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ORIGINAL

Rubicon in pancreatic beta cells plays a limited role in maintaining glucose homeostasis following increased insulin resistance

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Abstract. Autophagy has been reported to play a crucial role in the maintenance of intracellular homeostasis, including in pancreatic beta cells. Rubicon, which interacts with the phosphoinositide 3-kinase (PI3K) complex, through autophagy-related 14 (ATG14), is among the few autophagy regulators that have been reported to inhibit autophagic flux to date and the deletion of Rubicon has been shown to increase autophagic flux. Based on previous results showing a causal relationship between autophagic dysfunction and pancreatic beta-cell impairment, we hypothesized that the deletion of Rubicon in pancreatic beta cells would improve cell integrity and confer protective effects. To test this hypothesis, we first confirmed that Rubicon knockdown (KD) promoted autophagic flux in βTC3 pancreatic beta-cell line. Next, we generated pancreatic beta-cell-specific Rubicon knockout (βKO) mice, by administering tamoxifen to Rubicon$^{flox/flox}$:MIP-Cre-ERT mice, which showed normal glucose tolerance and insulin secretion under a normal chow diet, despite successful gene recombination. We also attempted to increase insulin resistance by feeding the mice with a high-fat diet for an additional 2 months to find little differences among the parameters evaluated for glucose metabolism. Finally, severe insulin resistance was induced with insulin receptor antagonist treatment, which resulted in comparable glucose homeostasis measurements between Rubicon βKO and control mice. In summary, these results suggest that in pancreatic beta cells, Rubicon plays a limited role in the maintenance of systemic glucose homeostasis.

Key words: Autophagy, PI3K, Rubicon, Pancreatic beta cells, Insulin resistance

DIABETES MELLITUS is a growing global health concern, associated with microvascular and macrovascular complications [1]. Insulin resistance, followed by pancreatic beta-cell failure, has been postulated to play a canonical role in the development of type 2 diabetes [2]. Pancreatic beta-cell-specific autophagy defects have been reported to result in decreased insulin secretion and glucose intolerance [3, 4]. In addition, autophagy impairment deteriorates pancreatic beta-cell function, *via* accumulation of toxic human islet amyloid polypeptide (IAPP), also suggesting that autophagy in pancreatic beta cells is necessary for the preservation of homeostasis [5-7]. These findings imply that activation of autophagy in pancreatic beta cells can mitigate cell failure caused by insulin resistance and accumulation of toxic fibrils. The administration of rapamycin, a potent mammalian target of rapamycin (mTOR) inhibitor that activates autophagy, improved glucose tolerance in Akita mice by reducing their endoplasmic reticulum (ER)-stress-mediated pancreatic beta-cell failure through activation of autophagy [8]. However, autophagy-activating drugs, such as rapamycin, can also have side effects, such as pancreatic islet toxicity [9]; therefore, autophagy regulation can be difficult to target using normal treatment modalities, making the genetic manipulation of autophagic activity a potentially favorable alternative.

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Rubicon (Run domain Beclin-1 interacting and cysteine-rich containing protein) has been identified as a protein that interacts with Beclin-1, a component of the Vps34 complex involved in initiation of isolation membrane formation, and negatively regulates autophagic activity [10, 11]. For example, liver-specific deletion of Rubicon ameliorated high-fat diet (HFD)-induced steatohepatitis by activating autophagy [12]. In addition, Rubicon knockout (KO) worms showed prolonged life spans, suggesting that inactivation of Rubicon has beneficial effects mediated by increased autophagic activity [13]. Based on these findings, we hypothesized that activation of autophagy in pancreatic beta cells, mediated by deletion of Rubicon, could be beneficial for preserving pancreatic beta-cell homeostasis against insulin resistance. In contrast with our prediction, no significant improvements in glucose tolerance were observed, even after induction of severe insulin resistance *in vivo*, although Rubicon KD in the pancreatic beta-cell line effectively increased autophagic flux.

