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The role of glucokinase and insulin receptor substrate-2 in the proliferation of pancreatic beta cells induced by short-term high-fat diet feeding in mice

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Abstract

Objective: We investigated whether glucokinase and insulin receptor substrate-2 were required for beta cell proliferation induced by short-term high-fat (HF) diet feeding, as has been shown for long-term HF diet.

Methods: Eight-week-old C57BL/6J mice were exposed to either a standard chow (SC) or HF diet. After 1 week on the diet, histopathological beta cell proliferation and gene expression in isolated islets were examined. Additionally, 8-week-old beta cell-specific glucokinase haploinsufficient (*Gck*$^{+/-}$) and *Irs2* knockout (*Irs2*$^{-/-}$) mice were exposed to either an SC or HF diet.

Results: Immunohistochemical analysis revealed that short-term HF diet feeding resulted in a significant increase in BrdU incorporation rate compared with SC consumption in wild-type mice. Western blot analysis demonstrated that Irs2 expression levels did not differ between the two diets. Moreover, there was a significant increase in the BrdU incorporation rate in the HF diet group compared with the SC group in both *Gck*$^{+/-}$ and *Irs2*$^{-/-}$ mice. Gene expression profiling of isolated islets from mice fed an HF diet for 1 week revealed that the expression levels of downstream genes of Foxm1 were coordinately upregulated. One week of HF diet feeding stimulated beta cell proliferation with Foxm1 upregulation in 48-week-old mice as well as in 8-week-old.

Conclusions: The mechanism of pancreatic beta cell proliferation induced by short-term HF diet feeding in mice could involve a glucokinase- and Irs2-independent pathway. Our results suggest that the pathways that induce beta cell proliferation in response to short-term HF diet feeding may differ from those in response to sustained HF diet feeding.

Key words: beta cell proliferation, glucokinase, high-fat diet, insulin receptor substrate-2

Abbreviations:

Aurkb, aurora kinase B; Ccna2, cyclin A2; Ccnb1, cyclin B1; Ccnd1, cyclin D1; Ccnd2, cyclin D2; Ccnd3, cyclin D3; Cdc20. cell division cycle 20; Cenpa, centromere protein A; Cenpb, centromere protein B; Cenpf, centromere protein F; Foxm1, forkhead transcription factor M1; Gck, glucokinase; GKA, glucokinase activator; HF, high-fat; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; Plk1, polo-like kinase 1; SC, standard chow

1. Introduction

Type 2 diabetes mellitus is characterised by two major features: peripheral insulin resistance and impaired insulin secretion from pancreatic beta cells [1]. The condition occurs when the pancreatic beta cells are unable to compensate for increased insulin demand due to insulin resistance in peripheral tissues [2]. Thus, insufficient insulin secretion is a central component in the pathophysiology not only of type 1 diabetes, but also of type 2 diabetes [3]. Given that one of the main causes of insulin deficiency is a reduction in pancreatic beta cell mass [4-6], its expansion could represent an ideal therapeutic approach in the treatment of diabetes [7, 8]. Proliferation of existing beta cells is an important mechanism that promotes postnatal expansion of beta cell mass in humans and rodents [9, 10]. Therefore, it is important to elucidate the mechanisms underlying beta cell proliferation for increased beta cell mass.

Beta cell proliferation can be induced experimentally in postnatal mice by several methods, including high-fat (HF) diet feeding, partial pancreatectomy and pregnancy [11-15]. The mouse model of HF diet-induced beta cell proliferation has been widely used in research [16]. We [17] previously demonstrated that wild-type mice fed an HF diet for 20 weeks showed marked beta cell hyperplasia, whereas mice with beta cell-specific glucokinase haploinsufficiency ($Gck^{+/-}$) exhibited reduced beta cell hyperplasia, decreased beta cell proliferation, and impaired upregulation of Irs2, despite having a similar degree of insulin resistance to wild-type mice. These results suggested that a combination of glucokinase and Irs2 is critical for beta cell proliferation to occur in response to 20 weeks of HF diet feeding [17, 18]. However, it has been unclear whether beta cell proliferation in this model is triggered by the HF diet itself or by the sustained metabolic changes related to HF diet-induced insulin resistance.

In recent years, beta cell proliferation induced by short-term HF diet feeding has been reported [19-21]. Stamateris et al. [19] found that beta cell proliferation began within the first 7 days of HF diet feeding, concurrent with the onset of metabolic changes. Mosser et al. [20] showed that HF

diet feeding induced beta cell proliferation as early as 3 days after HF diet initiation, and that enhanced beta cell proliferation occurred without the development of insulin resistance. These findings prompted us to examine the hypothesis that the mechanisms mediating beta cell proliferation in response to short-term HF diet feeding might be different from those activated by long-term. In the present study, we investigated whether glucokinase and Irs2 were required for beta cell proliferation induced by short-term HF diet feeding, as has been shown in long-term HF diet.

2. Methods

2.1. Animals Animal feeding protocols have been summarized in Supplementary Fig. 1. Seven-week-old male C57BL/6J mice were purchased from Clea Japan, Inc. (Tokyo, Japan). Since 8 weeks old, they were given free access to either a standard chow (SC) or HF diet for 2, 4 or 7 days. *Gck*$^{+/-}$ and Irs2-knockout (*Irs2*$^{-/-}$) mice were generated as described elsewhere [22, 23]. We backcrossed these mice with C57Bl/6J mice more than ten times. Male littermates derived from the intercrosses were fed an SC diet until 8 weeks of age and then were given free access to either the SC diet or an HF diet for 1 week. Additionally, the same experiment was performed using 48-week-old male C57BL/6J mice. All mice were housed on a 12-h light-dark cycle and maintained in accordance with standard animal care procedures based on institutional guidelines.

2.2. Diet protocol Standard chow (MF; Oriental Yeast Co. Ltd., Tokyo, Japan) and an HF diet (High Fat Diet 32; Clea Japan, Inc.) were used, as described previously [24].

2.3. Measurement of biochemical parameters Blood glucose was measured using a Glutestmint portable glucose meter (Sanwa Chemical Co., Nagoya, Japan). Insulin levels were determined with an insulin ELISA kit (Morinaga, Yokohama, Japan). Plasma free fatty acid, total cholesterol, and triglycerides were assayed by enzymatic methods (Wako Pure Chemical Industries Ltd., Osaka, Japan).

2.4. Insulin tolerance test and oral glucose tolerance test Insulin tolerance tests were performed under non-fasting conditions. Human regular insulin (0.75 mU/g body weight) was injected intraperitoneally, and blood samples were collected before and 30, 60, 90 and 120 min after the injection. For the oral glucose tolerance test, mice were denied access to food for more than 16 h before the test and then given an oral glucose load of 1.5 mg/g body weight. Blood samples were

collected before and 15, 30, 60 and 120 min after glucose loading.

2.5. Treatment of mice with glucokinase activator (GKA) and rapamycin A GKA was prepared by Tsukuba Research Institute, Banyu Pharmaceutical Co. Ltd. (Tsukuba, Japan), as described previously [23]. Male C57BL/6J mice aged 8 weeks were administered vehicle or GKA (56 mg/kg body weight) orally, twice daily for 3 days. Rapamycin (LC Laboratories Inc., Woburn, MA) was reconstituted in ethanol at 20 mg/ml and then diluted in 5% Tween-80 (Sigma-Aldrich Co. LLC., St. Louis, MO) and 5% PEG-400 (Hampton Research Co., Aliso Viejo, CA). Mice received rapamycin (4 mg/kg body weight) by intraperitoneal injection every other day for 1 week.

2.6. Beta cell morphology and immunohistochemistry Isolated pancreata were immersion-fixed in 4% paraformaldehyde. Tissue was then routinely processed for paraffin embedding, and 5-μm sections mounted on glass slides were immunostained with rabbit anti-human insulin antibody (diluted 1:1000; Santa Cruz Biotechnology Inc., Santa Cruz, CA). BrdU incorporation was analysed as described previously [25]. The sections were double-immunostained with anti-BrdU antibody (diluted 1:10; BD Biosciences, Franklin Lakes, NJ, USA) and anti-insulin antibody (diluted 1:1000). BrdU-positive beta cells were quantitatively assessed as a percentage of the total number of beta cells and at least 50 islets per mouse were counted in each group.

2.7. Islet isolation Islets were isolated using collagenase from *Clostridium histolyticum* (Sigma-Aldrich) according to the manufacturer's instructions.

2.8. Real-time quantitative PCR Total RNA was isolated with an RNeasy Mini Kit (Qiagen, Hilden, Germany) and used as the starting material for cDNA preparation. Real-time

PCR was performed in duplicate using a 7500 Fast Real Time PCR system with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). The primer sequences used are listed in Supplementary Table 1.

2.9. Western blot analysis Anti-Irs2 antibody was purchased from Cell Signaling Technology Inc. (Beverly, MA). Protein bands were visualised with an ImmunoStar LD western blotting detection kit (Wako), and images were obtained using an LAS-4000 UV mini CCD-camera system (Fujifilm Co., Tokyo, Japan).

2.10. Microarray analysis Total RNA from mouse islets was isolated with an RNeasy Mini Kit (Qiagen). The mRNA expression profiles were determined using a GeneChip Mouse Gene 2.0 ST array (Thermo Fisher Scientific Inc., Waltham, MA). Differentially expressed genes were defined as genes that showed at least a 1.5 fold change. The readings obtained were analysed by a hierarchical clustering method using MeV analysis software (version 4.9.0).

