Alterations in β-cell calcium dynamics and efficacy outweigh islet mass adaptation in compensation of insulin resistance and prediabetes onset

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Running Title: β-cell function outweighs islet mass adaptation

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Abstract

Emerging insulin resistance is normally compensated by increased insulin production of pancreatic β-cells, thereby maintaining normoglycemia. However, it is unclear whether this is achieved by adaption of β-cell function, mass, or both. Most importantly it is still unknown which of these adaptive mechanisms fail when type 2 diabetes develops. We performed longitudinal in vivo imaging of β-cell calcium dynamics and islet mass of transplanted islets of Langerhans throughout diet-induced progression from normal glucose homeostasis, over compensation of insulin resistance to pre-diabetes. The results show that compensation of insulin resistance is predominated by alterations of β-cell function, while islet mass only gradually expands. Hereby, functional adaptation is mediated by increased calcium efficacy, which involves Epac signaling. Prior to prediabetes, β-cell function displays decreased stimulated calcium dynamics, whereas islet mass continues to increase through prediabetes onset. Thus, our data reveal a predominant role of islet function with distinct contributions of triggering and amplifying pathway in the in vivo processes preceding diabetes onset. These findings support protection and recovery of β-cell function as primary goal for prevention and treatment of diabetes and provide insight into potential therapeutic targets.

Obesity and insulin resistance are important risk factors for type 2 diabetes (T2D) (1). However, the majority of obese and insulin resistant subjects do not develop hyperglycemia (2). This is attributed to the compensatory capacity of the islets of Langerhans, which meet the increased insulin demand by an elevated hormone output (3). Two processes have been implicated in islet compensation in response to obesity and insulin resistance: Firstly, an increased functionality and insulin secretion rate of the individual β-cells, e.g. by enhancing glucose metabolism, insulin gene expression or translation (4-6). In addition, increased systemic insulin output can be achieved by a morphological enlargement of β-cell mass through proliferation (7; 8), hypertrophy (5; 8) or neogenesis (9; 10). Whereas in rodents β-cell mass has been reported to increase substantially in response to insulin resistance (7; 11) this seems to be less pronounced in humans (12-14).

Failure of β-cell compensation in insulin resistance and obesity is believed to be a cause of T2D. Both, altered β-cell function and mass have been suggested to contribute to hyperglycemia. Decreased β-cell function was observed in islets isolated from type 2 diabetic organ donors (15) and animal models (16). In addition, rodent T2D models were shown to exhibit a reduction in β-cell mass (11; 17-19). Data on alterations of β-cell mass in type 2 diabetic humans are inconsistent and vary from no significant changes up to a 63% decrease in comparison to non-diabetic subjects (12; 14; 20; 21). However, the relative contribution of β-cell mass and function to the maintenance of normoglycemia in insulin resistance and their distinct roles in the onset of insulin deficiency and hyperglycemia are unclear. It remains elusive if the observed β-cell changes in T2D patients and hyperglycemic animal models are cause or consequence of the altered glucose homeostasis. To a large part this is due to the inability to separately assess β-cell mass and function in vivo.

In this study, we monitored for the first time in vivo dynamics and mechanisms of islet and β-cell mass in relation to systemic glucose homeostasis during long-term diet-induced obesity and insulin resistance. This allowed us to assess the kinetics and relative contribution of β-cell mass during the compensation phase and pre-diabetic stage of diabetes pathogenesis. Our results show that the functional response of β-cells dominates over islet mass adjustment in compensation of insulin resistance and during the development of prediabetes. To study the underlying mechanism of functional adaptation we monitored intracellular calcium dynamics of β-cells longitudinally in vivo. This revealed increased calcium efficacy as a crucial adaptive mechanism of β-cell function, which was dependent on Epac signaling in isolated islets. Meanwhile intracellular calcium dynamics in vivo showed an early decline preceding the onset of prediabetes. Therefore, our study provides novel insight into the pathogenesis of T2D and potential therapeutic targets.

Research Design and Methods

Mice

All experiments were conducted in accordance with the German Animal Welfare Act and approved by the Committee on the Ethics of Animal Experiments of the State Directory of Saxony. C57BL/6N albino mice (B6N-$^{Tyrc\text{-}Brd}$/BrdCrCrl, Charles River) were used as recipients for islet transplantation. Mice expressing green fluorescent protein under control of the mouse insulin I promoter (MIP-GFP) (22) and Pdx1CreER-GCaMP3 mice, both on C57Bl/6J background (Jackson Laboratories, ME, USA), were used as islet donors. Pdx1CreER-GCaMP mice were generated by crossing Pdx1CreER mice (23) with homozygous GCaMP3 mice (24) (Jackson Laboratories). To induce β-cell specific GCaMP3 protein expression, Pdx1CreER-

GCaMP3 mice were injected with tamoxifen (3 x 4 mg in corn oil in 5 days, sc) two weeks before islet isolation.

Diet-induced obesity model

Recipient mice received either standard normal diet (ND; Ssniff) or rodent high-fat diet with 60% kcal% fat (HFD; D12492, Research Diets) for 17 weeks before switching back to ND in the HFD group. Body weight and intraperitoneal glucose tolerance tests (IPGTTs) were assessed at indicated time points. For IPGTTs, mice were fasted for 6 h and injected intraperitoneal with 2 g glucose per kg body weight. Blood glucose was measured at 0, 30, 60 and 120 minutes after glucose injection using a glucometer (AccuCheck Aviva, Roche). Plasma insulin was measured at 0 and 30 minutes using a mouse Ultrasensitive Insulin ELISA kit (ALPCO). Pre-diabetes was defined as significantly increased 2hr blood glucose values at unchanged non-fasting blood glucose levels.

Islet isolation, transplantation and in vivo imaging of islet and β-cell volume

Islet isolation and transplantation to the anterior chamber of the eye was performed as previously described (25-28). Transplanted islets were allowed to engraft fully for at least 4 weeks before starting any in vivo experiments. In vivo imaging was performed as published before (26). Briefly, islet recipients were intubated and anesthetized by 2% isoflurane in 100% oxygen. A drop of 0.4% pilocarpine (Pilomann; Bausch&Lomb, NY, USA) in saline was placed on the cornea shortly before imaging to limit pupil dilation and iris movement. Animals were fixated and kept on a heating pad during the imaging procedure. Repetitive in vivo imaging was performed at indicated time points on an upright laser scanning microscope (LSM780 NLO; Zeiss, Germany) with a water dipping objective (W Plan-Apochromat 20x/1.0 DIC M27 75mm; Zeiss) using vidisic eye gel (Bausch&Lomb) as immersion. The total volume of transplanted

islets was assessed by detection of 633 nm laser backscatter, which allows assessment of islet morphology based on its light scattering properties (27). Total volume of each islet was calculated using surface rendering after 3D reconstruction of collected image stacks (Imaris 7.6, Bitplane AG, Switzerland). β-cell volume in MIP-GFP islets was assessed by two-photon laser excitation of GFP at 910 nm and detection at 500 - 550 nm using a non-descanned Gallium Arsenide Phosphate (GaAsP) detector. β-cell volume was then quantified by automatic surface rendering from median filtered z-stacks in Imaris 7.6 (Bitplane AG, Switzerland). Qtracker® 655 (0.4 μM in 100 μl PBS; Life Technologies) was injected into the tail vein to visualize blood vessels. Qtracker were excited by two-photon laser at 910 nm and detected at 635 - 675 nm. Vessel volume was calculated by automatic surface rendering. Vessel network length and vessel diameter were measured by filament-tracing within the vessel surface using Imaris software.