Materials and Methods

Cell culture and experiments for loss-of-function of Rubicon

MIN6 cells were kindly provided by Prof. Junichi Miyazaki (Osaka University) and were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, Carlsbad, CA), supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin (Nacalai Tesque, Tokyo, Japan), and 0.0005% β-mercaptoethanol. βTC3 cells were cultured in DMEM, supplemented with 10% fetal bovine serum, 15% horse serum, and 1% penicillin-streptomycin. Amino acid starvation was performed by replacing the media with Hank's Balanced Salt Solution (HBSS, Gibco) for 8 hours. Bafilomycin A1 (BafA1) was added at the concentration of 100 nM. To generate Rubicon knockdown (Rubicon KD) βTC3 cells, 25 pmol of small interfering RNA (siRNA), with the sequence CUAUGCCGCCUUCAGUACA [11], was transfected with RNAiMAX (ThermoFisher Scientific, Waltham, MA), and the cells were incubated for 72 hours. Rubicon KO MIN6 cells were established by transducing guide RNA (gRNA), targeted to mouse Rubicon, into a MIN6 clone efficiently expressing Cas9, as described previously [14]. The gRNA target sequence is 5'-GGCTCAG ATCGATGCATCCA-3', as described previously [15].

Examination of glucose-stimulated insulin secretion

We prepared Krebs Ringer Buffer (KRB), containing 2.8 mM glucose (low-glucose) or 16.8 mM glucose (high-glucose), as previously described [16]. MIN6 cells were incubated in low-glucose KRB, at 37°C for 30 min, before being stimulated with high-glucose KRB. Insulin secretion was evaluated using an insulin enzyme-linked immunosorbent assay (ELISA) kit (Morinaga, Tokyo, Japan) and was standardized by the total protein amount of the secretory cells.

Animal experiments

Rubicon$^{flox/flox}$ mice have been previously described [12]. MIP-Cre-ERT mice were kindly provided by Dr. Louis H. Philipson (The University of Chicago, Chicago) [17]. For induction of Cre-mediated recombination, we administered 6 mg of tamoxifen, by subcutaneous injection, 3 times at the age of 6 weeks. HFD (#D12492), consisting of 60% kcal fat, 20% kcal carbohydrate and, 20% kcal protein, was purchased from RESEARCH DIET (New Brunswick, NJ). To perform intraperitoneal glucose tolerance test (IPGTT), 2 g/kg of glucose was intraperitoneally injected and blood glucose and plasma insulin were measured at the indicated times. Insulin tolerance test (ITT) was performed by intraperitoneally administering 0.75 U/kg of human insulin following 6-hr starvation and blood glucose were evaluated at the indicated times. All mouse experiments were performed in accordance with Juntendo University School of Medicine regulations and with the permission of the Animal Care and Use Committee of Juntendo University.

Insulin receptor antagonist

An insulin receptor antagonist, S961, was kindly provided by Dr. Lauge Schaeffer (Novo Nordisk) [18]. S961 was administered using a subcutaneously implanted ALZET osmotic pump (ALZET Osmotic Pumps, Cupertino, CA), which released S961 at a rate of 20 nmol per week.

Sample preparation for western blotting

Pancreatic islets were isolated by collagenase digestion and collected under a microscope, as previously described [3]. Protein samples were suspended in radioimmunoprecipitation assay (RIPA) buffer, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed, followed by western blotting, using a standard protocol. Antibodies against Rubicon (Cell Signaling #8465), microtubule-associated proteins 1A/1B light chain 3 (LC3, Sigma Aldrich #L7543), p62 (PROGEN #GP62-C), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Cell Signaling #2118) were purchased from Nacalai Tesque (Tokyo, Japan). Band intensities were detected by chemiluminescence and quantified with FUSION FX system (VILBER, Collégien, France).

Statistical analysis

Significant differences between two groups of data were assessed by Student's *t*-test. All data are presented as the mean ± SEM.