2.11. Statistical analysis Results are expressed as mean ± SD. Differences between two groups were assessed using Student's *t* tests. Individual comparisons between more than two groups were performed using ANOVA. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of short-term HF diet feeding on metabolism in 8-week-old C57BL/6J mice. The effects of short-term HF diet exposure on metabolism and beta cell proliferation were tested in 8-week-old C57BL/6J male mice, based on the report that HF diet feeding induced beta cell proliferation within the first 7 days after HF diet initiation [19-21]. Body weight was significantly higher in the mice fed the HF diet than those fed SC on days 1, 3 and 6 (Fig. 1a and Supplementary Fig. 2a, d), and fed blood glucose levels were significantly higher in the HF group on days 3 and 6 (Fig. 1b and Supplementary Fig. 2b, e). Caloric intake in the HF group was significantly higher than that in the SC group (data not shown). Although visceral fat weight was significantly higher in the HF group than in the SC group, there were no significant differences in liver weight nor pancreatic weight between the two groups on days 2, 4 or 7 (Fig. 1c and Supplementary Fig. 2c, f). No significant differences in plasma triglyceride levels were observed between two groups, whereas plasma non-esterified fatty acid levels were significantly lower in the HF group than in the SC group (Fig. 1d, e). The glucose-lowering effect of insulin in the HF group was equivalent to that in the SC group on day 6 (Fig. 1f and Supplementary Fig. 3). The oral glucose tolerance test revealed that mice fed the HF diet showed mild hyperglycemia when compared with mice fed the SC diet, and that insulin levels before and 15 min after glucose loading were similar between the two groups (Fig. 1g, h).

3.2. Beta cell proliferation induced by short-term HF diet feeding in 8-week-old C57BL/6J mice. Immunohistochemical analysis revealed a significant increase in the BrdU incorporation rate in the HF group in comparison with the SC group on day 7 and an appreciable but non-significant increase on day 4, although there was no difference between the two groups on day 2 (Fig. 2a). Real-time quantitative PCR showed that Ki-67 levels were significantly increased in the HF group compared with the SC group on day 7. However, notably, the gene expression and protein levels

of Irs2 did not differ between the two groups (Fig. 2b-d). No increases in the expression level of genes involved in beta cell function, including pancreatic and duodenal homeobox-1, glucokinase, insulin-1 and insulin-2, were seen in the HF group compared with the SC group (Fig. 2e). We also examined beta cell function, and found no differences in glucose-stimulated insulin secretion or islet insulin content between the two groups on day 7 (Supplementary Fig. 4a, b). These results indicate that beta cell proliferation was induced by HF diet exposure for 7 days in the absence of Irs2 upregulation.

3.3. Beta cell proliferation induced by short-term HF diet feeding in $Gck^{+/-}$ and $Irs2^{-/-}$ mice. The above results prompted us to investigate whether glucokinase and Irs2 were required for beta cell proliferation induced by short-term HF diet feeding. First, eight-week-old wild-type and $Gck^{+/-}$ male mice were exposed to either an SC or HF diet for 1 week. Both the wild-type and $Gck^{+/-}$ mice showed slightly increased body weight in the HF diet group compared with the SC group (Fig. 3a). Since fed blood glucose levels were markedly higher in $Gck^{+/-}$ mice than in wild-type mice in the SC condition, the increase in blood glucose levels in response to HF diet did not reach statistical significance in the $Gck^{+/-}$ mice (Fig. 3b). The BrdU incorporation rate increased significantly in the HF group compared with the SC group in both wild-type and $Gck^{+/-}$ mice to a similar extent (Fig. 3c). Real-time quantitative PCR from isolated islets showed that Ki-67 expression was significantly increased in the HF group compared with the SC group in $Gck^{+/-}$ mice, consistent with the result seen in wild-type mice (Fig. 3d). However, there were no differences in the gene expression levels of Irs2, glucose-stimulated insulin secretion or islet insulin content between the SC and HF groups in either the $Gck^{+/-}$ or the wild-type mice (Fig. 3e and Supplementary Fig. 5). Next, eight-week-old wild-type and $Irs2^{-/-}$ male mice were exposed to either an SC or HF diet for 1 week. The $Irs2^{-/-}$ mice yielded similar results to those observed in $Gck^{+/-}$ mice (Fig. 3f–h). These findings indicate that HF diet feeding for 1 week induced beta cell

proliferation in the glucokinase and Irs2 independent fashion.

3.4. Additive effect of GKA on short-term HF diet-induced beta cell proliferation. We examined the effect of GKA in combination with short-term HF diet feeding on beta cell proliferation, since GKA stimulates beta cell proliferation [22, 23]. As summarised in Fig 4a, we divided the 8-week-old C57BL/6J male mice into four groups: mice fed the SC diet for 7 days (SC group), mice fed the HF diet for 7 days (HF group), mice fed the SC diet for 7 days with GKA on the final 3 days (SC+GKA group), and mice fed the HF diet for 7 days with GKA on the final 3 days (HF+GKA group). Body weight did not differ significantly between the four groups, but the body weight was slightly increased in the HF and HF+GKA groups on day 7 (Fig. 4b). On day 7, blood glucose levels in the HF+GKA group were significantly lower than those in the HF group, and blood glucose levels in the SC+GKA group tended to be lower than those in the SC group (Fig. 4c). The BrdU incorporation rate in the HF group tended to be increased compared with that in the SC group. Moreover, there was a marked increase in the BrdU incorporation rate in the HF+GKA group compared with the other three groups (Fig. 4d).

3.5. Changes in gene expression in islets isolated from mice fed an HF diet for 1 week. To screen the gene expression profiles of the islets, we performed a microarray analysis of genes expressed in the islets from mice fed an SC or HF diet for 1 week. We identified 62 genes expressed differentially (fold change ≥ 1.5) between the HF diet group and the SC group (Fig. 5a); however, glucokinase and Irs2 showed no group differences in expression levels. According to gene ontology annotation, these 62 genes were mainly related to the cell cycle category (Supplementary Table 2). When we clustered placentas according to the gene expression profiles for these 62 genes, three genes, cyclin A2 (Ccna2), cyclin B1 (Ccnb1) and centromere protein A (Cenpa), were relatively close to each other (Fig. 5b). Real-time quantitative PCR revealed that expression

levels of forkhead transcription factor M1 (Foxm1) and its downstream genes, such as Ccna2, Ccnb1, Cenpa, were significantly increased in the HF group compared with the SC group (Fig. 5c, d). Conversely, there were no significant differences in the gene expression levels of cyclin D1 (Ccnd1), cyclin D2 (Ccnd2) or cyclin D3 (Ccnd3) between the two groups (Fig. 5d). Additionally, Foxm1 expression levels were significantly increased in the HF group compared with the SC group in the *Gck*$^{+/-}$ mice (Supplementary Fig. 6).

3.6. Effect of rapamycin on short-term HF diet-induced beta cell proliferation. To examine the effect of a mammalian target of rapamycin (mTOR) inhibitor, rapamycin, on short-term HF diet-induced beta cell proliferation, we divided 8-week-old C57BL/6J male mice into four groups: mice fed the SC diet for 7 days (SC group), mice fed the HF diet for 7 days (HF group), mice fed the SC diet for 7 days with rapamycin (SC+Rapa group), and mice fed the HF diet for 7 days with rapamycin (HF+Rapa group) (Fig. 6a). Body weight did not differ significantly between the four groups (Fig. 6b). Blood glucose levels in the HF, SC+Rapa and HF+Rapa groups were significantly higher than those in the SC group on day 7 (Fig. 6c). Although the BrdU incorporation rate in the HF group was significantly increased compared with that in the SC group, there was no difference in the BrdU incorporation rate between the SC+Rapa group and the HF+Rapa group (Fig. 6d).

3.7. Beta cell proliferation induced by short-term HF diet feeding in 48-week-old C57BL/6J mice. To examine whether short-term HF diet feeding stimulates beta cell proliferation in aged mice, 48-week-old C57BL/6J male mice were exposed to either an SC or HF diet for 1 week. Body weight and fed blood glucose levels were significantly higher in the mice fed the HF diet than in the mice fed the SC diet (Fig. 7a, b). A significant increase in the BrdU incorporation rate was observed in the HF group compared with the SC group (Fig. 7c). The expression levels of Ki-67,

Foxm1, Ccna2 and Ccnb1 were significantly increased in the HF group compared with the SC group, but Irs2 levels did not differ between the groups (Fig. 7d). These results were interpreted that short-term HF diet feeding stimulated beta cell proliferation not only in 8-week-old mice but also in 48-week-old mice.

4. Discussion

In the current study, we found that beta cell proliferation was induced by HF diet feeding for only 1 week in 8-week-old male C57Bl/6J mice. Body weight, fed blood glucose levels and visceral fat weight were higher in mice fed an HF diet for 1 week than in mice fed SC. However, the glucose-lowering effect of insulin was equivalent in both groups of mice. Thus, the initiation of beta cell proliferation coincided with the onset of increases in body and visceral fat weight and hyperglycaemia, but not with the onset of insulin resistance in this model. These data are in keeping with previous reports of beta cell proliferation induced by HF diet feeding for 1 week [19, 20]. Although insulin resistance was assessed by insulin tolerance test in one of these previous investigations and in our current study [19], Mosser's group [20] confirmed the absence of insulin resistance by hyperinsulinemic euglycaemic clamps and measurements of alpha-hydroxybutyrate. Therefore, beta cell proliferation in this model is not caused by a compensatory response to HF diet-induced insulin resistance.