In vivo imaging of β-cell cytosolic free calcium dynamics

Pdx1CreER-GCaMP3 islet recipient mice were fasted overnight and anesthetized with an intraperitoneal injection of a mixture of fluanisone (12.5 μg/g body weight; CHEMOS, Germany), fentanyl (0.788 μg/g body weight; Hameln pharma plus, Germany), and midazolam (12.5 μg/g body weight; Ratiopharm, Germany). The anesthetized mice were intubated and respiration was maintained by use of a small animal ventilator with room air (270 μl stroke volume, 250 strokes / min; Hugo Sachs, Germany). Animals were fixed and positioned as described above. In vivo imaging was performed using confocal laser scanning microscopy. The volume of islets was assessed by detection of 633 nm backscatter laser light. The functional index of islets was calculated by dividing stimulated plasma insulin over fold changes in islet mass at corresponding time points in the same mice. GCaMP3 GFP was excited at 488 nm and detected at 468 - 607 nm. Z-stacks of the islet were acquired at 1.5 μm intervals and time-series recording of GCaMP3 GFP signal from the same islets were carried out with an image sampling rate of 2

seconds. After acquiring a baseline recording for 5 minutes, glucose was injected intravenously (1 g/kg body weight) via the tail vein. Changes in β-cell intracellular calcium (Ca^{2+}_i), as reflected by changes in GCaMP3 GFP fluorescence intensity, were recorded for 30 minutes after glucose injection. After the recording mice were maintained on ventilator breathing until autonomous respiration was restored. Z-stacks and time-series were processed using Imaris 8.1 software (Bitplane, Switzerland). The GCaMP3 expressing β-cell area was defined by applying a threshold acquired in the GFP negative area. Mean GCaMP3 GFP fluorescence per β-cell area was calculated for each frame and normalized to the minimal value in the baseline. Finally, total β-cell Ca^{2+}_i dynamics was quantified by calculating the area under curve of the fluorescence trace for 5 minutes before glucose injection (basal Ca^{2+}_i dynamics) and 30 minutes after glucose injection (stimulated Ca^{2+}_i dynamics). In vivo calcium efficacy was calculated by dividing stimulated plasma insulin over the product of stimulated Ca^{2+}_i dynamics per minute and fold changes in islet mass at corresponding time points in the same mice. 1st phase peak amplitude was defined by the peak intensity over mean basal intensity within 5 minutes after glucose injection.

In vitro culture and insulin secretion

After overnight culture in standard RPMI 1640 medium, islets from female C57Bl/6J mice were handpicked and cultured in 24-well plates in culture medium with a 0.1 mM BSA, 8 mM glucose and 0.5 mM BSA-conjugated palmitate ($G_8P_{0.5}$). Control islets were cultured in RPMI 1640 media in the presence of 5.5 mM glucose without palmitate ($G_{5.5}P_0$). The EPAC specific inhibitor ESI-09 (1 to 5 μM; Sigma) was applied in both, basal culture and GSIS when indicated. Basal insulin secretion was assessed from twenty-four hour culture medium and corrected for release per hour. For assessment of stimulated insulin release islets were placed for 1 hour in their respective culture medium, containing elevated glucose concentrations (25 mM). Islets were

lysed in acid/ethanol buffer for insulin content. Total insulin content was defined by the sum of insulin secreted into medium upon stimulation and insulin content in the lysate. Collected medium and islet lysate were stored at -20 ℃ until insulin content was measured using a mouse Ultrasensitive Insulin ELISA kit (ALPCO).

In vitro imaging of β-cell cytosolic free calcium dynamics

For in vitro imaging of cytosolic free calcium dynamics in β-cells, Pdx1CreER-GCaMP islets were handpicked after overnight culture and embedded in fibrin gels on coverslips according to a previously published protocol (29). Briefly, fibrin gels were prepared by mixing 3 µl HBSS with 1 µl human fibrinogen (10 mg/ml in HBSS; Sigma), after which 6 islets were placed individually in the gel. Fibrinogen polymerization was induced by adding 1 µl human thrombin (50 units/ml in HBSS; Sigma). Subsequently, the gel-embedded islets were cultured for 24 hours in indicated conditions. Calcium imaging was performed in a custom-made perifusion system (30). Briefly, coverslips with gel-embedded islets were moved into a temperature-controlled perifusion chamber where the temperature was set at 37 ℃. In vitro imaging was performed in the above described LSM system. GCaMP3 GFP was excited at 488 nm and detected at 493 - 598 nm. Time-series recording of the GCaMP3 GFP signal from the same islets were carried out with an image sampling rate of 2 seconds. A basal recording was acquired for 3 minutes when the islets were perifused in their respective culture medium. For stimulation islets were perifused with their respective culture medium, containing elevated glucose concentrations (25 mM). Changes in β-cell Ca^{2+}_i were recorded for 25 minutes after stimulation. Mean GCaMP3 GFP fluorescence per β-cell area and total β-cell GCaMP3 GFP fluorescence was processed and quantified using the same protocol as for the in vivo imaging. Basal and stimulated Ca^{2+}_i dynamics are displayed as area under the curve per minute in basal and stimulated conditions, respectively. Calcium

efficacy was calculated by dividing stimulated insulin over stimulated Ca^{2+}_i dynamics per minute in corresponding experiments.

Pancreatic β-cell area

Fractional pancreatic β-cell area was assessed in cryosections (10 µm) stained for insulin (1:200 dilution; Dako, Germany), glucagon (1:500; Millipore, MA, USA) and DAPI (2.5 µg / ml; Sigma-Aldrich). Immunostaining was visualized by Alexa 488, 633 secondary antibodies (1:200; Life Technologies). Images were acquired in a slide scanner (Axio Scan.Z1; Zeiss, Germany). Quantification of immunohistochemistry was done manually using Fiji software. Fractional β-cell area was calculated from at least forty pancreas sections (with a minimal distance of 150 µm between sections) per mouse.

Statistical analysis

Data are expressed as mean ± SEM. Statistics were analyzed using Prism 6 (GraphPad Software, CA, USA) or SPSS 21 (IBM, NY, USA). To properly acknowledge the two sources of correlation that are inherent to the repeated longitudinal measurements, longitudinal in vivo data were analyzed by Linear Mixed Models (26). Significant differences are indicated by * ($P < 0.05$).