Results

Effects following Rubicon loss-of-function in pancreatic beta-cell lines

Several reports have shown that Rubicon loss-of-function promotes autophagic flux in various cell lines [10, 11]; however, whether Rubicon also regulates autophagic flux in pancreatic-beta-cell-derived cell lines has not been determined. First, we attempted to knockdown Rubicon in βTC3 cells, a pancreatic-beta-cell-derived cell line compatible with siRNA transfection using lipofection and, evaluated autophagic flux by western blotting. The effective knockdown of Rubicon was confirmed following the transfection of Rubicon siRNA, and the LC3-II band and LC3-II/I ratio were slightly decreased in Rubicon KD cells compared with control cells, after starvation for 8 hours in HBSS media (Fig. 1A and B). In addition, starvation using HBSS media containing 100 nM bafilomycin A1, a specific inhibitor for vacuolar-type H^+-ATPase, which can be used to evaluate autophagic flux [19], showed that autophagic flux increased in the Rubicon KD cells, accompanied by increased LC3-II levels (Fig. 1A and B). These data suggested that Rubicon negatively regulates autophagic flux in response to starvation also in βTC3 cells. Next, to investigate whether deletion of Rubicon affects insulin secretion, we established pancreatic-beta-cell-derived MIN6 cells constitutively expressing Cas9 and transduced gRNA targeted to Rubicon [14], to successfully generate Rubicon KO MIN6 cells (Fig. 1C). The levels of glucose-stimulated insulin secretion, which is induced by replacing low-glucose media with high-glucose media, were comparable between control and Rubicon KO cells (Fig. 1D). These data suggested that Rubicon does not directly regulate insulin secretion in pancreatic beta-cell lines, despite its negative regulatory role in autophagic flux.

Generation of pancreatic beta-cell-specific Rubicon KO mice

To further investigate Rubicon's role in the maintenance of pancreatic beta-cell homeostasis *in vivo*, we next generated beta-cell-specific Rubicon KO (βKO) mice, by crossing $Rubicon^{flox/flox}$ mice [12] with MIP-Cre-ERT mice, which express Cre recombinase under the control of mouse insulin1 promotor, in a tamoxifen-dependent manner [17]. First, we investigated whether beta-cell-specific Rubicon deletion affects glucose tolerance in mice fed with normal chow (NC) for 6 weeks, followed by 2 months of HFD, to induce gradual insulin resistance (Fig. 2A). Control and βKO mice demonstrated similar increases in body weights (BWs) following both NC and HFD, although the increased BW for βKO mice was slightly less after HFD than that for control mice (Fig. 2B). Western blotting for Rubicon in isolated islets showed that the beta-cell-specific KO was effective; however, the amounts of LC3-II, p62, and LC3-II/I ratio were comparable between βKO and control mice, suggesting no huge differences in autophagic activity (Fig. 2C and D).

Analysis of glucose metabolism in βKO mice under conditions of normal and increased insulin resistance

Glucose tolerance in control and βKO mice being fed with NC and HFD was evaluated by IPGTT, as shown in Fig. 2A. Under NC conditions, glucose tolerance was comparable between Control and βKO mice, at both 2 weeks and 6 weeks (Fig. 3A and B). Peripheral insulin resistance, as estimated by ITT, also showed no differences between the two groups (Fig. 3A and C). Next, we performed IPGTT and ITT after 4 weeks and 8 weeks of HFD. Although 4 weeks of HFD significantly increased insulin resistance and blood glucose levels in both control and βKO mice, no differences were observed between the two groups, for either IPGTT or ITT (Fig. 3A–C). After 8 weeks of HFD, glucose tolerance continued to remain comparable between control and βKO mice (Fig. 3A–C). To further evaluate glucose-stimulated insulin secretion, plasma insulin concentrations were measured 15 min after glucose injection. As shown in Fig. 3D, plasma insulin concentrations were similar between Control and βKO mice, although the concentrations after 4 weeks of HFD were higher than those under NC conditions. However, after 8 weeks of HFD, insulin secretion in βKO mice was reduced compared with control mice (Fig. 3D). This result may suggest that pancreatic beta-cell-specific Rubicon KO resulted in reduced protective effects for pancreatic beta cells in response to increased insulin resistance. Alternatively, the nonsignificant improvements in insulin sensitivity that were observed in βKO after 8 weeks of HFD (Fig. 3A) may suppress unnecessary insulin secretion from pancreatic beta cells. In any case, the reduced insulin secretion observed in βKO mice after 8 weeks of HFD did not affect systemic glucose tolerance.