The main aim of our study was to identify whether glucokinase and Irs2 were required for beta cell replication induced by short-term HF diet feeding. Glucokinase plays a critical role in regulating blood glucose levels by catalysing the rate-limiting biochemical reaction of glycolysis. [26, 27]. In pancreatic beta cells, phosphorylation of glucose by glucokinase is the rate-limiting step in insulin secretion. Thus, $Gck^{+/-}$ mice have been reported to exhibit mild diabetes due to impaired secretion of insulin in response to glucose [28], which was confirmed in our results (Fig. 3b). Furthermore, $Gck^{+/-}$ mice fed an HF diet for 20 weeks have been shown to exhibit decreased beta cell proliferation compared with wild-type mice [17], and Porat et al. [29] reported a dramatic drop in beta cell proliferation rate in adult beta cell-specific glucokinase knockout mice in response to tamoxifen injection. Taken together with previous reports [30, 31], these findings indicate that glucose signalling mediated by glucokinase in beta cells plays an important role in beta cell proliferation. However, we observed a significant and similar increase in beta cell

proliferation rate in both wild-type and *Gck*$^{+/-}$ mice fed an HF diet for 1 week in our study (Fig. 3c), indicating that the mechanism underlying short-term HF diet-induced beta cell proliferation involves a glucokinase-independent pathway.

Glucose metabolism by glucokinase increases Irs2 expression, activating a signalling cascade that leads to beta cell proliferation [18]. A previous study demonstrated that *Gck*$^{+/-}$ mice fed an HF diet for 20 weeks showed decreased beta cell proliferation and impaired upregulation of Irs2 compared with wild-type mice, and that overexpression of Irs2 in *Gck*$^{+/-}$ mice fed the HF diet rescued beta cell proliferation [17]. Recently, it has been reported that Irs2 is required for glucose-induced beta cell proliferation *in vivo* and *ex vivo* [32]. Taken together with further insights obtained from studies of genetically modified mice [33-35], these findings indicate that Irs2 also plays a crucial role in beta cell proliferation. However, our results revealed that beta cell proliferation was induced by HF diet feeding for 1 week without upregulation of Irs2, and that there was a significant and similar increase in beta cell proliferation rate in both wild-type and *Irs2*$^{-/-}$ mice fed the HF diet for 1 week (Fig. 3h). These results suggest that the mechanism underlying short-term HF diet-induced beta cell proliferation involves an Irs2-independent pathway.

These findings raise the question of why glucokinase and Irs2 contribute to the HF diet-induced proliferative response in islets after 20 weeks of HF diet loading [17], but not after 1 week of HF diet loading. We speculate that HF diet-induced insulin resistance could affect the contribution of glucokinase and Irs2 to this proliferative response. To investigate further, we performed an insulin tolerance test and measured beta cell proliferation in mice challenged with HF diet for different time periods (4, 8, 12 and 20 weeks). There was a significant increase in the BrdU incorporation rate in mice on the HF diet compared with those on the SC diet for all time periods, while the glucose-lowering effect of insulin was impaired in HF-fed mice after 8 weeks (data not shown). From these results, it is possible that after 8 weeks on the HF diet, the

short-term HF diet-induced proliferative mechanism (glucokinase- and Irs2-independent pathway) is switched to or added to the long-term HF diet-induced proliferative mechanism (glucokinase- and Irs2-dependent pathway). To confirm this, further studies using *Gck*$^{+/-}$ and *Irs2*$^{-/-}$ mice on a HF diet for different time periods are needed.

GKA is a glucose-like activator of beta cell metabolism. Glucokinase activation by GKA has been shown to increase Irs2 expression, and GKA-stimulated Irs2 expression has been reported to affect beta cell proliferation [22, 23]. Thus, beta cell proliferation induced by long-term HF diet feeding and glucokinase activation by GKA would share common pathways. To explore our hypothesis that the mechanisms of beta cell proliferation induced by short-term HF diet feeding differ from the above glucokinase and Irs2 pathway, we investigated the combined effect of short-term HF diet feeding and GKA on beta cell proliferation. This combined administration markedly increased beta cell proliferation (Fig 4d), suggesting that the glucokinase- and Irs2-independent pathway induced by short-term HF diet feeding and the glucokinase- and Irs2-dependent pathway induced by GKA could act additively to promote beta cell proliferation.

These findings raise the question of what mechanism of beta cell proliferation is induced by short-term HF diet feeding. Gene expression profiling of isolated islets from mice fed a HF diet for 1 week revealed that expression levels of downstream genes of Foxm1 were coordinately upregulated (Fig. 5). Foxm1 is a transcription factor that stimulates cell proliferation and exhibits a proliferation-specific expression pattern [36]. It also appears to be a key transcriptional regulator of cell-cycle progression in pancreatic beta cells. Indeed, it has been reported that Foxm1 is necessary for adult beta cell proliferation in response to pancreatectomy, pregnancy and obesity [37-39]. Foxm1 stimulates the transcription of many mitotic genes, including Plk1, Aurkb, Survivin, Cenpa, Cenpb, Cenpf, and Cdc20, to ensure correct regulation of mitosis [39]. These increased gene expression levels were confirmed by real-time quantitative PCR in our study. Recently, it has been reported that Foxm1 acts via Plk1 to regulate Cenpa expression and

deposition on centromeres, and that Cenpa deficiency impairs adaptive beta cell proliferation [40].

The transcription factor Foxm1 is thought to play a role in all phases of the cell cycle, including G1/S-phase and G2/M-phase transitions. Our results show that expression of Ccna2 and Ccnb1, but not Ccnd, was significantly upregulated in islets from mice fed an HF diet for 1 week compared with those fed SC (Fig. 5d). The effect of Foxm1 on cyclin family members has been explored previously: Ccna2 and Ccnb1 expression levels were increased in isolated mouse islets overexpressing Foxm1b, whereas Foxm1b overexpression had no effect on Ccnd1, Ccnd2 nor Ccnd3 expression [39]. Moreover, increased expression levels of Foxm1, Ccna2 and Ccnb1, but not Ccnd2, were observed in mouse islets after partial pancreatectomy [13, 37]. These results are consistent with our data. In contrast, Irs2-mediated signalling, which is enhanced by glucokinase, is linked with Ccnd2 [17], indicating that the cyclin expression profiles in islets from mice fed an HF diet for 1 week are quite different from those induced by the enhancement of the glucokinase- and Irs2-dependent pathway. Thus, the pancreatic beta cell proliferation induced by short-term and long-term HF diet feeding might be mediated by different pathways.

The mechanism of Foxm1 upregulation by short-term HF diet feeding remains unknown. Rapamycin has been shown to block increases in Foxm1 signalling and beta cell proliferation in response to a 72-hour coinfusion of glucose and Intralipid in Wister rats [41], which is in accordance with our results for short-term HF diet-induced beta cell proliferation (Fig.6d). Given that the mTOR complex 1 (mTORC1) signalling pathway integrates signals from growth factors and nutrients [42], further studies are underway to identify the factors augmenting this mTORC1/Foxm1 pathway.

Basal beta cell proliferation decreases with age in mouse models [43, 44] as well as in humans [10]. Moreover, adaptive beta cell proliferation is strongly restricted with age in mice [44]. Our results show that HF diet feeding for 1 week stimulated beta cell proliferation with upregulation of Foxm1, Ccna2 and Ccnb1 not only in 8-week-old mice, but also in 48-week-old mice (Fig.7). In

support of this finding, Galson and colleagues [45] demonstrated that expression of activated Foxm1 in aged beta cells triggers cell-cycle progression, leading to elevated beta cell proliferation. From a clinical point of view, given that the onset of type 2 diabetes increases with age in humans, the ability to increase beta cell proliferation in aged islets is particularly important.

In conclusion, the mechanism of the pancreatic beta cell proliferation induced by short-term HF diet feeding in mice would involve a glucokinase- and Irs2-independent pathway. Our results suggest as follows; the pathways that induce beta cell proliferation in response short-term HF diet feeding may differ from those in response to sustained HF diet feeding. These findings might provide new therapeutic approaches to enhance beta cell mass and thereby prevent or delay the occurrence of type 2 diabetes.

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Contribution statement

All authors conceived and designed the study, and participated in the analysis and interpretation of the data. N.K. and A.N. drafted the manuscript and all other authors revised it critically for intellectual content. All authors approved the final version of the paper.

Duality of interest

The authors declare that there is no duality of interest associated with this manuscript.

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Figure legends

Fig. 1. Effect of short-term high-fat (HF) diet feeding on metabolism in 8-week-old C57/BL6J mice.

a, b: Changes in body weight (a) and fed blood glucose level (b) in mice fed a standard chow (SC) or HF diet (SC: open circles, HF: filled circles) for 6 days (n = 10). c: Weight of liver, pancreas and visceral fat in mice fed the SC or HF diet (SC: white bars, HF: grey bars) on day 7 (n = 10). d, e: Plasma triglycerides (d) and nonesterified fatty acids (e) in mice fed the SC or HF diet (SC: white bars, HF: grey bars) on day 6 (n = 10). f: Insulin tolerance test in mice fed the SC or HF diet (SC: open circles, HF: filled circles) on day 6 (n = 10). g, h: Blood glucose (g) and insulin (h) levels during the oral glucose tolerance test in mice fed the SC or HF diet (SC: open circles and white bars, HF: filled circles and grey bars) on day 6 (n = 5). Values are means ± SD. ** $p < 0.01$.