Results

High fat diet induces impaired glucose tolerance and prediabetes despite the compensational release of insulin

We transplanted reporter islets for in vivo imaging into the anterior chamber of the eye of albino C57Bl/6N mice. After engraftment recipient mice were fed with normal diet (ND; 11% kcal from fat) or high fat diet (HFD; 60% kcal from fat) for 17 weeks, followed by 2 weeks ND. HFD mice showed continuously increasing bodyweight (37.3 ± 0.6 vs. 26.2 ± 0.5 g, HFD vs. ND, 17 weeks;

Fig. 1A). Furthermore, fasting blood glucose levels were slightly, but significantly increased at several time points during the study (Fig. 1B). Fasting and stimulated plasma insulin levels in response to an intraperitoneal glucose tolerance test increased during HFD, reaching a plateau at 9 weeks (fasting: 0.68 ± 0.11 vs. 0.16 ± 0.06 ng / ml, HFD vs. ND, 9 weeks; stimulated: 1.28 ± 0.16 vs. 0.34 ± 0.06 ng / ml, HFD vs. ND, 9 weeks ; Fig. 1C,D). Also the fasting and stimulated plasma insulin levels in relation to the given glucose concentration were increased in HFD, indicating an increased sensitivity of β-cells to glucose (Fig. 1E,F). However, despite the elevated insulin release glucose tolerance continuously deteriorated in HFD mice (Fig. 1G). In addition, the 2-hour glucose tolerance test value was significantly higher after 9 weeks HFD feeding, indicating a stage of prediabetes in these animals (Fig. 1H).

Islet and β-cell mass gradually increases in response to HFD

Islets engrafted in the anterior chamber of the eye of the above described mice allowed longitudinal assessment of total islet volume by backscatter laser light during long-term ND and HFD feeding. The acquired backscatter volume included all endocrine cells and the vascular network of an islet (Fig. 2A,B). While the total volume of islets in ND fed mice remained unchanged, islets within HFD fed mice showed a slow but continuous increase in size, doubling their volume after 16 weeks HFD diet exposure (1.97 ± 0.14 vs 1.07 ± 0.08 fold increase, HFD vs. ND, week 16; Fig. 2A,C).

Utilizing MIP-GFP reporter islets in the anterior chamber of the eye during HFD demonstrated that the observed islet mass enlargement was due to β-cell expansion (1.88 ± 0.17 fold increase, week 16; Fig. 3A,B). This was the result of an increase in β-cell number (1.92 ± 0.15 fold increase, week 16; Fig. 3C), whereas no significant change in the individual β-cell size was observed (Fig. 3D). Intra-islet vessels adapted to islet growth and contributed to total islet volume increase, by a lengthening of the vascular network (2.11 ± 0.15 fold increase, week 16; Fig.

3A,E), thereby maintaining a constant fractional vessel volume within the growing islets (data not shown). In addition, intra-islet vessel diameter increased in response to HFD presumably to match the increased secretory activity (8.79 ± 0.09 vs 8.22 ± 0.11 μm, HFD vs. ND, week 16; Fig. 3F). Pancreatic β-cell area showed a similar ca. 2-fold increase after 16 weeks HFD feeding in our setting (Fig. 3G and S1), verifying the validity of the anterior chamber of the eye platform to study islet biology during HFD.

Because the kinetics of islet mass expansion (Fig. 2C) and rising plasma insulin levels (Fig. 1D) within the same animal showed obvious differences we calculated an approximate index of the relative contribution of β-cell function by dividing stimulated plasma insulin by the increase in islet cell mass. β-cell functional index peaked within one week of HFD feeding with a 3-fold increase over ND (3.2 ± 1.0 vs 1.1 ± 0.2, HFD vs. ND; Fig. 4). Thereafter, it remained elevated over ND throughout HFD feeding period at slightly reduced levels (2.6 ± 0.6, 2.8 ± 0.3, and 2.5 ± 0.5 fold at weeks 4, 8 and 16, respectively; Fig. 4). This revealed that β-cell function showed an immediate strong compensatory response to HFD feeding, which in contrast to islet mass did not further increase during the course of pre-diabetes development.

β-cell functional adaptation to high fat diet is associated with increased basal and decreased glucose-stimulated Ca^{2+}_i

We examined the role of intracellular calcium (Ca^{2+}_i) dynamics in the functional compensation of β-cells during prolonged HFD feeding by longitudinal in vivo imaging. To this end we transplanted Pdx1CreER;GCaMP3 islets into albino C57Bl/6N mice. Prior to islet isolation and transplantation CreER-LoxP mediated recombination of GCaMP3 expression was induced by tamoxifen application to the islet donor. As previously reported in neurons (24), Ca^{2+}_i dynamics in β-cells was assessed by monitoring GFP fluorescence of the GCaMP3 reporter in response to an intravenous glucose challenge at indicated time points before, during and after long-term ND

or HFD feeding (Fig. 5). Under control conditions β-cells showed little or no basal Ca^{2+}_i dynamics (Fig. 5A - panel 1). Upon glucose injection into the tail vein of the recipient mouse, β-cells displayed a strong peak of Ca^{2+}_i dynamics (Fig. 5A - panel 2), followed by a lower plateau and/or oscillations in Ca^{2+}_i (Fig. 5A - panels 3-5). Islets either displayed a continuous plateau, regular oscillations or a mix of both after the initial peak. The respective pattern of Ca^{2+}_i dynamics of an islet was comparable at the various recording time points and was synchronized throughout the image plane. Islets in ND mice showed little alterations in Ca^{2+}_i dynamics during repetitive imaging over 19 weeks (Fig. 5B). However, in response to HFD feeding GCaMP3 GFP fluorescence traces displayed considerable changes in basal and stimulated Ca^{2+}_i dynamics (Fig. 5C).

Quantification revealed that minimum values of GCaMP3 GFP fluorescence intensity were comparable between islets in ND and HFD mice throughout the study. This suggests that there were no major changes in expression of the fluorescent reporter or resting intracellular calcium levels. However, basal islet Ca^{2+}_i dynamics, assessed as area under the curve of the GCaMP3 GFP fluorescence trace prior to intravenous glucose injection, continuously increased in islets of the HFD fed mice (7.93 ± 0.45 vs 6.25 ± 0.27 AU; HFD vs. ND, week 16; Fig. 6A). This parallels the observed rising levels of basal plasma insulin (Fig. 1C) which is most likely the result of elevated basal glucose levels (Fig. S2A) and increasing glucose sensitivity (Fig. 1E). In contrast, stimulated Ca^{2+}_i dynamics in response to intravenous glucose decreased early and remained significantly lower in the HFD group (42.05 ± 2.32 vs 52.83 ± 2.60 AU; HFD vs. ND, week 16; Fig. 6B). This was associated with a reduced 1st phase peak amplitude of calcium activity after glucose injection (Fig. 6D), while 2nd phase oscillation amplitude and frequency were highly variable in both groups and showed no significant effect by HFD feeding. Increased β-cell function without elevation in stimulated Ca^{2+}_i dynamics suggested an augmentation of

insulin release by amplification of calcium induced secretion in vivo to overcome impaired glucose tolerance (Fig. 1G and S2B). This was illustrated by the calculated enhanced efficacy of calcium to induce insulin release during HFD feeding (Fig. 6D).