Effects of acute insulin resistance induction in βKO mice

The data from the previous section prompted us to investigate whether acute and more robust induction of

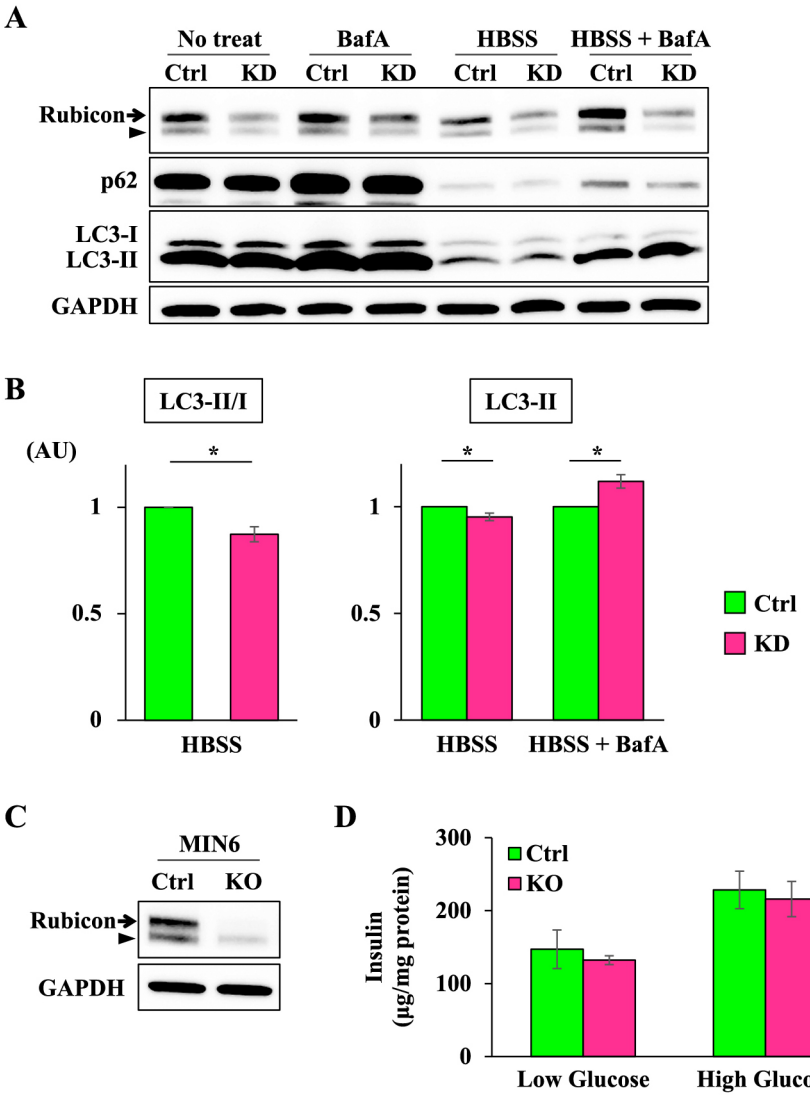

Fig. 1 The role of Rubicon in regulation of autophagic flux and glucose-stimulated insulin secretion in pancreatic beta-cell lines. A. Representative figures of western blots used to estimate autophagic flux in βTC3 cells transfected with Rubicon (KD) or control (Ctrl) siRNA under the conditions of normal media (No treatment), normal media with 100 nM bafilomycin A1 (BafA), HBSS, and HBSS with BafA. The cells were stimulated for 8 hours in each condition. The arrowhead indicates non-specific bands. B. Quantification of the bands shown in panel A. The intensity of the LC3-I or LC3-II bands, for the groups treated with either HBSS or HBSS containing BafA, were normalized against the GAPDH intensity, and LC3-II/I ratio was calculated (left). The intensity of the LC3-II bands was expressed as a ratio to the control (right). Data shown are representative of three separate experiments. * indicates $p < 0.05$. C. Confirmation of Rubicon knockout (KO) in MIN6 cells using CRISPR/Cas9-mediated gene deletion by western blotting. The arrowhead indicates non-specific bands. D. Comparison of glucose-stimulated insulin secretion (GSIS) between control and Rubicon KO MIN6 cells. Data shown are representative of three independent experiments.

