βADKO mice placed on a HFD. After only two-weeks of HFD, female βADKO mice displayed lower fasting glucose levels but similar glucose tolerance (Fig. 2E). Following six-weeks and 18-weeks of HFD, female βADKO mice demonstrated substantial improvement in their ipGTT (Fig.
A similar improvement in ipGTT was also observed in HFD-fed male βADKO mice (Fig. 2H). Fasting and random fed glucose values were also significantly improved in HFD-fed βADKO mice (Fig. 2,I,J). Thus, βADKO animals were resistant to HFD-dependent impairment of glucose homeostasis.

**HFD-Fed βADKO Mice Demonstrate Enhanced Insulin Sensitivity and Insulin Secretion.**

To assess the physiologic basis of improved ipGTT in HFD-fed βADKO mice we performed i.p. insulin tolerance testing (ipITT; Fig. 3A,B). As anticipated, βADKO fasting glucose values were lower than controls (154±19 vs. 214±34, p<0.01). Consequently, we compared glucose clearance by βADKO and Rip-Cre animals with (Fig. 3A) and without (Fig. 3B) normalizing to starting glucose values. Interestingly, βADKO mice displayed more robust insulin responsiveness at 30 and 60 minutes after insulin injection. However, the absolute drop in blood glucose levels in response to insulin was greater for Rip-Cre mice compared to βADKO mice (Rip-Cre 156±29 vs. βADKO 124±18, p<0.05). These results indicated that HFD-fed βADKO mice had modestly enhanced insulin sensitivity but, given the subtlety of this difference, other factors were likely contributing to the improved glucose tolerance of the βADKO mice.

Next we evaluated whether enhanced insulin secretion contributed to the improved glucose tolerance of βADKO mice by measuring glucose-stimulated insulin secretion (GSIS) in NC- and HFD-fed Rip-Cre and βADKO mice (Fig. 3C). As anticipated HFD-fed mice displayed increased insulin secretion relative to NC-fed mice. Interestingly, both NC- and HFD-fed βADKO mice displayed increased insulin secretion compared to Rip-Cre controls (Fig. 3C,D). The increased GSIS of NC-fed mice was unexpected since no difference in ipGTT was detected. However, this difference was subtle (NC AUC Rip-Cre 8.1±2.1 vs.
βADKO 13.6±1.9, p<0.05) compared to HFD-fed mice (HFD AUC Rip-Cre 32.31±3.7. vs. βADKO 61.4±9.9, p<0.05).

β-Cell Expansion Protects βADKO Mice from HFD-Induced Glucose Intolerance.

Islet isolations from HFD-fed βADKO mice demonstrated increased yield (1.3±0.2-fold; p<0.01) compared to Rip-Cre mice (Fig. 4A). This observation led us to consider two potential explanations: (1) HFD-fed βADKO mice had more islets or (2) HFD-fed βADKO mice had larger islets that were more efficiently isolated. To assess these possibilities, islet size and number were analyzed in pancreatic sections from HFD-fed Rip-Cre and βADKO animals. Visual inspection of insulin-stained sections gave the impression that βADKO islets were enlarged (Fig. 4B). To quantitate this impression, we measured the insulin-stained area. Consistent with visual observation, βADKO animals demonstrated a right shift in insulin cluster size (Fig. 4C). Additionally, the average insulin-positive cluster size was increased in βADKO mice (average insulin area, βADKO 7,790±694µm² vs. Rip-Cre 3857±952µm², p<0.01) (Fig. 4D). Furthermore, the insulin-positive area and β-cell mass but not pancreatic area or pancreatic mass were increased in βADKO mice (Fig. 4E-I). Notably, no increase in the number of insulin-positive clusters was detected (Rip-Cre 6.34±1.9 vs. βADKO 7.6±0.4; p=0.4) (Fig. 4J). No change in alpha-cell area was detected (Fig. 4K) and β-cell proliferation in HFD-fed mice, as measured by BrdU incorporation, demonstrated a trend toward increase in the βADKO mice (Fig. 4L). Taken together, these results indicated that β-cell expansion rather than increased islet number was present in HFD-fed βADKO mice.
Disruption of ADK Expression in Mature β-Cells Protects Against HFD-Induced Hyperglycemia and Promotes β-Cell Replication.