Inhibition of Epac signaling blocks amplified calcium efficacy

To reveal the mechanism underlying amplified calcium efficacy, we mimicked the acute functional compensation by 24 hour culture of isolated mouse islets in 8 mM glucose plus 0.5 mM palmitate ($G_8P_{0.5}$). This condition resembled the slightly elevated glucose levels and the presence of increased free fatty acids observed during HFD in vivo. The control islets were cultured in 5.5 mM glucose without palmitate ($G_{5.5}P_0$) to resemble ND feeding. Compared to control, $G_8P_{0.5}$ islets had significantly higher basal and glucose stimulated insulin secretion (Fig. 7A). At the same time, $G_8P_{0.5}$ islets showed significantly higher basal Ca^{2+}_i dynamics in vitro, while glucose-stimulated Ca^{2+}_i dynamics was slightly but significantly reduced (Fig. 7B). This revealed a significantly higher calcium efficacy of $G_8P_{0.5}$ islets upon stimulation (Fig. 7C) as observed during HFD in vivo (Fig. 6C). Previous data indicate cAMP mediated signaling via the exchange protein activated by cAMP (Epac) to potentiate insulin secretion and mediate the β-cell response to increased metabolic demand (31; 32). We therefore assessed the role of Epac in the observed elevated calcium efficacy in our conditions. ESI-09, an Epac specific inhibitor, dose-dependently (1, 3 and 5 μM) reduced glucose stimulated insulin secretion when supplemented in $G_8P_{0.5}$ islet culture and stimulation, while basal insulin secretion was not significantly affected (Fig. 7D). On the other hand, 5 μM ESI-09 had no effect on basal or stimulated calcium dynamics (Fig. 7E), revealing a significant inhibition of calcium efficacy by 5 μM ESI-09 (Fig. 7F) and indicating a crucial role of Epac in the amplification of insulin secretion by increased calcium efficacy.

Changes in glucose homeostasis and β-cell function are reversed by normal diet feeding

Interestingly, after 17 weeks HFD a subsequent 2-week period of ND was sufficient to reverse the observed phenotype. Body weight decreased but remained significantly elevated (Fig. 1A). Fasting blood glucose and glucose tolerance returned to control values (Fig. 1B,G,H). Also, basal and stimulated plasma insulin levels dropped markedly (Fig. 1C,D). Furthermore, islet mass enlargement stopped when HFD mice were switched back to ND (Fig. 2C). Finally, β-cell functional index (Fig. 4) as well as basal and stimulated calcium dynamics (Fig. 6A,B) were restored to pre-diet values.

Discussion

In our study we assessed for the first time islet mass and β-cell function longitudinally throughout the progression from normal glucose tolerance, over compensation to prediabetes. For this purpose we correlated systemic glucose homeostasis with longitudinal in vivo imaging of transplanted islets of Langerhans during diet-induced obesity. Although we cannot exclude potential effects of the different local environment on intraocular islets, our own data and previous studies suggest that islets in the anterior chamber of the eye closely resemble the behavior of endogenous pancreatic islets (26; 28; 33-35).

Our results reveal that HFD feeding, a model of early diabetes pathogenesis (36), leads to distinct dynamics of β-cell mass and function preceding the onset of diabetes. Initially, enhanced β-cell function compensates insulin resistance by elevated plasma insulin levels in the absence of any significant islet mass increase. During a second phase gradual islet mass expansion contributes to rising plasma insulin levels in combination with elevated β-cell function. However, despite a continuous islet mass increase, ongoing HFD feeding subsequently leads to prediabetes as a result of insufficient β-cell function. In our study, islet mass is not decreased at the time of

prediabetes onset, but, on the contrary, is still increasing. This supports the hypothesis that β-cell function and not islet mass decline is the crucial islet related mechanism in early T2D pathogenesis. This is in line with the observation that the majority of known T2D susceptibility genes are associated with β-cell function (37). Therefore, observed reduced β-cell mass in type 2 diabetic individuals might be a result of stress-induced cell death or dedifferentiation after onset of hyperglycemia (20). However, our results do not rule out a potential role of β-cell mass at later stages of diabetes pathogenesis or the influence of diminished inherent β-cell mass for diabetes development (38).

Investigating the mechanisms underlying functional compensation our findings suggest that increasing fasting plasma insulin levels in HFD are the result of enhanced β-cell function illustrated by the rising basal Ca^{2+}_i dynamics. Higher fasting insulin has also been observed in other mouse models of T2D (39) and is most likely due to an increased glucose sensitivity of β-cells in the presence of elevated free fatty acid levels (40). Conversely, whereas plasma insulin levels were increased in response to glucose, we found stimulated Ca^{2+}_i dynamics to be reduced under HFD in vivo, which was associated with a decreased 1st phase response. In dispersed cells or isolated islets diminished stimulated Ca^{2+}_i dynamics was observed in several models of β-cell stress and linked to altered gene expression of Ca^{2+}-channel subunits (41), impaired glucose metabolism (42), or abnormal intracellular Ca^{2+}-handling (43). Importantly, blunted stimulated Ca^{2+}_i dynamics might be directly related to the increased basal Ca^{2+}_i, as both have been observed to occur simultaneously in pathophysiological conditions (39; 44). Our observation that stimulated insulin release is elevated despite reduced Ca^{2+}_i dynamics suggests that functional compensation in HFD is accomplished by the amplifying pathway of insulin secretion and augmented calcium efficacy (45-47). This is probably mediated by free fatty acids which rise during HFD feeding (48) and have been shown to potentiate glucose stimulated insulin secretion

(49). Mimicking HFD feeding conditions in vitro by culture of isolated islets in palmitate and slightly elevated glucose we found Epac to be involved in amplified calcium efficacy. These findings underline the potential of lipid derived signals to activate the amplifying pathway (50) and the role of Epac in elevated β-cell function during HFD (31).

Interestingly, islet mass expansion stopped but did not reverse after cessation of HFD feeding. However, given the observed slow adaptation of β-cell mass to the changed metabolic environment, this might take place within a longer time frame than assessed in our study. In contrast, HFD induced alterations in glucose homeostasis and Ca^{2+}_i dynamics were quickly reversed by ND feeding. This is in agreement with the rapid recovery of mouse and human islets isolated from diabetic subjects (51; 52) and might explain diverse findings in isolated islets in vitro in comparison to the here presented in vivo results (5).