insulin resistance might reveal the effects of Rubicon KO in pancreatic beta cells. S961, an insulin receptor antagonist, is a potent inducer of insulin resistance [18]; therefore, we examined the effects of S961-induced insulin resistance on glucose metabolism in βKO mice (Fig. 4A). We successfully confirmed the deletion of Rubicon in βKO mice, whereas autophagic activity, as estimated by western blotting of levels of LC3-II, p62, and LC3-II/I ratio, was comparable between the control and βKO mice (Fig. 4B and C). After 1 week of continuous S961 infusion, both control and βKO mice demonstrated significantly increased plasma insulin levels, due to hypersecretion of insulin following robust insulin resistance; however, no significant difference was observed between the two groups in contrast to the data shown in HFD-treated mice (Fig. 4D). Blood glucose levels, evaluated by IPGTT, were also comparable between control and βKO mice, despite the significant increase in blood glucose levels after S961 treatment (Fig. 4E and F).

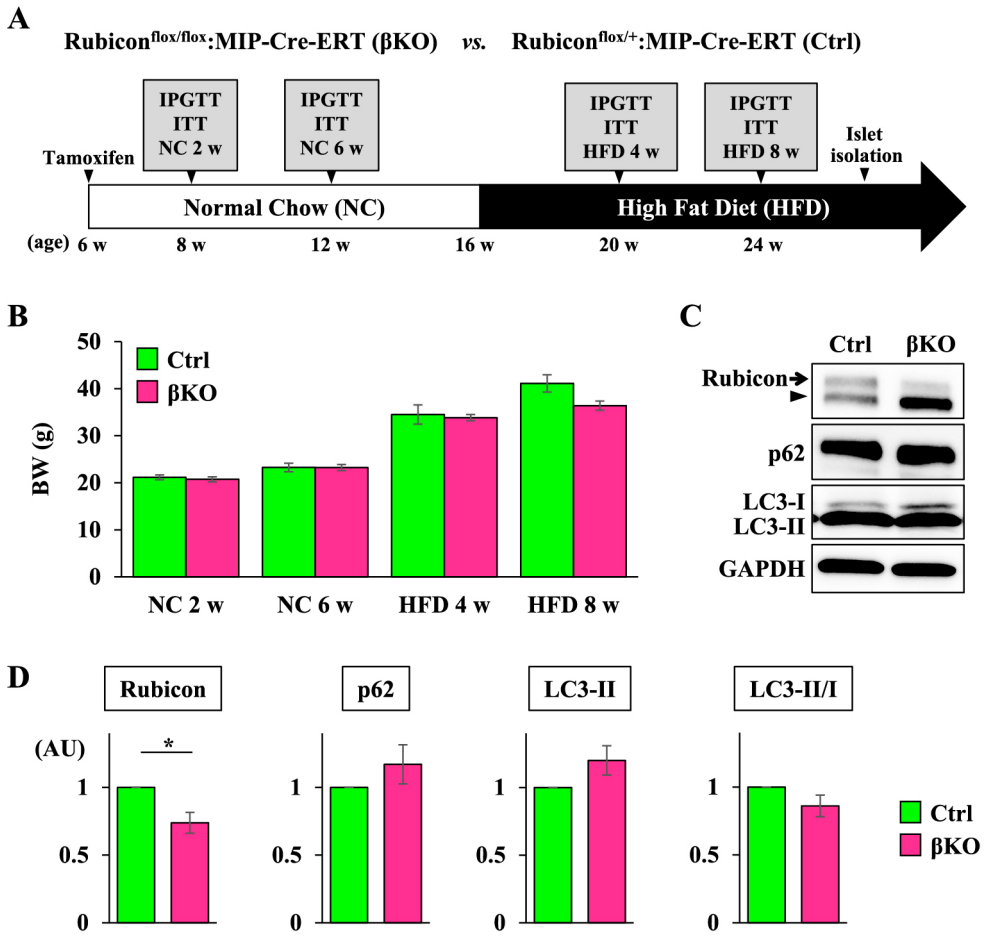

Fig. 2 Generation of pancreatic beta-cell-specific Rubicon KO (βKO) mice by crossing Rubicon$^{flox/flox}$ with MIP-Cre-ERT mice. A. A scheme for evaluating glucose metabolism in βKO mice. βKO mice were generated by injecting tamoxifen at the age of 6 weeks, to induce Cre-mediated recombination, and glucose tolerance and insulin sensitivity were evaluated at 8 and 12 weeks, under normal chow (NC) conditions. At the age of 16 weeks, NC was replaced with high-fat diet (HFD). IPGTT and ITT were performed at the indicated times, and islets were isolated for further analysis. B. Changes in body weight for βKO (n = 4) and control mice (n = 5), following the experiment. C. Representative western blot of isolated islets derived from βKO and control mice. The