To mitigate deficiencies of the Rip-Cre transgene, \( \text{Tg(Ins2-Cre}\text{)}^{25\text{Mgn}} \), we reevaluated our findings with the inducible and β-cell specific Cre-expressing Mip-Cre/ERT mouse line \( \text{Ins1-cre/ERT}^{1\text{Lphi}} \) (31; 32). Because Mip-Cre/ERT mice have tamoxifen-independent growth hormone expression, we used vehicle-treated iβADKO mice as control subjects (33).

We evaluated the impact of tamoxifen-induced β-cell specific ADK disruption on glucose homeostasis and β-cell replication in mature animals (Fig. 5A). Tamoxifen-dependent disruption of ADK-expression was confirmed by induction of β-galactosidase activity (Fig. 5B). Similar to NC-fed βADKO mice, NC-fed vehicle- and tamoxifen-treated iβADKO mice demonstrated comparable ipGTTs (Fig 5C). Consequently, tamoxifen-treatment had no impact on the ipGTT. Also consistent with the βADKO phenotype, HFD-fed tamoxifen-treated mice demonstrated significantly lower fed glucose values (Fig. 5D) and enhanced ipGTT following 3 weeks (Fig. 5E) and 11 weeks (Fig. 5F) of HFD. By contrast, insulin sensitivity measured by ipITT was unchanged (Fig. 5G); perhaps indicating an impact of ectopic recombinase activity in βADKO mice. Finally, we assessed whether disruption of ADK enhanced \textit{in vivo} GSIS. Indeed, tamoxifen-treated mice demonstrated increased insulin secretion following glucose challenge (15 minute vehicle-treated 0.15±0.02 ng/mL vs. tamoxifen-treated 0.24±0.04 ng/mL, \( p=0.01 \); Fig. 5H). These data indicated that β-cell-specific disruption of ADK in adult mice was protective against HFD-induced glucose intolerance and enhanced GSIS \textit{in vivo}.

Next, we assessed the impact of ADK disruption on mature β-cell proliferation. To perform this analysis, we determined the percentage of insulin-positive cells that co-expressed ki67 in vehicle- and tamoxifen-
treated mice fed either NC or HFD. Interestingly, loss of ADK expression had no effect on β-cell replication in NC-fed mice (% replication of vehicle 0.36±0.1 vs. tamoxifen 0.50±0.10, p=0.38); however, ki67 expression was significantly increased in HFD-fed tamoxifen-treated mice (vehicle 1.59±0.10 vs. tamoxifen 2.70±0.32, p<0.05) (Fig. 5I). We confirmed this finding using distinct β-cell (PDX1) and replication (BrdU incorporation) markers (vehicle 1.00±0.26 vs. tamoxifen 2.33±0.26, p<0.05)(Fig. 5J). Representative images used to quantify β-cell replication are shown (Fig. 5K). Finally, we compared the in vitro basal and compound-induced replication index of β-cells obtained from vehicle- and tamoxifen-injected iβADKO mice (Fig. 5L). Islet ADK deletion was confirmed by western blot (Fig. 5M). Notably, ADK-deficient β-cells (tamoxifen-injected) displayed increased basal replication compared to ADK-expressing β-cells (Fig. 5L). Additionally, ADK-expressing β-cells displayed a ~two-fold replication increase following treatment with 5-IT (an ADK and DYRK1A/B inhibitor) (Fig.5L) (19; 20). Similarly, 5-IT treatment of ADK-deficient β-cells demonstrated a ~1.5-fold increase in β-cell replication. Therefore, disruption of ADK expression in β-cells increases the basal β-cell replication index in vitro but does not eliminate 5-IT-dependet replication induction. These results indicate that ADK negatively regulates β-cell replication in vitro and that the β-cell replication-promoting activity of 5-IT is not mediated entirely via ADK-inhibition.
DISCUSSION

Identification of novel strategies to enhance \textit{in vivo} \(\beta\)-cell proliferation and mass while retaining optimum function is an attractive therapeutic strategy for diabetes. Recently, we discovered that short-term treatment with ADKI’s stimulated rodent and porcine \(\beta\)-cell replication (19). Herein, we used a novel genetic mouse model to study the function of ADK in \(\beta\)-cells and to contemplate the potential utility of ADK inhibition. This study addressed two critical questions: (1) is long-term disruption of ADK detrimental to \(\beta\)-cell function? (2) Are mice lacking ADK expression in their \(\beta\)-cells protected against diabetogenic insults such as aging and HFD? Using two different mouse models, we found \(\beta\)-cell selective disruption of ADK expression to be well tolerated and protective against HFD challenge.