In conclusion, our data provide first in vivo evidence that β-cell function outweighs the adaptive response of islet mass during compensation of insulin resistance and progression to prediabetes. Thereby, the amplifying pathway of insulin secretion, via increased calcium efficacy and Epac signaling, plays a crucial role in the β-cells' capacity to compensate for the higher insulin demand. At the onset of prediabetes β-cell function is still enhanced but shows signs of weakened compensation. If this is primarily the result of reduced stimulated Ca^{2+}_i dynamics or additionally decreasing calcium efficacy, needs further investigation. While caution has to be taken, when translating mouse data to the human situation, it is likely that due to the low proliferative potential of adult human β-cells (13) the role of β-cell function in human diabetes pathogenesis will be even more significant. Thus, we believe that T2D prevention and treatment should aim at protecting and improving β-cell function by addressing calcium dynamics and efficacy.

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Author Contributions

C.C. and S.S. designed the study, analyzed data and wrote the manuscript. C.C. performed the experiments. H.C., C.M.C., J.A.C., S.J., J.S. and I.U. analyzed data and provided intellectual input. Funding was provided by S.S. S.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes full responsibility for the work as a whole, including the decision to submit and publish the manuscript.

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Figure Legends

Figure 1 – HFD leads to impaired glucose tolerance and pre-diabetes despite increased insulin secretion. Effect of 17 weeks normal diet (ND) or high-fat diet (HFD) feeding, followed by 2 weeks ND, on body weight (**A**), fasting blood glucose (**B**), fasting insulin (**C**), stimulated insulin (**D**), insulin to glucose ratio in fasting (**E**) and stimulated (**F**) state during IPGTT, glucose tolerance (**G**) and 2hr glucose tolerance blood glucose levels (**H**). n=3-8 for ND and 6-8 for HFD; Mean ± SEM; $^{*}P < 0.05$ vs ND.

Figure 2 – Prolonged HFD feeding induces a continuous increase of islet volume (**A**) *In vivo* backscatter images of a representative islet engrafted in the anterior chamber of the eye of a mouse at indicated time points during HFD feeding. (**B**) Islet volume (quantified by laser backscatter; μm^3) of individual islets at the initial time point (prediet). (**C**) Total islet volume (quantified by laser backscatter; fold change) of intraocular islets during prolonged ND and HFD feeding. n=11-27 islets in 3-8 mice; Mean ± SEM; $^{*}P < 0.05$ vs ND; Scale bars: 50 μm.

Figure 3 – HFD induced islet growth is the result of increased β-cell number and vessel network adaptation. (**A**) *In vivo* fluorescence images of an intraocular MIP-GFP islet at indicated time points of HFD feeding (Green: MIP-GFP; Magenta: vessels). (**B-G**) Longitudinal effect of HFD feeding on β-cell volume (**B**), number (C) and size (**D**); as well as on islet vessel network length (**E**), diameter (**F**). n=18-33 islets in 4-7 mice; Mean ± SEM; $^{*}P < 0.05$ vs pre-diet; Scale bars: 50 μm. (**G**) Fractional β-cell area (percentage of total pancreatic area) of mice with ND feeding (ND) or 16 weeks of HFD feeding. n=6 mice; Mean ± SEM; $^{*}P < 0.05$ vs ND.

Figure 4 – Islets compensate HFD feeding with an increased β-cell functional index. Islet functional index, calculated as stimulated plasma insulin levels over total islet volume adaptation of the same animal. n=9-24 islets in 3-8 mice; Mean ± SEM; $^{*}P < 0.05$ vs ND.

Figure 5 – Longitudinal in vivo imaging of β-cell Ca^{2+}_i dynamics during prolonged ND and HFD feeding. (**A**) Intensity–encoded images from indicated time points of the Pdx1CreER-GCaMP3 islet GFP fluorescence recording shown in B (pre-diet). (**B** and **C**) Representative repetitive recordings of *in vivo* Ca^{2+}_i dynamics of a Pdx1CreER-GCaMP3 islet in response to an intravenous glucose injection (0 min) at indicated time points during 17 weeks ND (**B**) or HFD (**C**) feeding and after subsequent 2 weeks on ND ('recovery').

Figure 6 – HFD induces increased basal and decreased glucose-stimulated β-cell Ca^{2+}_i dynamics in vivo. (**A** and **B**) Quantification of basal (**A**) and stimulated (**B**) β-cell Ca^{2+}_i dynamics during prolonged ND and HFD feeding and subsequent recovery with ND. (**C**) Quantifications of β-cell Ca^{2+}_i peak amplitude ($\Delta F/F_{basal\ min}$ over basal mean) in the first phase (0'-5') after glucose injection. Dash line represents the switching from HFD to ND. (**D**) In vivo calcium efficacy of β-cells during ND or HFD, calculated by correlation of stimulated plasma insulin levels and β-cell Ca^{2+}_i dynamics and corrected for islet volume adaptation. n=4-8 islets and mice; Mean ± SEM; $*P < 0.05$ vs ND; $\wedge P < 0.05$ vs prediet.

Figure 7 – Palmitate induced increased calcium efficacy in isolated islets involves Epac signaling. (**A**) Basal and stimulated insulin secretion from isolated islets after 24 hour culture in 5.5 mM glucose without palmitate ($G_{5.5}P_0$) or in 8 mM glucose plus 0.5 mM palmitate ($G_8P_{0.5}$); n=5. (**B**) β-cell Ca^{2+}_i dynamics of Pdx1CreER-GCaMP3 islets under identical conditions as in

(B); n=18-21 islets from 5-6 experiments. (**C**) In vitro calcium efficacy calculated from islets shown in (B) and (C); n=4. (**D-F**) Effect of indicated concentration of the Epac specific inhibitor ESI-09 on insulin secretion (**D**) (n=4-5), Ca^{2+}_i dynamics (**E**) (n=17-19 islets from 6 experiments) and calcium efficacy (**F**) (n=4) of islets cultured for 24 hours in $G_8P_{0.5}$. Mean ± SEM; **P* < 0.05.