Fig. 2. Beta cell proliferation and changes in gene expression levels in islets of 8-week-old C57/BL6J mice exposed to short-term high-fat (HF) diet feeding.

a: Proliferation rates of beta cells, assessed by BrdU incorporation, were determined in mice fed a standard chow (SC) or HF diet (SC: white bars, HF: grey bars) for 2, 4 and 7 days (n = 10: ten mice were used per group). b, e: mRNA levels of *Ki67*, *Pcna*, and *Irs2* (b), and mRNA levels of *Pdx1*, *Gck*, *Glut2*, *Ins1*, *Ins2*, and *Mafa* (e) in islets of mice fed the SC or HF diet (SC: white bars, HF: grey bars) for 7 days measured by real-time quantitative PCR. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression (n = 9). c: Western blot analysis of Irs2 levels in islets of mice fed the SC or HF diet for 7 days. Equal amounts of lysates were blotted with anti-Irs2 and anti-beta actin antibodies. d: Expression level of Irs2 in islets. The intensity of Irs2 was normalized to beta actin expression (SC: white bars, HF: grey bars) (n = 6). Values are means ± SD. * $p < 0.05$.

Fig. 3. Effect of short-term high-fat (HF) diet feeding on beta cell proliferation in 8-week-old *Gck*$^{+/-}$ or *Irs2*$^{-/-}$ mice.

a, b: Changes in body weight (a) and fed blood glucose level (b) in wild-type mice fed a standard chow (SC) or HF diet (SC: open circles, HF: filled circles) and *Gck*$^{+/-}$ mice fed the SC or HF diet (SC: open squares, HF: filled squares) for 7 days (n = 10). c: Proliferation rates of beta cells, assessed by BrdU incorporation, were determined in wild-type mice fed the SC or HF diet (SC: white bars, HF: light grey bars) and *Gck*$^{+/-}$ mice fed the SC or HF diet (SC: dark grey bars, HF: black bars) for 7 days (n = 10: ten mice were used per group). d, e: mRNA levels of *Ki67* (d) and *Irs2* (e) in islets of wild-type mice fed the SC or HF diet (SC: white bars, HF: grey bars) and those of *Gck*$^{+/-}$ mice fed the SC or HF diet (SC: dark grey bars, HF: black bars) for 7 days measured by real-time quantitative PCR. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression (n = 7–8). f, g: Changes in body weight (f) and fed blood glucose level (g) in wild-type mice fed the SC or HF diet (SC: open circles, HF: filled circles) and *Irs2*$^{-/-}$ mice fed the SC or HF diet (SC: open squares, HF: filled squares) for 7 days (n = 10). h: Proliferation rates of beta cells, assessed by BrdU incorporation, were determined in wild-type mice fed the SC or HF diet (SC: white bars, HF: light grey bars) and *Irs2*$^{-/-}$ mice fed the SC or HF diet (SC: dark grey bars, HF: black bars) for 7 days (n = 10: ten mice were used per group). Values are means ± SD. * $p < 0.05$; ** $p < 0.01$.

Fig. 4. Effect of glucokinase activator (GKA) on beta cell proliferation induced by short-term high-fat (HF) diet feeding in 8-week-old C57/BL6J mice.

a: Experimental protocol. Arrows show the days of GKA administration (days 4, 5, and 6). b, c: Changes in body weight (b) and fed blood glucose level (c) in mice fed a standard chow (SC) or HF diet without GKA (SC: open circles, HF: filled circles) and mice fed the SC or HF diet with GKA (SC+GKA: open squares, HF+GKA: filled squares) (n = 10). d: Proliferation rates of beta

cells, assessed by BrdU incorporation, were determined in mice fed the SC or HF diet without GKA (SC: white bars, HF: light grey bars) and mice fed the SC or HF diet with GKA (SC+GKA: dark grey bars, HF+GKA: black bars) (n = 10: ten mice were used per group). Values are means ± SD. * $p < 0.05$; ** $p < 0.01$.

Fig. 5. Microarray analysis and changes in gene expression levels in islets of 8-week-old C57/BL6J mice exposed to short-term high-fat (HF) diet feeding.

a, b: Total (a) and focused (b) data for visualisation of differentially expressed genes using hierarchical clustering (fold change > 1.5) in islets of mice fed a standard chow (SC) or HF diet for 7 days (n = 3). c, d: mRNA levels of *Foxm1* and its downstream genes (c), and mRNA levels of *Ccnds*, *Ccna2* and *Ccnb1* (d) in islets of mice fed the SC or HF diet (SC: white bars, HF: grey bars) for 7 days measured by real-time quantitative PCR. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression (n = 9–11). Values are means ± SD. * $p < 0.05$; ** $p < 0.01$.

Fig. 6. Impact of rapamycin on short-term high-fat (HF) diet-induced beta cell proliferation in 8-week-old C57/BL6J mice.

a: Experimental protocol. Arrows show the days of rapamycin administration (days 0, 3, and 5). b, c: Changes in body weight (b) and fed blood glucose level (c) in mice fed a standard chow (SC) or HF diet without rapamycin (SC: open circles, HF: filled circles) and mice fed the SC or HF diet with rapamycin (SC+Rapa: open squares, HF+Rapa: filled squares) (n = 10). d: Proliferation rates of beta cells, assessed by BrdU incorporation, were determined in mice fed the SC or HF diet without rapamycin (SC: white bars, HF: light grey bars) and mice fed the SC or HF diet with rapamycin (SC+Rapa: dark grey bars, HF+Rapa: black bars) (n = 5: five mice were used per group). Values are means ± SD. ** $p < 0.01$.

Fig. 7. Beta cell proliferation and changes in gene expression levels in islets of 48-week-old C57/BL6J mice exposed to short-term high-fat (HF) diet.

a, b: Changes in body weight (a) and fed blood glucose level (b) in mice fed a standard chow (SC) or HF diet (SC: open circles, HF: filled circles) for 6 days (n = 8-9). c: Proliferation rates of beta cells, assessed by BrdU incorporation, were determined in mice fed the SC or HF diet (SC: white bars, HF: grey bars) for 7 days (n = 8–9: eight or nine mice were used per group). d: mRNA levels of *Ki67*, *Irs2*, *Foxm1*, *Ccna2* and *Ccnb1* in islets of mice fed the SC or HF diet (SC: white bars, HF: grey bars) for 7 days measured by real-time quantitative PCR. Data were normalised to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression (n = 4). Values are means ± SD. * $p < 0.05$; ** $p < 0.01$.

Supplementary Table 1. The primer sequences used for real-time quantitative PCR

Gene (Forward/Reverse)	Sequence
GAPDH forward	GGCCCCTCTGGAAAGCTGTGGTGT
GAPDH reverse	GTTGGGGGCCGAGTTGGGATAGG
Ki67 forward	CTGCCTGCGAAGAGAGCATC
Ki67 reverse	AGCTCCACTTCGCCTTTTGG
Pcna forward	ACCTGCAGAGCATGGACTCG
Pcna reverse	GCAGCGGTATGTGTCGAAGC
Irs2 forward	AACCTGAAACCTAAGGGACTGG
Irs2 reverse	CGGCGAATGTTCATAAGCTGC
Pdx1 forward	CTCCGGACATCTCCCCATAC
Pdx1 reverse	ACGGGTCCTCTTGTTTTCCT
Gck forward	AGAAGGCTCAGAAGTTGGAGAC
Gck reverse	GGATGGAATACATCTGGTGTTTCG
Glut2 forward	TGTGGTGTCGCTGTTTGTTG
Glut2 reverse	AATGAAGTTTGAGGTCCAGTTGG
Ins1 forward	GACCAGCTATAATCAGAGACC
Ins1 reverse	AGTTGCAGTAGTTCTCCAGCTG
Ins2 forward	AGCCCTAAGTGATCCGCTACAA
Ins2 reverse	AGTTGCAGTAGTTCTCCAGCTG
Mafa forward	CTTCAGCAAGGAGGAGGTCATC
Mafa reverse	GCGTAGCCGCGGTTCTT
Foxm1 forward	CTCAAGCCGCCTGCTATCCT
Foxm1 reverse	CAGCCCTTGCCTGCTAGCTT
Plk1 forward	GGTCCGGGAGACAAATGAGG
Plk1 reverse	TTGGAGGCGTTGACACTGGT
Aurkb forward	TCGGGGTGCTCTGCTATGAA
Aurkb reverse	CCACCTTGACAATCCGACGA
Survivin forward	GCAGCCCATGGGTAAGTGTG
Survivin reverse	CCGTTACCCCGTGGTAGGAA
Cenpa forward	AGTTCTGGCGGTCCTGACG
Cenpa reverse	CCGCCTTCCCTAAGCCTTCT
Cenpb forward	GGAGTCACCACCCAGGCTCT
Cenpb reverse	GCAGAAGGACCCGACGAGAT
Cenpe forward	TCATACACATCGGACGCCACTGAA

Cenpe reverse	ACTCTTTCTTAGCGTCAAGGGCCA
Cenpf forward	CGGAGGACAAAAACCCAGGAC
Cenpf reverse	GAACATCCATGGGCACCAAA
Cdc20 forward	TGACCGCTTTATCCCCCAAC
Cdc20 reverse	CTTCCGGCTGGTTTTCCTTG
Nek2 forward	TGACCGAACCAACACAACCCTGTA
Nek2 reverse	TTCTGTGACACTCTTTCAGGGCCA
Ccnd1 forward	TAGGCCCTCAGCCTCACTC
Ccnd1 reverse	CCACCCCTGGGATAAAGCAC
Ccnd2 forward	AAGCCTGCCAGGAGCAAA
Ccnd2 reverse	ATCCGGCGTTATGCTGCTCT
Ccnd3 forward	CCAGCGTGTCCTGCAGAGTT
Ccnd3 reverse	CCTTTTGCACGCACTGGAAG
Ccna2 forward	TCCTTGCTTTTGACTTGGCT
Ccna2 reverse	ATGACTCAGGCCAGCTCTGT
Ccnb1 forward	TGGCCTCACAAAGCACATGA
Ccnb1 reverse	GCTGTGCCAGCGTGCTAATC

Supplementary Table 2. Gene ontology analysis of islets from wild-type mice exposed to short-term high-fat diet.