\textit{Is long-term disruption of ADK detrimental to \(\beta\)-cell function?} Currently available ADKI’s are not amenable to long-term \textit{in vivo} treatment because of associated toxicity (19; 34). Additionally, global disruption of ADK expression results in perinatal lethality (29). These findings raise concern about potential detrimental effects of ADK disruption on \(\beta\)-cell viability and function. We found that ADK expression in the islet was low relative to the exocrine pancreas and that disruption of ADK had limited impact on \(\beta\)-cell development, function and growth. NC-fed mice lacking \(\beta\)-cell expression of ADK, either constitutively or acutely, displayed glucose homeostasis parameters similar to control mice with no detectable difference in \(\beta\)-cell replication or mass. Hence, disruption of ADK in \(\beta\)-cells was well tolerated.

\textit{Are \(\beta\)-cells lacking ADK activity protected against diabetogenic insults such as aging and HFD?} Although NC-fed ADK-deficient mice were essentially indistinguishable from control animals, a subtle but consistent improvement in ipGTT emerged as animals aged beyond one year. To better gauge the
potential benefit of ADK disruption on glucose homeostasis, βADKO mice were challenged with a HFD. Indeed, HFD-fed mice harboring ADK-deficient β-cells displayed significantly enhanced ipGTT, enhanced \textit{in vivo} GSIS and increased β-cell mass. No change in α-cell mass was observed, indicating that the impact was cell-autonomous and/or lineage specific. Additionally, we found no evidence for islet neogenesis as islet density (insulin clusters per section) was unchanged in HFD-fed βADKO mice. Although βADKO mice maintained on HFD for several months displayed increased β-cell mass, active β-cell replication was not significantly increased in these animals. By contrast, β-cell replication was increased one week after conditional disruption of ADK expression. These observations are consistent with prior work demonstrating that β-cell replication rates initially increase in response to HFD but decline with prolonged exposure (35). Interestingly, ADK-deficient β-cells demonstrated an increased \textit{in vitro} replication index compared to control β-cells. Notably, the ADKI 5-IT that promotes human β-cell replication, in part through DYRK1A/B inhibition (25), further enhanced the replication of ADK-deficient β-cells; suggesting that inhibition of both ADK and DYRK1A/B contribute to rodent β-cell replication control. In summary, disruption of ADK expression in β-cells was protective against HFD-induced glucose intolerance in mice, in part as a result of an increased β-cell replication response, β-cell mass expansion and enhanced insulin secretion.

Numerous mouse models of β-cell specific gene disruption yield altered β-cell mass and/or glucose homeostasis (36). However, the vast majority of these models demonstrate an impact under basal conditions. Disruption of ADK expression in β-cells is distinct in that essentially no phenotype was identified in unchallenged animals. These finding suggest that ADK plays a role in dampening the adaptive β-cell response to metabolic challenge. Defining factors that specifically modulate the adaptive β-cell response is an important avenue for uncovering therapeutic strategies for T2D. Similar to ADK,
Connective Tissue Growth Factor (CTGF) contributes to the adaptive response of β-cells: over-expression of CTGF in mature β-cells has no impact on β-cell proliferation but does enhance β-cell expansion under diabetogenic conditions (37). Similarly, β-cell-specific deletion of the prolactin receptor has no impact on β-cell mass or function under basal conditions but predisposes female mice to gestational diabetes by dampening the β-cell replication response during pregnancy (38). Although the molecular links between CTGF, prolactin signaling and ADK are not immediately obvious, these models highlight the potential to therapeutically manipulate the adaptive response of β-cells. Perhaps more relevant, β-cell-specific deletion of the adenosine receptor 2a (Adora2a) in mice was recently shown to impair glucose regulation and β-cell proliferation in pregnancy while having no impact on these parameters under basal conditions (39); indicating a role for adenosine signaling in promoting the adaptive β-cell response to an insulin-resistant state. Notably, a primary function of ADK is to reduce extracellular adenosine levels via adenosine phosphorylation. Hence, disruption of ADK in β-cells might promote an adaptive β-cell response in vivo by increasing extracellular adenosine levels and augmenting Adora2-dependent signaling.