arrowhead indicates non-specific bands. D. Quantification of the western blotting analysis shown in panel C. The amount of protein derived from βKO (n = 4) mice is expressed as a ratio to control levels (n = 4). LC3-II/I ratio was calculated as described in Fig. 1B. * indicates $p < 0.05$.

Discussion

Maintaining pancreatic beta-cell homeostasis against various cellular stresses is a critical strategy for the prevention of diabetes onset and deterioration of diabetic symptoms, and improving autophagic function is an attractive strategy, based on recent findings that suggest that autophagy plays a protective role in pancreatic beta-cell function. Many drugs, including clinically approved drugs, have been reported to modulate autophagy [20]; however, successful administration of medicines that promote autophagy has not yet been achieved. We focused on Rubicon, which is among the few molecules known to suppress autophagic flux and, examined whether the protective effects of autophagy could be achieved by deleting Rubicon in pancreatic beta cells. Consistent with previous data [10, 11], Rubicon KD promoted autophagic flux also in pancreatic beta-cell line βTC3, demonstrating that Rubicon negatively regulates autophagic flux. In contrast, our data demonstrated that pancreatic beta-cell-specific KO of Rubicon showed no improvements for glucose tolerance under NC or HFD conditions, implying that Rubicon deletion does not play a protective role in pancreatic beta-cell homeostasis. Furthermore, even after the acute induction of robust insulin resistance, using insulin receptor antagonist S961, βKO mice showed no improvements in glucose tolerance. These results could be caused by the insufficient deletion of Rubicon gene in pancreatic beta cells, as the Rubicon bands were not completely deleted after tamoxifen