Rank	Term	*P* value
1	Cell cycle process	8.12E-16
2	Condensed chromosome	1.43E-14
3	Mitosis	1.65E-13
4	Nuclear division	1.65E-13
5	Organelle fission	4.83E-13
6	Cell cycle	5.85E-13
7	Cell division	8.26E-13
8	Chromosome segregation	6.01E-11
9	Condensed chromosome kinetochore	2.09E-10
10	Sister chromatid segregation	3.49E-10

The list shows the top 10 gene ontology analysis terms according to p-value. A total of 62 genes were significantly upregulated by short-term high-fat diet feeding.

Supplementary figure legends

Supplementary Fig. 1. Animal feeding protocols

Supplementary Fig. 2. Effect of short-term high-fat (HF) diet feeding on metabolism in 8-week-old C57/BL6J mice.

a, b, d, e: Changes in body weight (a, d) and fed blood glucose (b, e) in mice fed a standard chow (SC) or HF diet (SC: open circles, HF: filled circles) for 1 day (a, b) or 3 days (d, e) (n = 10). c, f: Weight of liver, pancreas and visceral fat in mice fed the SC or HF diet (SC: white bars, HF: grey bars) on day 2 (c) and day 4 (f) (n = 10). Values are means ± SD. * $p < 0.05$; ** $p < 0.01$.

Supplementary Fig. 3. The actual values from the insulin tolerance test in mice fed the SC or HF diet (SC: open circles, HF: filled circles) on day 6 (n = 10). Values are means ± SD.

Supplementary Fig. 4. Glucose-stimulated insulin secretion in isolated islets from C57/BL6J mice exposed to short-term high-fat (HF) diet feeding.

a: Glucose-stimulated insulin secretion was measured at 5.6 mM or 22 mM glucose in isolated islets from 8-week-old C57/BL6J mice fed a standard chow (SC) or HF diet (SC: white bars, HF: grey bars) for 7 days. Insulin concentration adjusted for insulin content of each well (n = 11–12).

b: Insulin content of isolated islets from 8-week-old C57/BL6J mice fed the SC or HF diet (SC: white bars, HF: grey bars) for 7 days (n = 11–12). Values are means ± SD. ** $p < 0.01$.

Supplementary Fig. 5. Glucose-stimulated insulin secretion in isolated islets from wild-type and *Gck*$^{+/-}$ mice exposed to short-term high-fat (HF) diet feeding.

a: Glucose-stimulated insulin secretion was measured at 5.6 mM or 22 mM glucose in isolated

islets from 8-week-old wild-type mice fed the SC or HF diet (SC: white bars, HF: light grey bars) and *Gck*$^{+/-}$ mice fed the SC or HF diet (SC: dark grey bars, HF: black bars) for 7 days. Insulin concentration adjusted for insulin content of each well (n = 6). b: Insulin content of isolated islets from 8-week-old wild-type mice fed the SC or HF diet (SC: white bars, HF: light grey bars) and *Gck*$^{+/-}$ mice fed the SC or HF diet (SC: dark grey bars, HF: black bars) for 7 days (n = 6). Values are means ± SD.

Supplementary Fig. 6. mRNA levels of *Foxm1* in islets of wild-type mice fed a standard chow (SC) or high-fat (HF) diet (SC: white bars, HF: grey bars) and those of *Gck*$^{+/-}$ mice fed the SC or HF diet (SC: dark grey bars, HF: black bars) for 7 days measured by real-time quantitative PCR. Data have been normalised to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression (n = 6–7). Values are means ± SD. ** $p < 0.01$.

Fig. 1

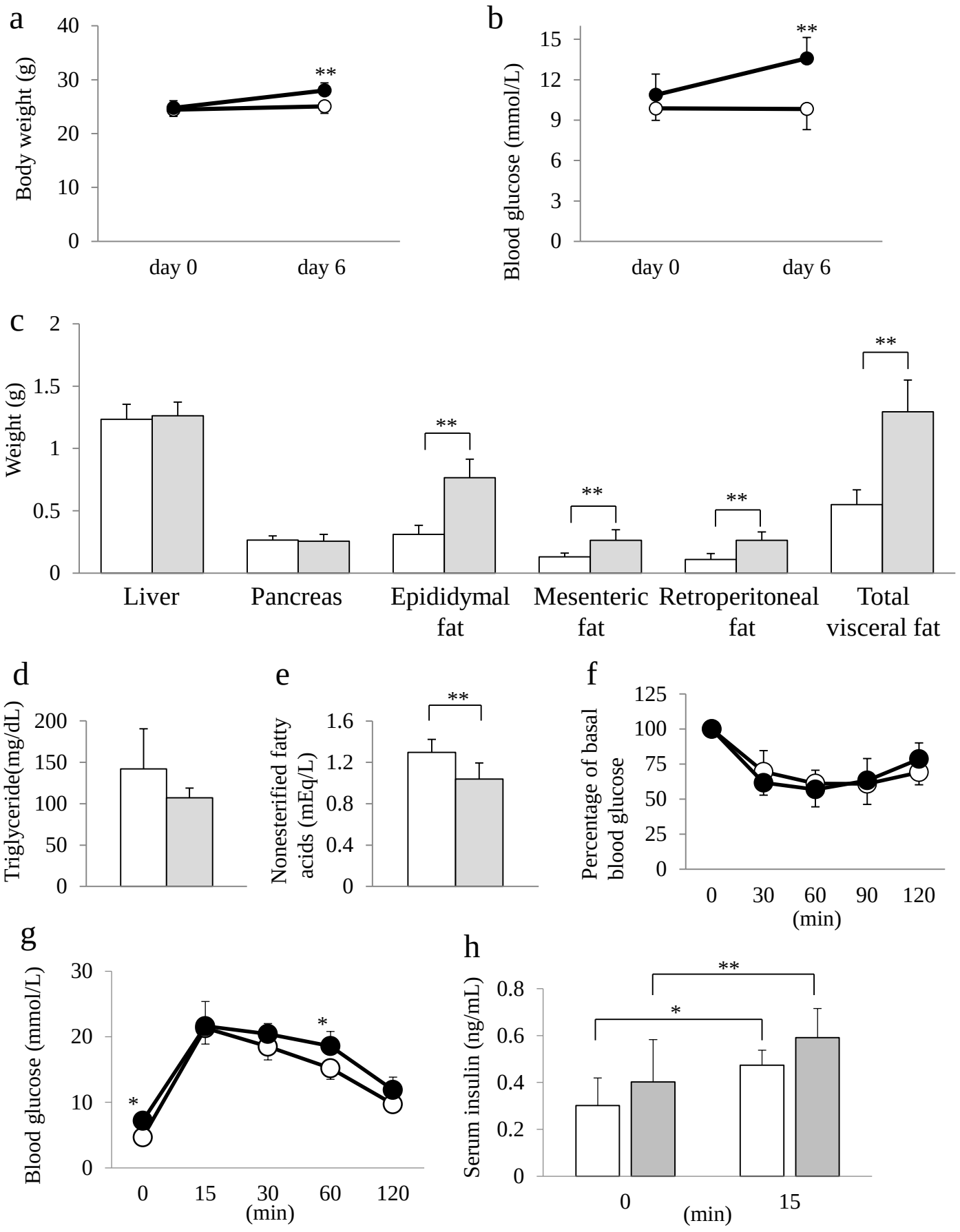

a
Body weight (g)
40
30
20
10
0
**
day 0
day 6
b
Blood glucose (mmol/L)
15
12
9
6
3
0
**
day 0
day 6
c
Weight (g)
2
1.5
1
0.5
0
**
**
**
**
Liver
Pancreas
Epididymal fat
Mesenteric fat
Retroperitoneal fat
Total visceral fat
d
Triglyceride(mg/dL)
200
150
100
50
0
e
Nonesterified fatty acids (mEq/L)
1.6
1.2
0.8
0.4
0
**
f
Percentage of basal blood glucose
125
100
75
50
25
0
0
30
60
90
120
(min)
g
Blood glucose (mmol/L)
30
20
10
0
*
*
0
15
30
60
120
(min)
h
Serum insulin (ng/mL)
0.8
0.6
0.4
0.2
0
**
*
0
15
(min)