There are notable limitations to our study. First, caution must be taken with use of the Rip-cre and MIP-Cre/ERT transgenic lines (32; 33). Prior studies have shown detrimental effects of the Rip-Cre cassette on glucose tolerance and insulin secretion; findings opposite to what we observed in the βADKO mice. Consequently, impacts of the Rip-Cre line are anticipated to bias results away from the observed protective effect of ADK-deletion on HFD-induced glucose intolerance. Additionally, complimentary findings obtained with the constitutive and inducible Cre driver strains exclude developmental impacts and ectopic gene deletion as the probable basis of the enhanced β-cell replication, mass and function we observed. Second, the applicability of our findings to human β-cells is unknown as adult human β-cells
have limited regenerative capacity (14; 40). Third, the potential applications of *in vivo* ADK inhibition may be limited by the function of ADK in other tissues such as the liver. Indeed, identifying methods for lineage-restricted drug delivery is likely to be a critical hurdle for developing regenerative therapies for diabetes. Future efforts will focus on better understanding the mechanism by which ADK disruption enhances the adaptive response of β-cells to HFD challenge.
**Contribution statement** GN conceived and designed experiments, performed experiments, analyzed data and approved the final version of the manuscript, YA conceived and performed experiments, analyzed data and assisted in writing of the manuscript, ZZ, HH and SL performed experiments, analyzed data and approved the final version of the manuscript, NAA analyzed data and and assisted in writing of the manuscript, JPA conceived and designed experiments, analyzed data and wrote the manuscript. JPA is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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**Duality of interest** The authors declare no duality of interest associated with their contributions to this work.
References


FIGURE LEGENDS

Figure 1 Conditional disruption of ADK gene expression. (A) Schematic representation of the Adk locus and targeting construct: mutagenic orientation (ADK¹), Flp recombinase-dependent non-mutagenic orientation (ADK²) and Cre recombinase-dependent mutagenic orientation (ADK³). Forward primer (Fwd), reverse primer (Rev), B32 primer, recombination sequences (Frt and LoxP), splice acceptor sequence (SA) and the β-galactosidase / neomycin resistance fusion gene (βgeo) are shown. (B) Histochemical staining of pancreatic sections from Adk-targeted mice for β-galactosidase activity (blue); counterstaining with eosin (pink, top row) or insulin (brown, bottom row) are shown. (C) ADK-directed western blot of hepatic tissue lysates from Adk-targeted and control mice. A non-specific upper band reflects sample loading (LC). (D) Liver (left) and islet (right) lysates probed for ADK (upper panels) and enolase (loading control, lower panels).

Figure 2 Constitutive deletion of ADK in β-cells enhanced glucose tolerance. (A) Body weights of normal chow (NC) and high fat diet (HFD)-fed Rip-Cre and βADKO female mice (n=8 mice per group). Statistical comparisons: RIP-Cre (NC) vs. βADKO (NC), p<0.05 for weeks 8 and 13 only; RIP-Cre (HFD) vs. βADKO (HFD) p>0.05 for all time points; RIP-Cre (NC) vs. RIP-Cre (HFD), p<0.05 weeks 15-20; βADKO (NC) vs. βADKO (HFD), p<0.05 weeks 15-20. (B) Weight gain of RIP-Cre and βADKO mice on HFD (no statistical differences detected). (C) ipGTT of 13 week NC-fed RIP-Cre and βADKO mice (n=15 each group; p>0.05 for all time points). (D) ipGTT of 52 week NC-fed RIP-Cre and βADKO mice (n=8 each group; p<0.05 at 120 minutes and p>0.05 for all other time points). (E) ipGTT of RIP-Cre and βADKO mice after two weeks of HFD (age 15 weeks; n=7 each group; p<0.05 at 0 minutes and p>0.05 for all other time points). (F) ipGTT of female RIP-Cre and βADKO mice after six weeks of HFD (age 19 weeks; n=7 each group; p<0.05 at 30, 60 and 90 minutes).
mice after 18 weeks of HFD (age 31 weeks; n=7 each group; p<0.05 at 30 and 60 minutes). (H) ipGTT of male Rip-Cre and βADKO mice after 6 weeks of HFD (age 19 weeks; n=7 each group; p<0.05 at 30, 60 and 120 minutes). (I) Fasting glucose values of female Rip-Cre and βADKO mice after 23 weeks of HFD (age 30 weeks, n=8 each group; *p<0.01) (J) Random glucose values of female Rip-Cre and βADKO mice after 12 weeks of HFD (age 25 weeks; n=8 each group; *p<0.01).