Figure 1

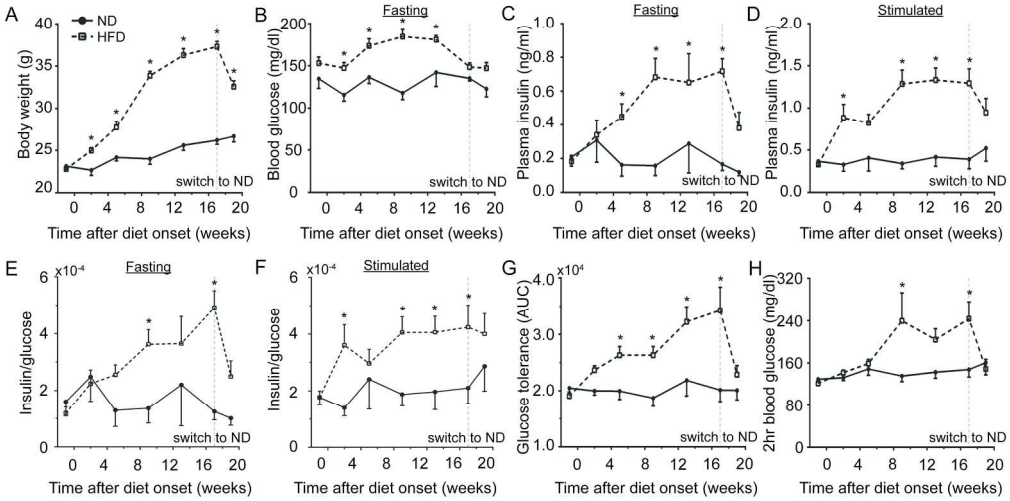

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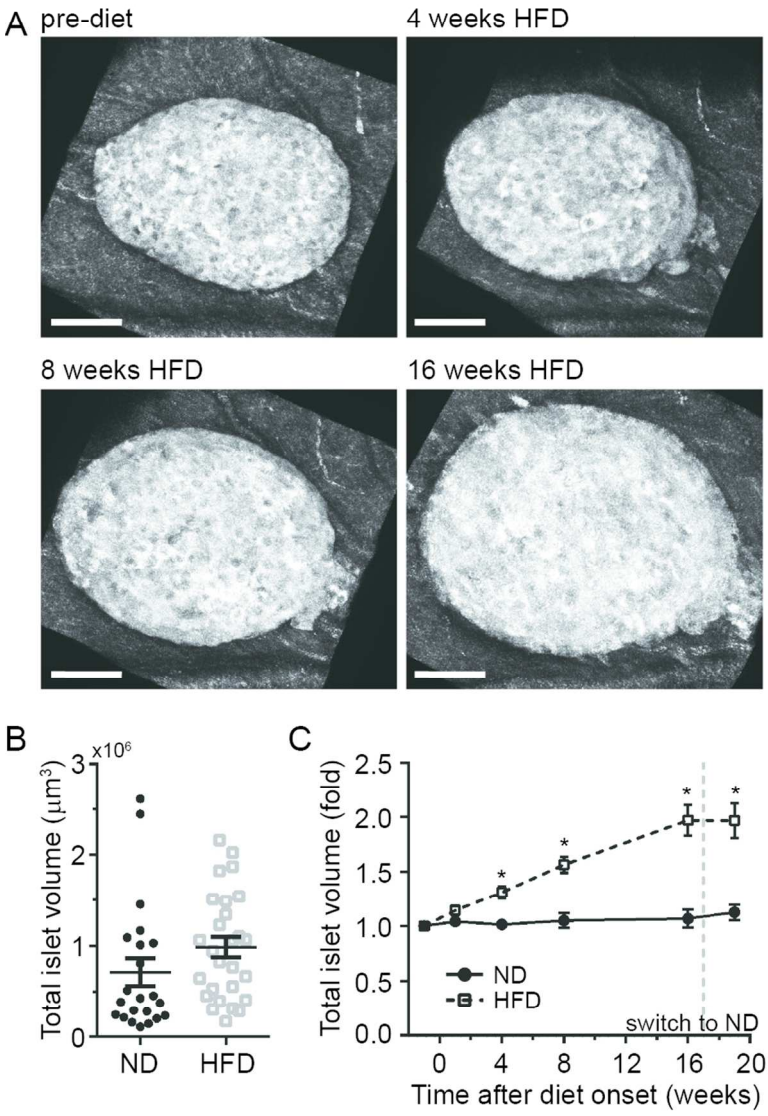
Figure 2



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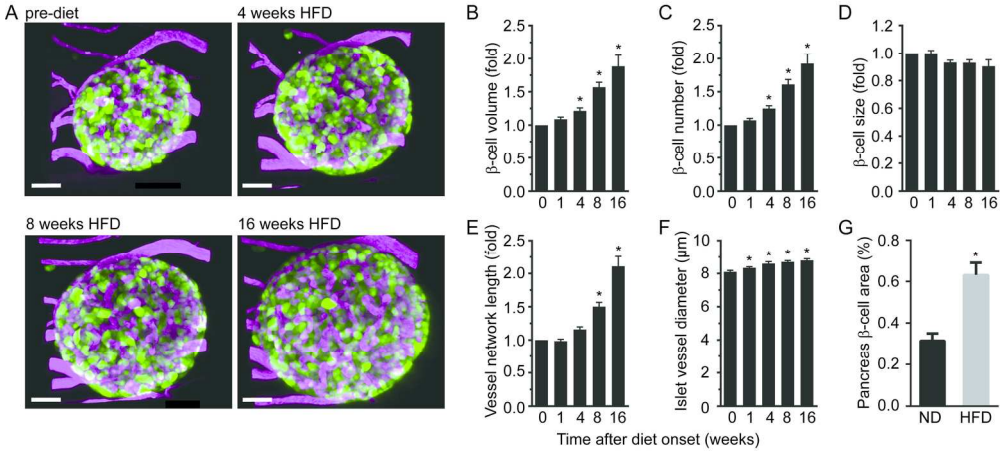

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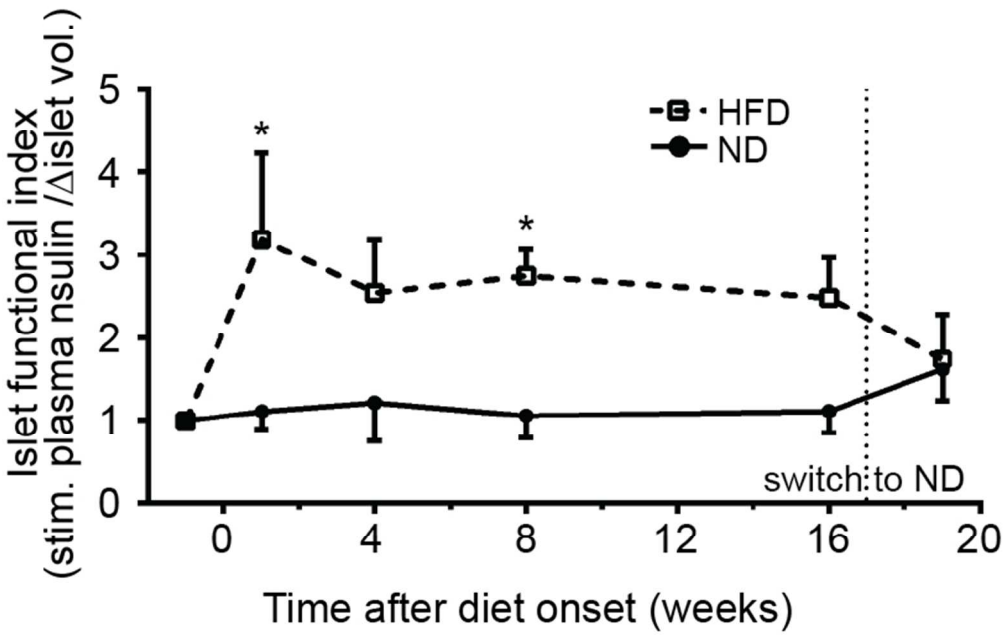