Fig. 2

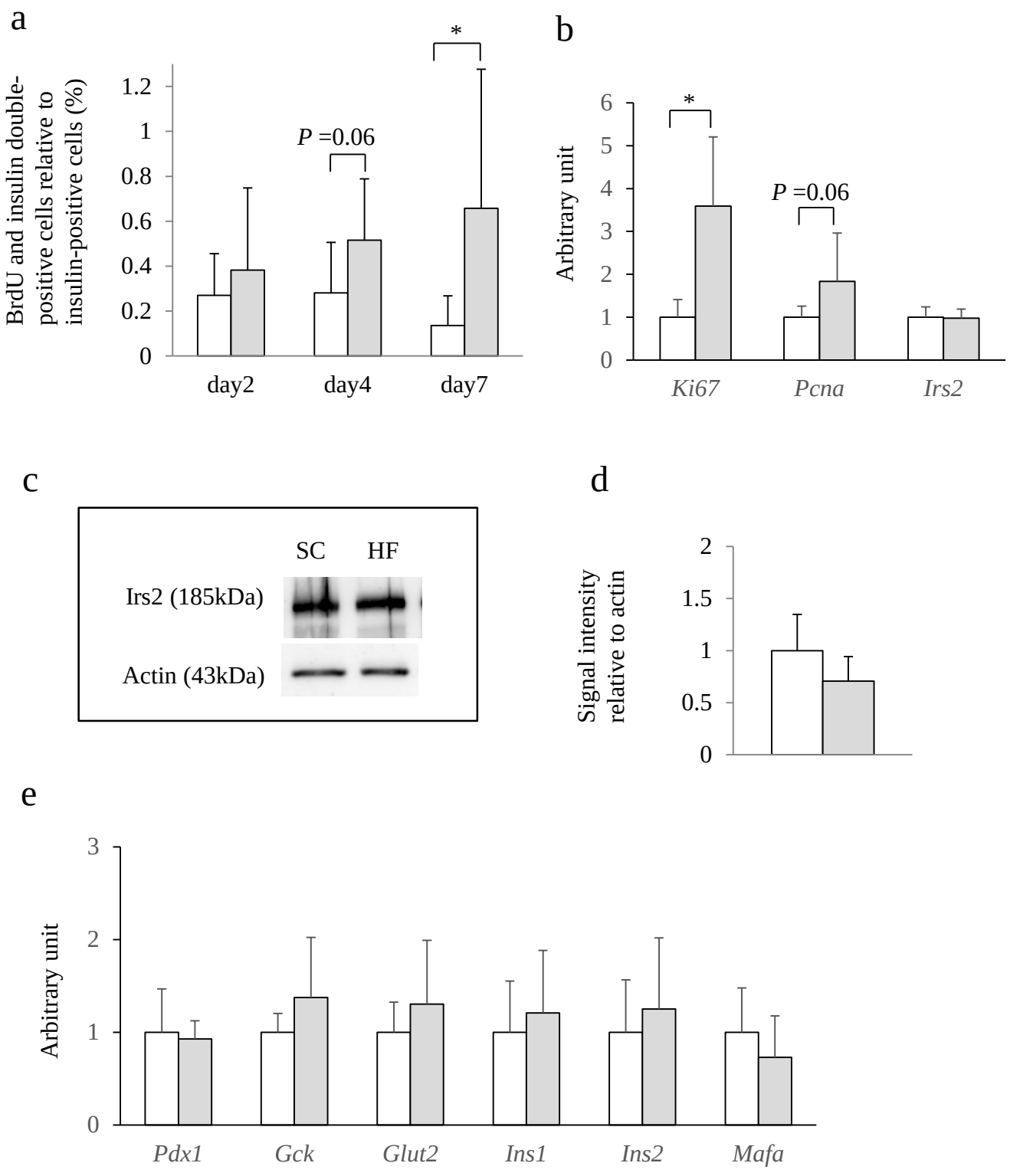

a
BrdU and insulin double-positive cells relative to insulin-positive cells (%)
1.2
1
0.8
0.6
0.4
0.2
0
P =0.06
*
day2
day4
day7
b
Arbitrary unit
6
5
4
3
2
1
0
*
P =0.06
Ki67
Pcna
Irs2
c
SC
HF
Irs2 (185kDa)
Actin (43kDa)
d
Signal intensity relative to actin
2
1.5
1
0.5
0
e
Arbitrary unit
3
2
1
0
Pdx1
Gck
Glut2
Ins1
Ins2
Mafa

Fig. 3

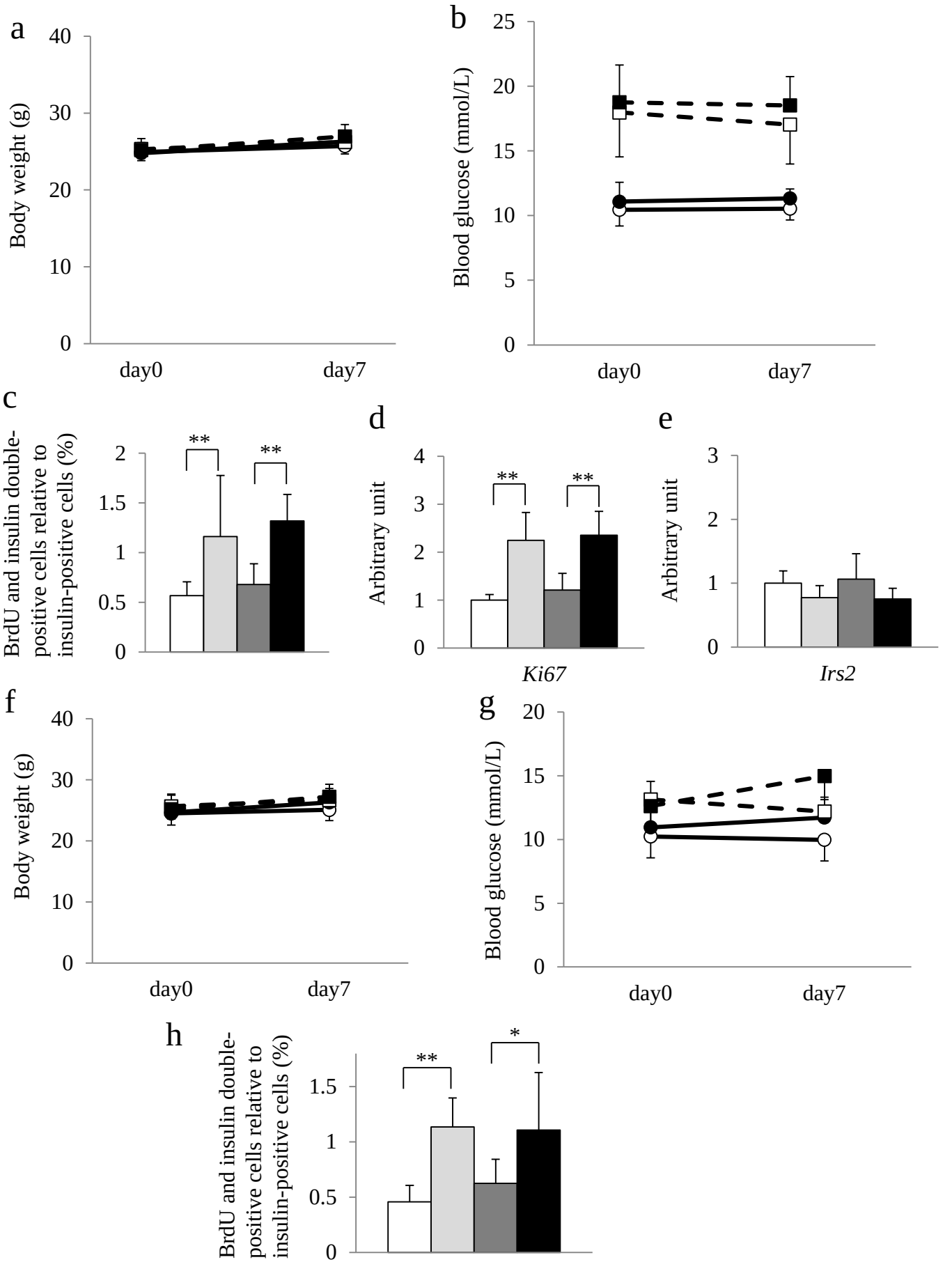
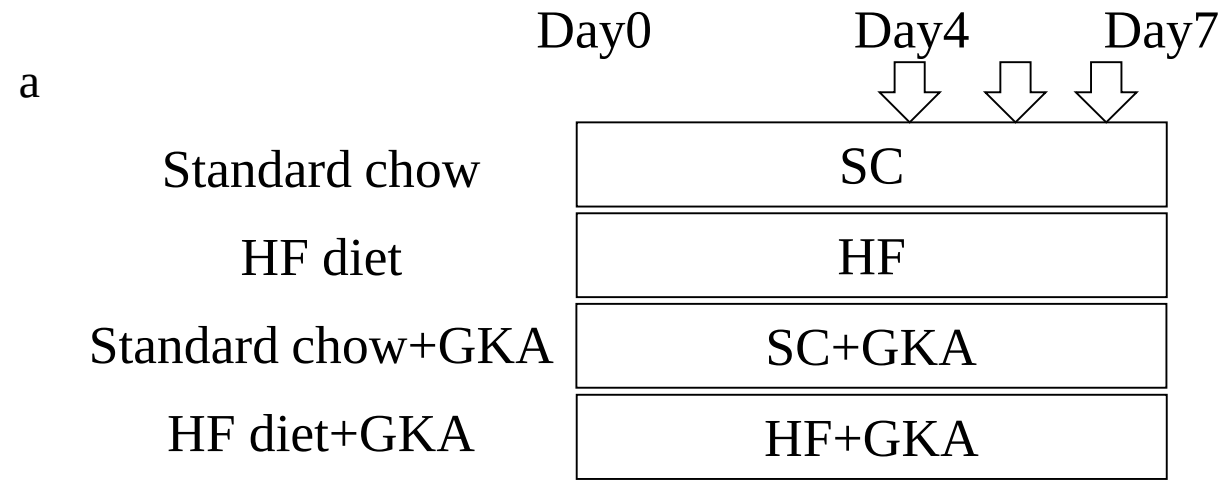
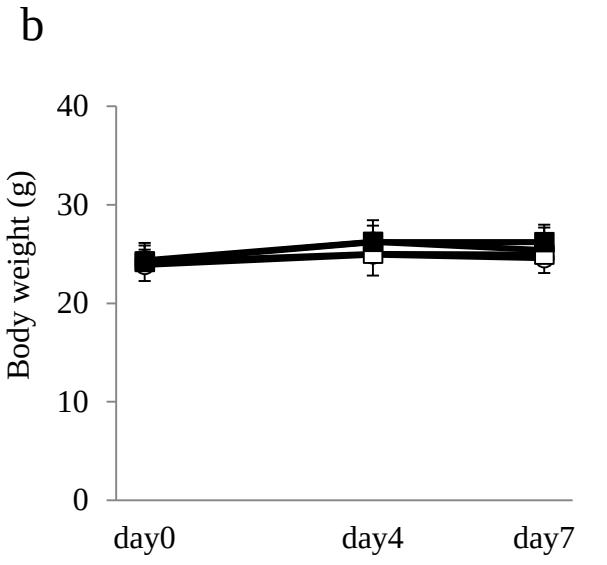
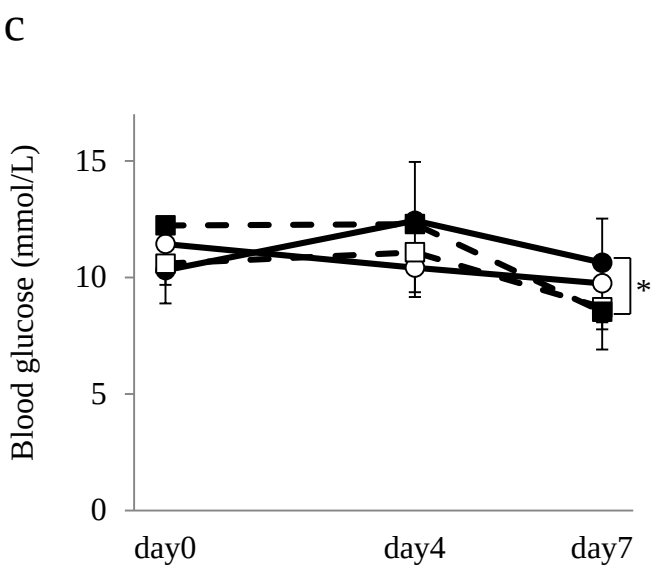