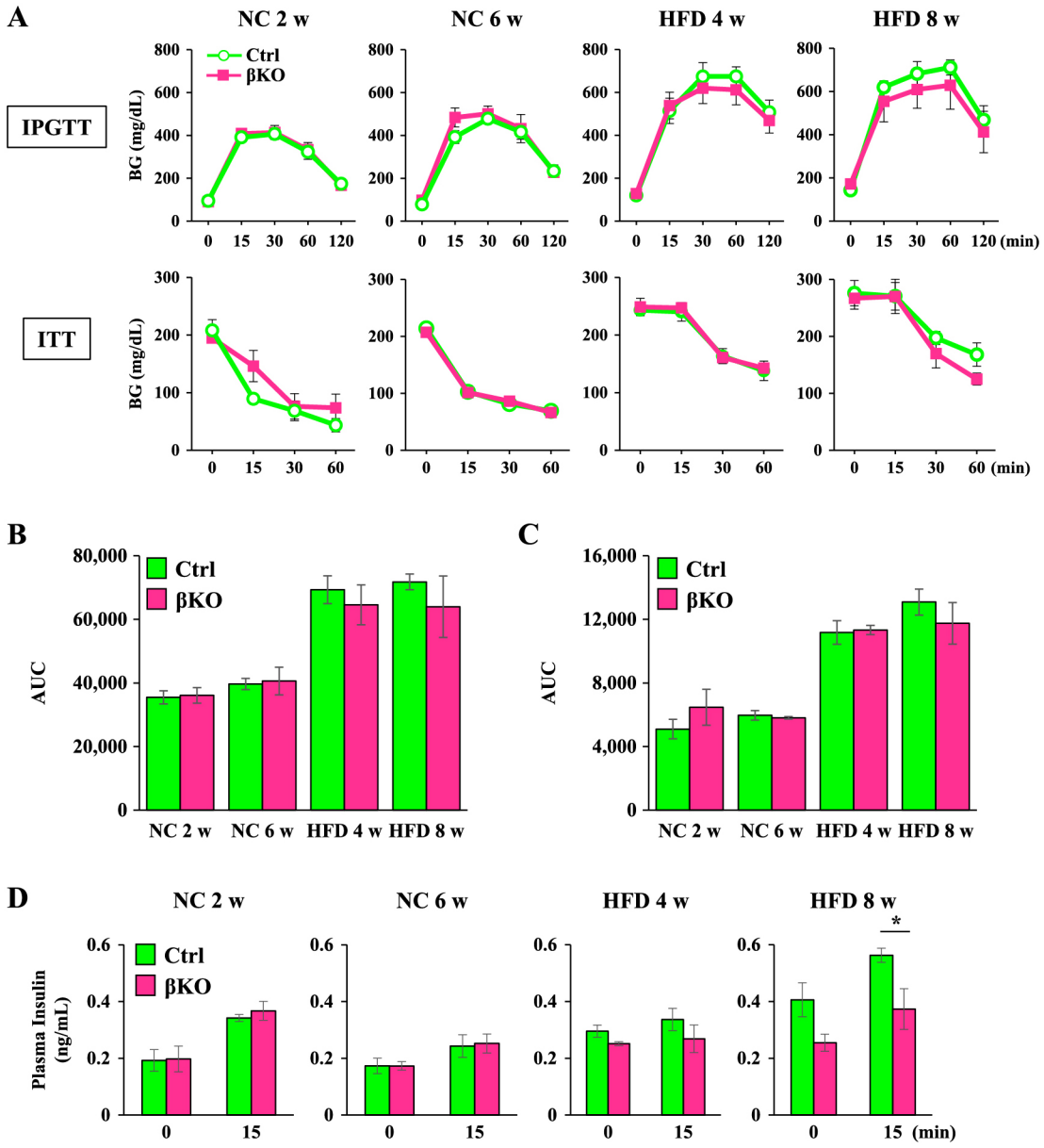

Fig. 3 Examination of glucose tolerance in βKO mice fed with NC, followed by HFD. A. IPGTT and ITT results at the indicated times, under NC and HFD conditions, between control (n = 4) and βKO (n = 5) mice. Note that one additional control mouse was evaluated after 2 weeks, under NC conditions. B and C. Area under the curve (AUC) estimation for IPGTT (B) and ITT (C). D. Plasma insulin concentrations of βKO and control mice, as assessed by IPGTT. * indicates $p < 0.05$.

treatment in western blotting analysis. However, the incomplete disappearance of Rubicon bands may derive from non-beta cells in pancreatic islets, such as alpha cells, delta cells, and pancreatic polypeptide (PP) cells, despite successful isolation of pancreatic islets, which was performed to avoid contamination of pancreatic acinar cells. On the other hand, pancreatic beta-cell dysfunction and glucose intolerance were manifested despite incomplete loss of ATG7 bands in western blots in pancreatic beta-cell-specific ATG7 KO mice, described in our previous report [3], implying that complete KO of autophagy regulatory genes is not necessarily required to evaluate their roles in pancreatic beta-cell homeostasis. Another interpretation is that Rubicon plays a limited role in autophagy regulation in pancreatic beta cells *in vivo*, suggesting that functional differences of Rubicon may exist between organs. Notably, the expression of Rubicon interacting molecules, such as Beclin-1 and ATG14 in pancreatic beta cells, has been reported using RNA-seq [21]. However, we also have to consider that evaluation of autophagic flux *in vivo* remains challenging and that its estimation in our experiments may be