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Figure 5

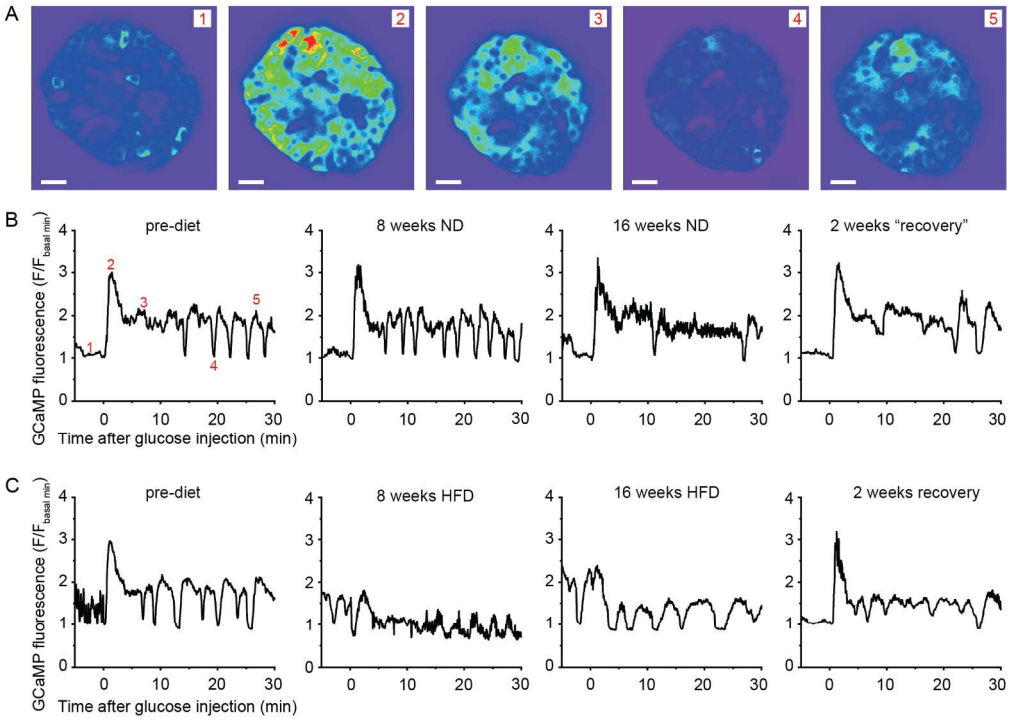

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Figure 6

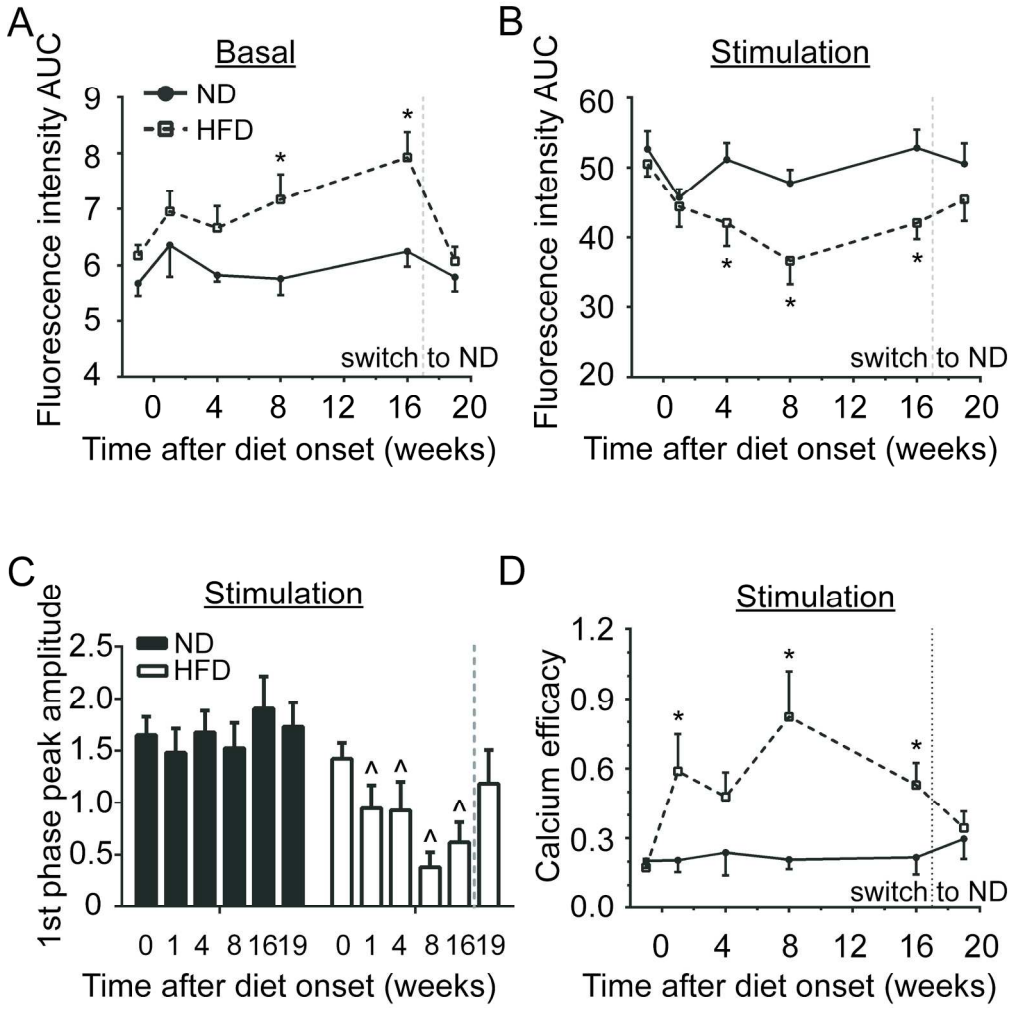

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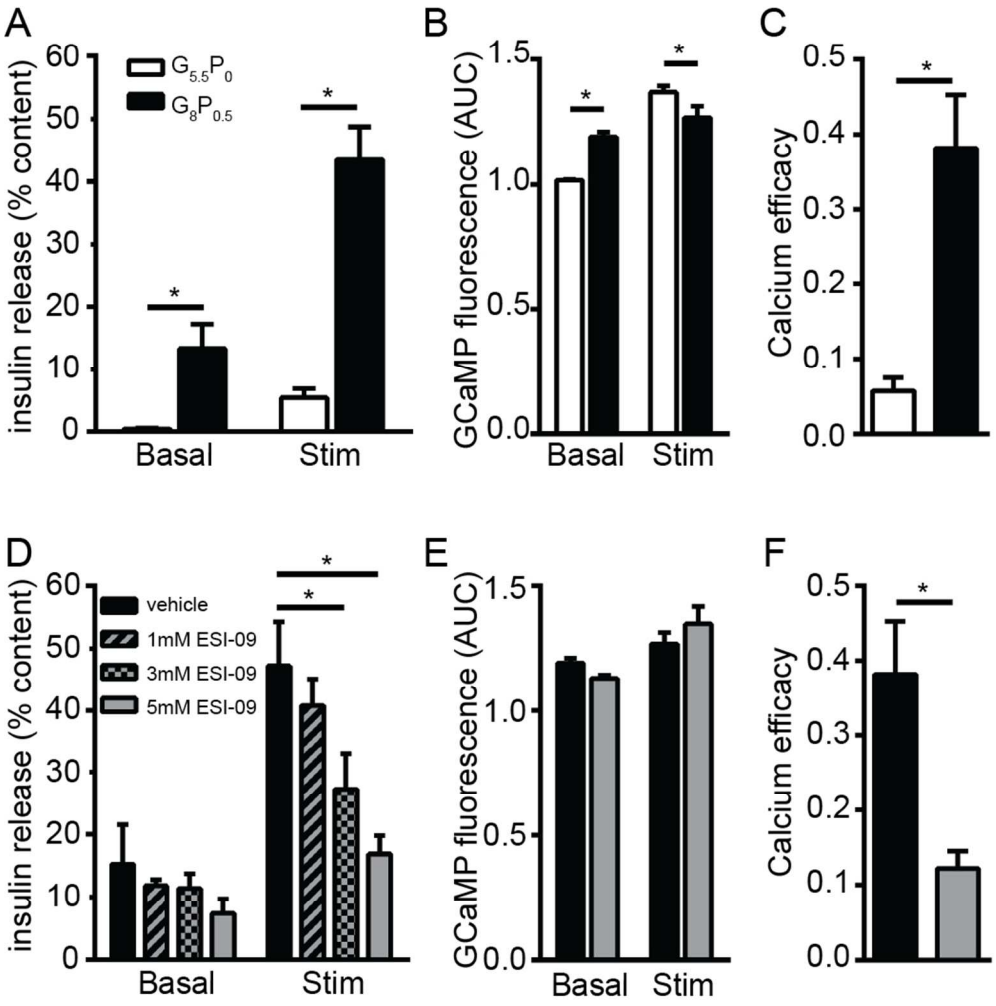
Figure 7



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Online Supplemental Data

Alterations in β-cell calcium dynamics and efficacy outweigh islet mass adaptation in compensation of insulin resistance and prediabetes onset

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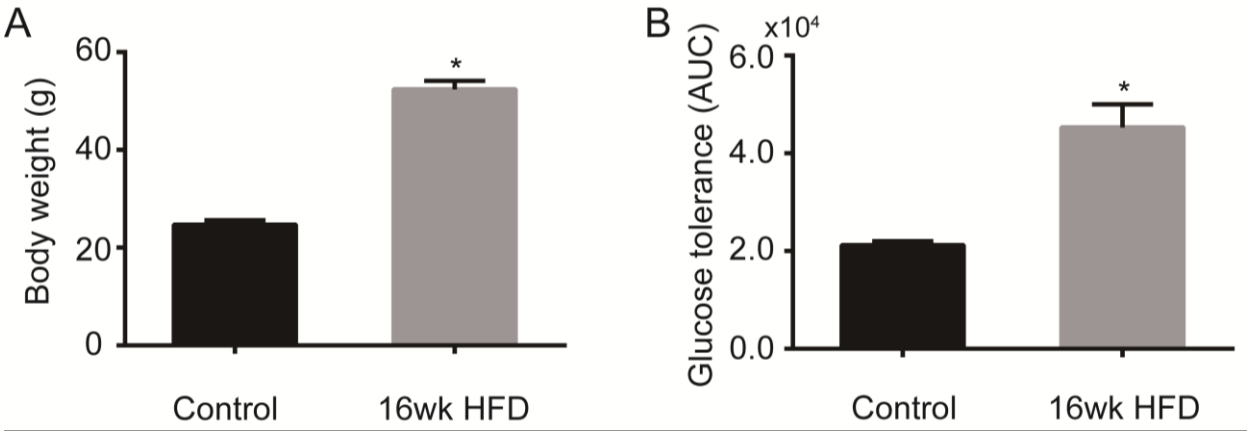