a
Body weight (g)
40
30
20
10
0
day0
day7
b
Blood glucose (mmol/L)
25
20
15
10
5
0
day0
day7
c
BrdU and insulin double-positive cells relative to insulin-positive cells (%)
2
1.5
1
0.5
0
**
**
d
Arbitrary unit
4
3
2
1
0
**
**
Ki67
e
Arbitrary unit
3
2
1
0
Irs2
f
Body weight (g)
40
30
20
10
0
day0
day7
g
Blood glucose (mmol/L)
20
15
10
5
0
day0
day7
h
BrdU and insulin double-positive cells relative to insulin-positive cells (%)
1.5
1
0.5
0
**
*

Fig. 4


a
Day0
Day4
Day7
Standard chow
SC
HF diet
HF
Standard chow+GKA
SC+GKA
HF diet+GKA
HF+GKA


b
Body weight (g)
40
30
20
10
0
day0
day4
day7


c
Blood glucose (mmol/L)
15
10
5
0
day0
day4
day7
*

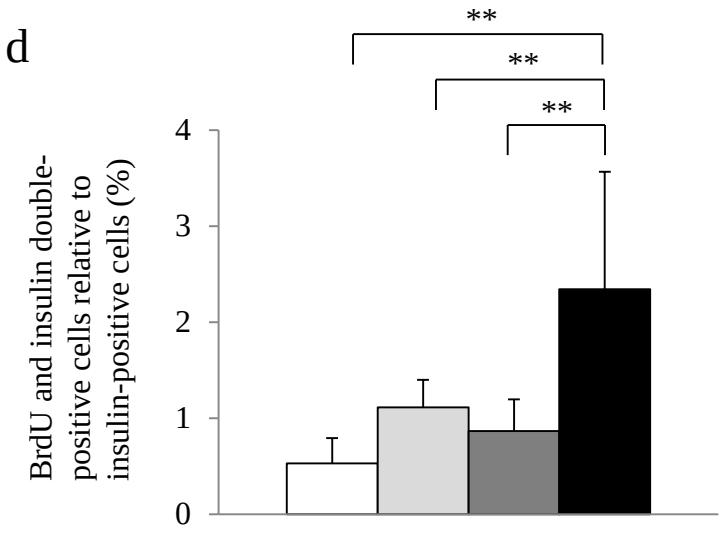

d
BrdU and insulin double-positive cells relative to insulin-positive cells (%)
**
**
**
4
3
2
1
0

Fig. 5

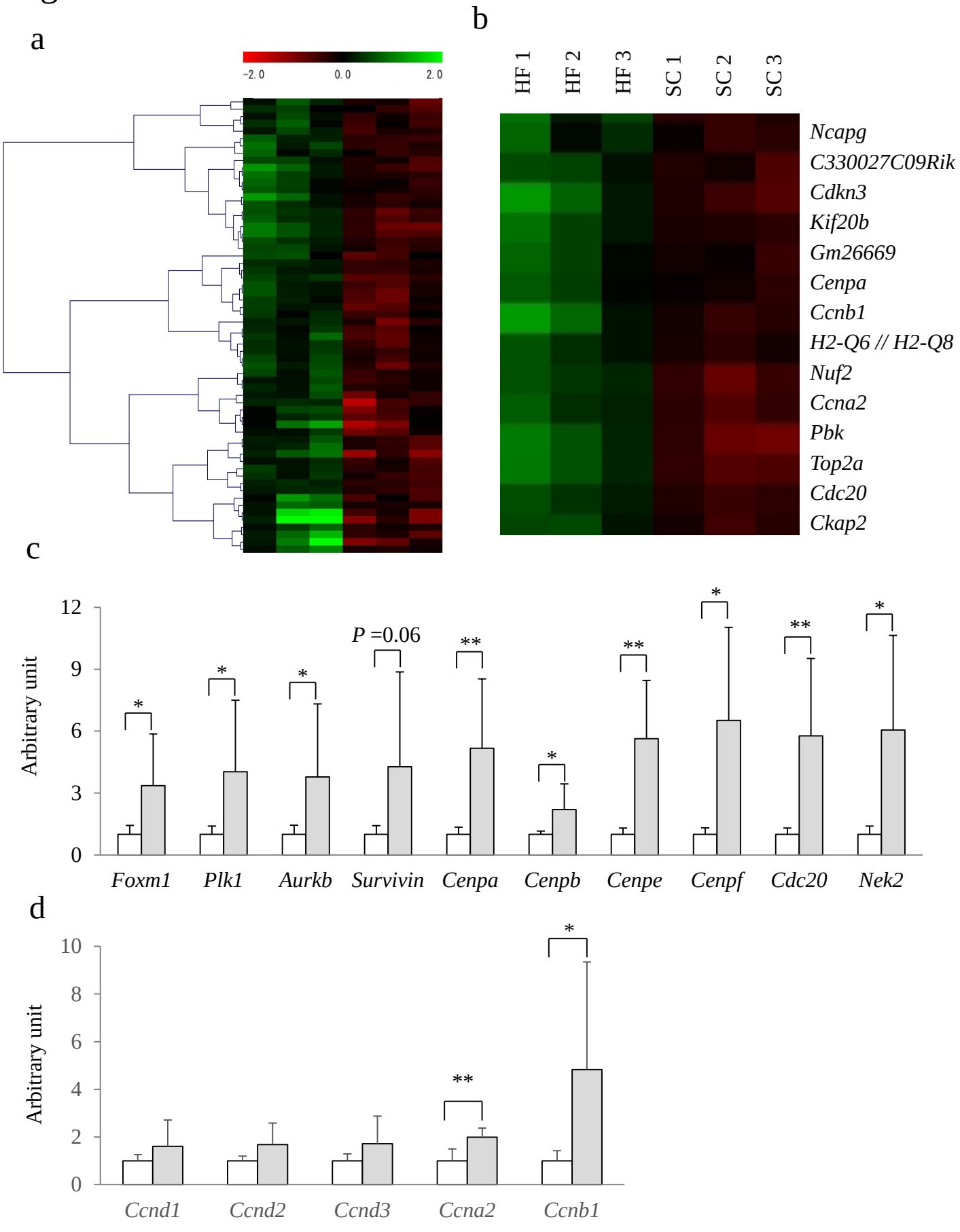
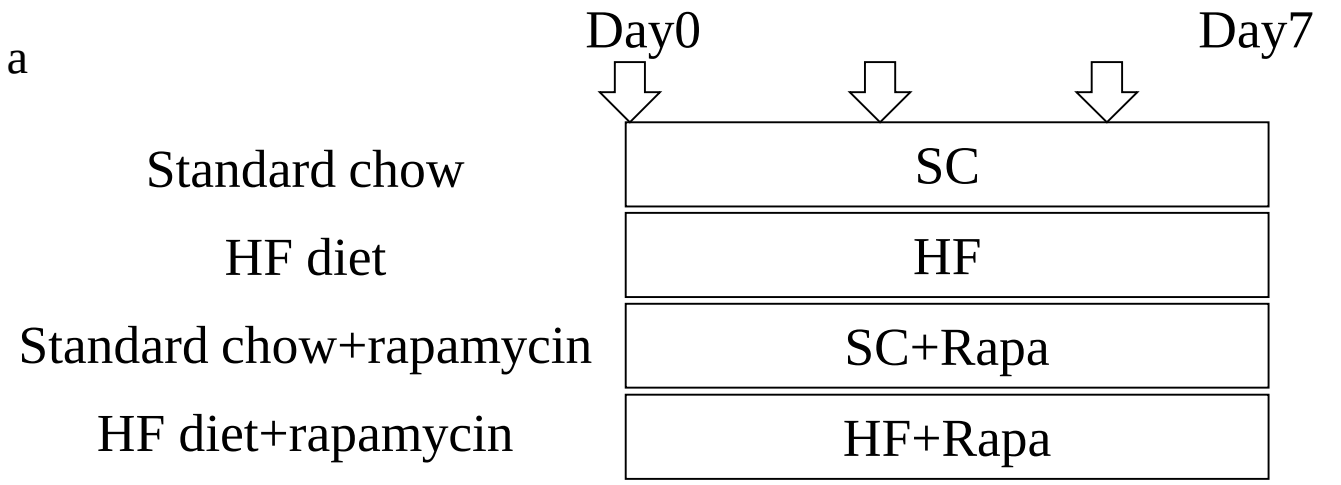
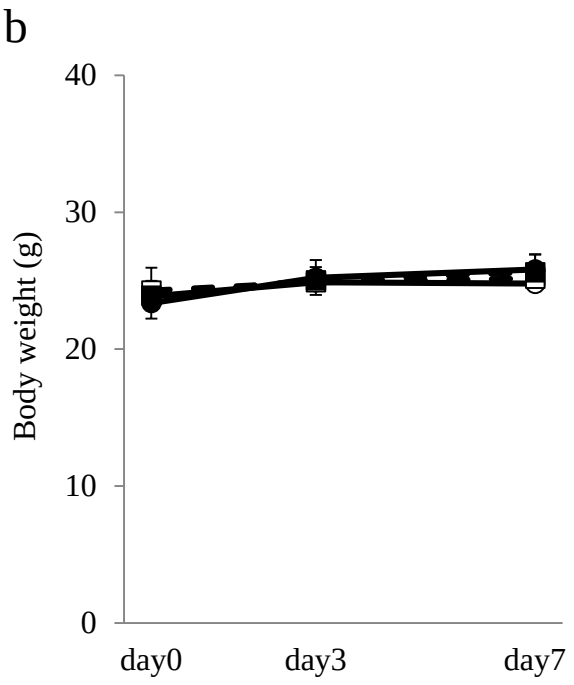
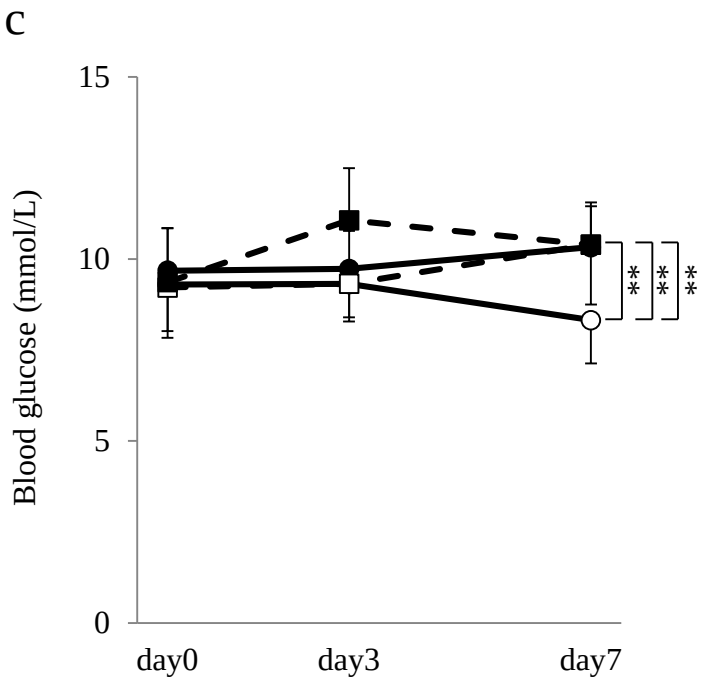