Figure 3 HFD-fed βADKO mice have enhanced insulin tolerance and glucose-stimulated insulin secretion in vivo. (A) Normalized and (B) raw glucose values from 20 week HFD-fed female Rip-Cre and βADKO mice subjected to an ITT (n=7 per group; *,p<0.05). (C) In vivo GSIS of 24 week NC- or HFD-fed female Rip-Cre and βADKO mice (n=5-8 mice per group). Significant differences are for comparisons made between Rip-Cre and βADKO mice on the same diet indicated (*p<0.05). Insulin levels are significantly higher at all time-points in HFD-fed mice (p<0.05). (D) The total insulin secretion (AUC) calculated from Fig. 3C (*, p<0.05).

Figure 4 HFD-fed βADKO animals demonstrate increased β-cell but not alpha-cell mass. (A) Relative number of islets isolated from 28 week (21 weeks of HFD) female HFD-fed Rip-Cre and βADKO mice (n=8; *,p<0.05). The average number of islets isolated per mouse was 107.5±10.1 and 141.7±17.0 from the HFD-fed Rip-Cre and βADKO mice, respectively. (B) Representative images of 24 week female HFD-fed Rip-Cre- and βADKO-derived pancreatic sections stained for DAPI (blue) and insulin (red). (C) Size distribution of insulin\(^+\) area obtained from Rip-Cre and βADKO pancreatic sections (minimum of 6 per mouse) stained as in Fig.4b (n=5 mice per group, *p<0.05). (D) Average and (E) total insulin\(^+\) cluster area (n=5 mice per group, *p<0.05). (F) Total pancreas area measured based upon DAPI staining (data are mean ±SEM). (G) Percentage of pancreatic area (DAPI) that is insulin\(^+\) (n=5, *p<0.05). (H) Average
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**Figure 5** Conditional deletion of ADK in the β-cells of HFD-fed mice enhances glucose tolerance and β-cell replication. (A) The temporal relationship of experiments performed on iβADKO mice (red text indicates male mice only). (B) Representative β-galactosidase histochemistry in pancreatic sections obtained from vehicle- and tamoxifen-injected animals one week post injection. (C) ipGTT of NC-fed female mice treated with vehicle or tamoxifen (n=8 per group; no significant differences observed). (D) Fed glucose values obtained from 22 week NC and HFD-fed (4 weeks) mice (n=8 per group; * p<0.05) (E) ipGTT of 21 week (3 weeks of HFD) and (F) 29 week (11 weeks of HFD) female mice (n=8 per group; * p<0.05). (G) ipITT of HFD-fed female mice treated with vehicle or tamoxifen (n=8 per group; no significant differences observed). (H) *In vivo* GSIS measurements of HFD-fed female mice treated with vehicle or tamoxifen (n=8 per group; *p<0.05). (I) β-cell replication index of male NC- and HFD-fed iβADKO mice that received vehicle or tamoxifen treatment (n=8 per group; *p<0.05). (J) β-cell replication index of vehicle- and tamoxifen-treated HFD-fed male iβADKO mice (n=8 per group; *p<0.05). (K) Representative images of pancreatic sections used for β-cell replication analysis. Images were obtained from vehicle- (left) and tamoxifen-treated (right) iβADKO mice stained for DAPI (blue), insulin (red) and ki67 (green, top panels) or DAPI (blue), PDX1 (red) and BrdU (green, lower panels). (L) *In vitro* β-cell replication index of DMSO- and 5-IT- (2µM) treated islet cultures obtained from vehicle-
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