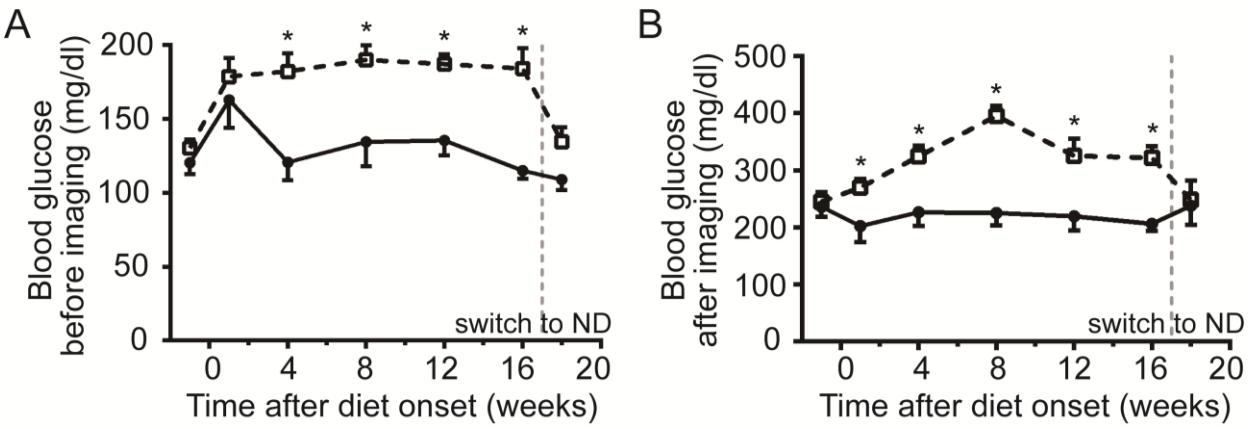
Supplemental Figure 1



Supplemental Figure 1: Body weight (**A**) and IPGTT (**B**) of female C57BL/6N albino mice fed ND (control) or 16 weeks of HFD prior to assessment of pancreatic beta cell fraction (Fig. 3G).

Supplemental Figure 2



Supplemental Figure 2: Blood glucose of anesthetized Pdx1CreER-GCaMP3 islet recipient mice immediately before (**A**) and after (ca. 35-40 min) (**B**) intravenous glucose challenge (1g/kg) during *in vivo* imaging of β-cell $Ca^{2+}{}_i$ dynamics at indicated time points.