a
b
c
d
-2.0
0.0
2.0
HF 1
HF 2
HF 3
SC 1
SC 2
SC 3
Ncapg
C330027C09Rik
Cdkn3
Kif20b
Gm26669
Cenpa
Ccnb1
H2-Q6 // H2-Q8
Nuf2
Ccna2
Pbk
Top2a
Cdc20
Ckap2
Arbitrary unit
P =0.06
Foxm1
Plk1
Aurkb
Survivin
Cenpa
Cenpb
Cenpe
Cenpf
Cdc20
Nek2
Ccnd1
Ccnd2
Ccnd3
Ccna2
Ccnb1

Fig. 6


a
Day0
Day7
Standard chow
SC
HF diet
HF
Standard chow+rapamycin
SC+Rapa
HF diet+rapamycin
HF+Rapa


b
Body weight (g)
40
30
20
10
0
day0
day3
day7


c
Blood glucose (mmol/L)
15
10
5
0
day0
day3
day7
**
**
**

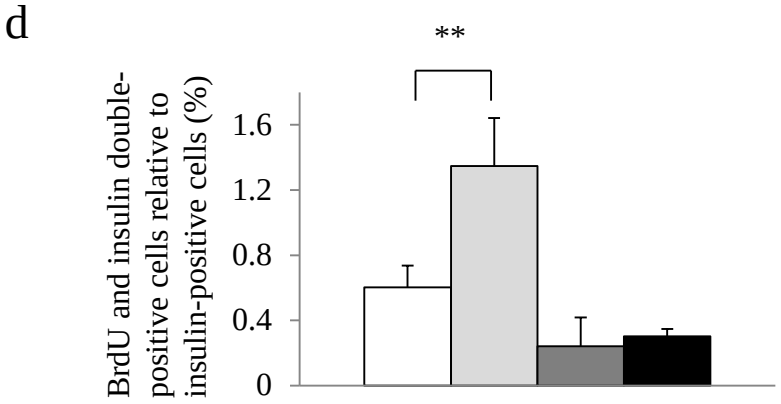
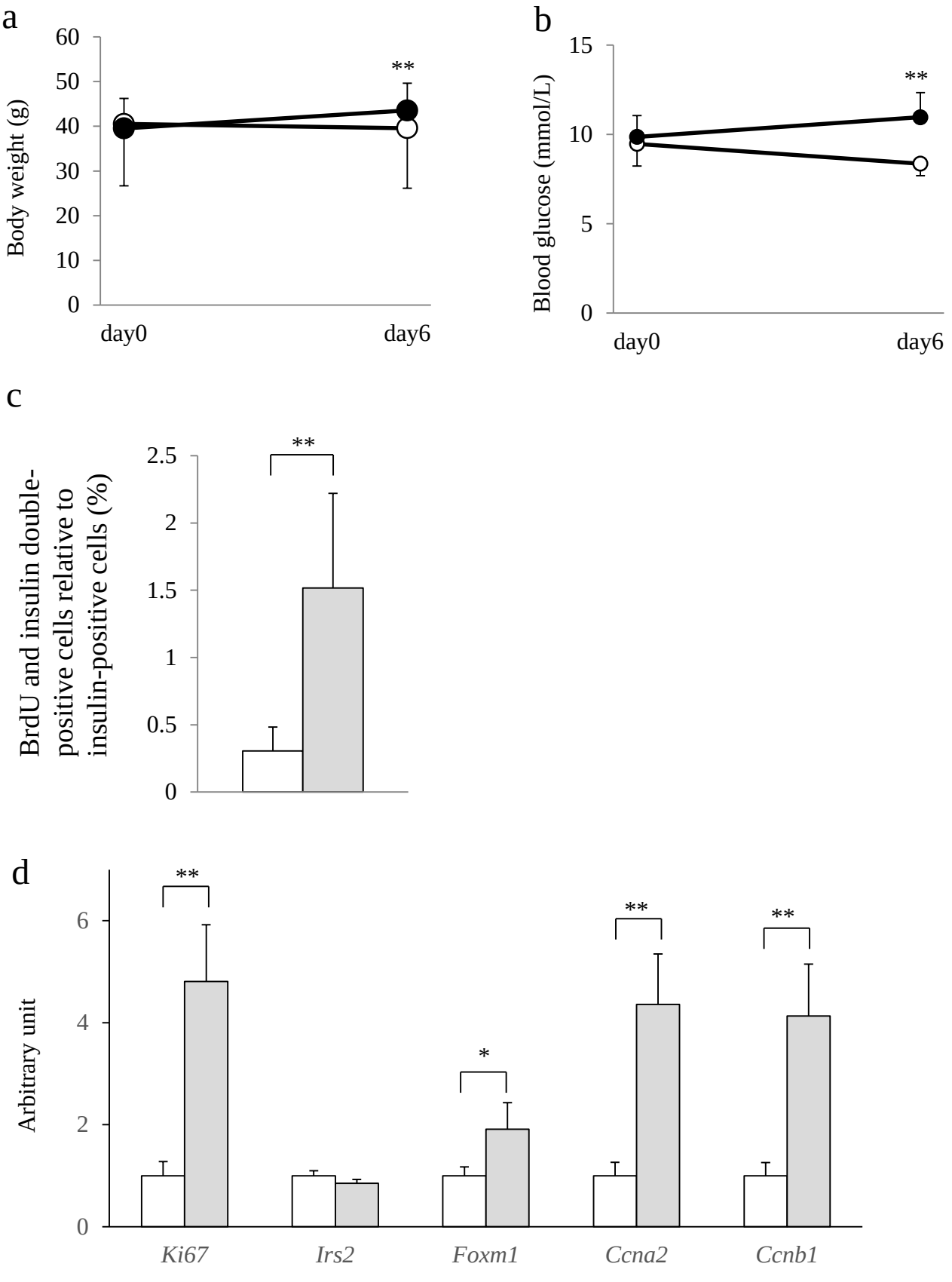

d
**
BrdU and insulin double-positive cells relative to insulin-positive cells (%)
1.6
1.2
0.8
0.4
0

Fig. 7


a
Body weight (g)
60
50
40
30
20
10
0
**
day0
day6
b
Blood glucose (mmol/L)
15
10
5
0
**
day0
day6
c
BrdU and insulin double-positive cells relative to insulin-positive cells (%)
2.5
2
1.5
1
0.5
0
**
d
Arbitrary unit
6
4
2
0
**
*
**
**
Ki67
Irs2
Foxm1
Ccna2
Ccnb1