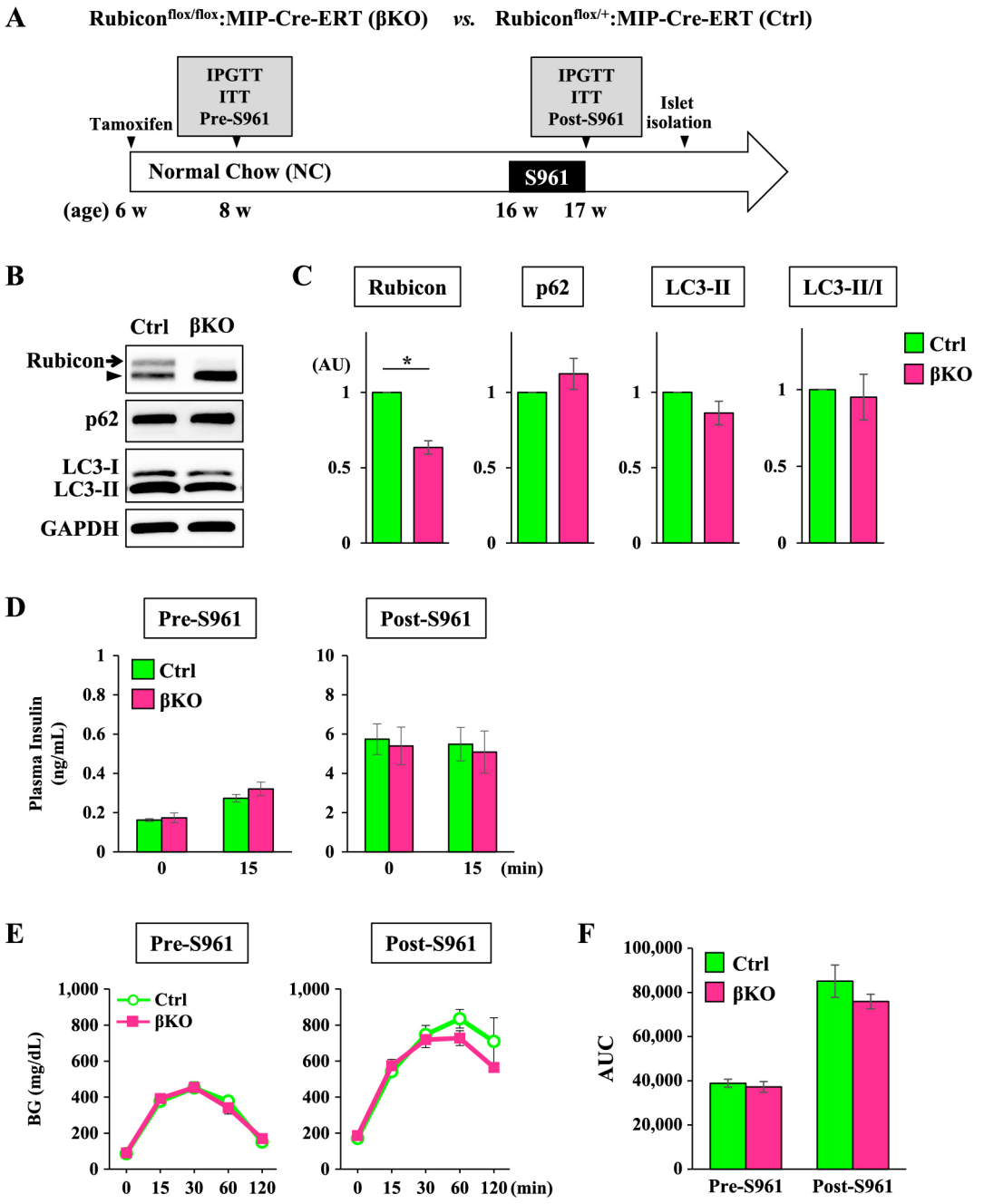

Fig. 4 Estimation of autophagy and glucose-tolerance levels in βKO mice after the administration of S961. A. Evaluation of βKO mice following the S961-mediated increase in insulin resistance. S961 was administered in control and βKO mice, using an ALZET osmolar pump, for a week. Glucose metabolism was compared before (Pre-S961) and after (Post-S961) S961 administration. B. Representative western blot of the isolated islets, after the administration of S961. The arrowhead indicates non-specific bands. C. Quantification of the western blotting analysis shown in panel B. The amount of the protein derived from βKO mice (n = 6) is expressed as a ratio to the amount of protein derived from control mice (n = 6). LC3-II/I ratio was calculated as described in Fig. 1B. * indicates $p < 0.05$. D. Estimation of insulin resistance, based on plasma insulin concentrations, after S961 treatment. E and F. IPGTT results, performed before and after S961 administration (E). AUC values were calculated based on panel E (F).

incomplete, although we demonstrated that Rubicon suppresses autophagic flux in βTC3 cell line. In summary, our results suggested that the role of Rubicon in pancreatic beta cells is limited in maintaining glucose homeostasis against increased insulin resistance.

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Conflicts of Interest

None of the authors have any potential conflicts of interest associated with this research.

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