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RESEARCH ARTICLE

Figures and figure supplements

Inhibition of mTORC1 by ER stress impairs neonatal β-cell expansion and predisposes to diabetes in the *Akita* mouse

Yael Riahi *et al*

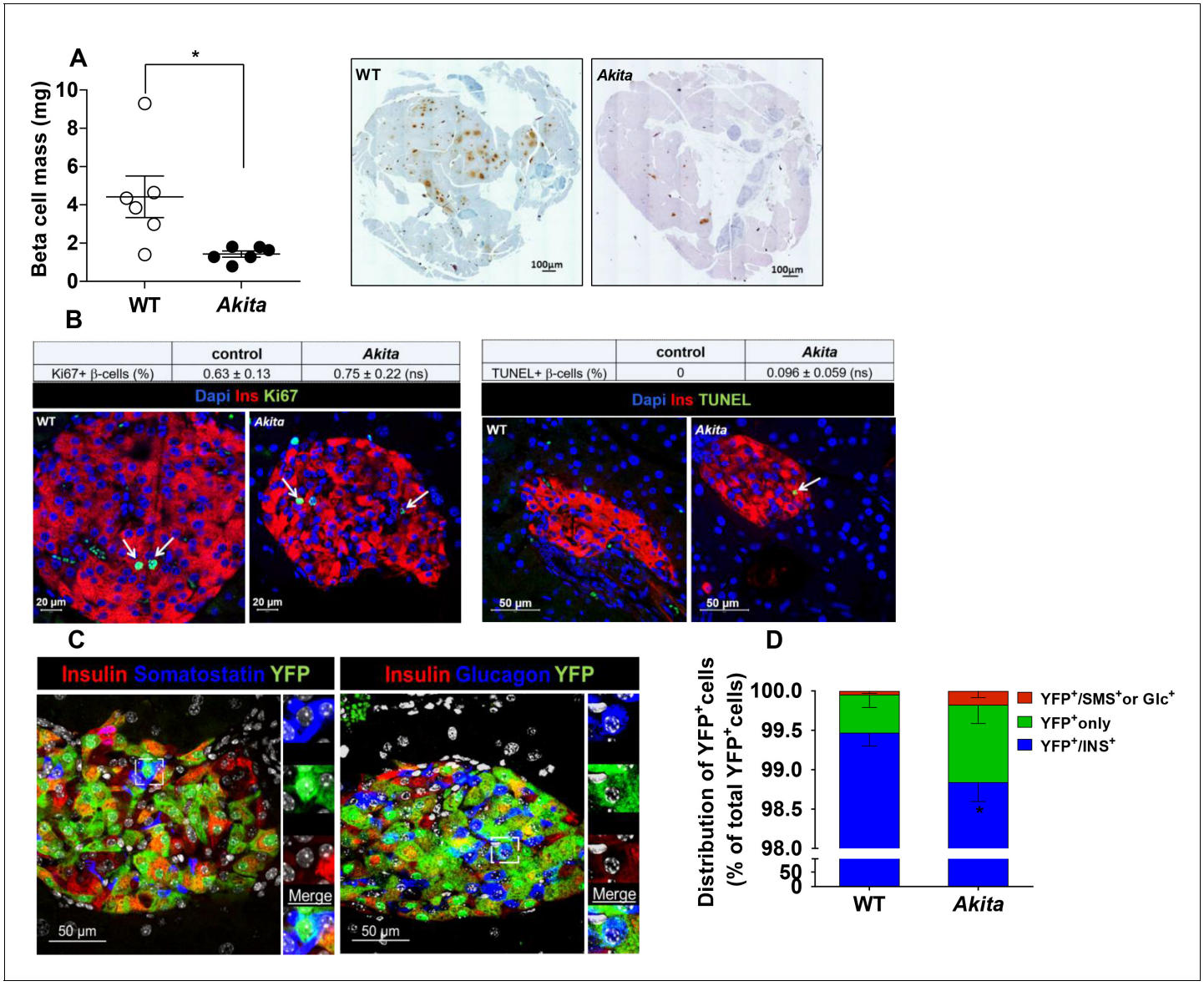

Figure 1. *β*-Cell mass, turnover and differentiation in adult *Akita* mice. Analyses were performed on 2- to 3-month-old *Akita* mice and age-matched controls. (**a**) *β*-cell mass (n = 6 in each group); (**b**) *β*-cell proliferation and apoptosis assessed by staining for insulin and Ki67 (n = 6–7 mice in each group; a total of 4909 wild type (WT) and 2523 *Akita* *β*-cells were quantified) or TUNEL (n = 4–5 mice in each group; 2592 WT and 1754 *Akita* *β*-cells). The percentage of Ki67^{+} and TUNEL^{+}*β*-cells is shown in the table above; (**c–d**) *β*-cell differentiation was assessed by lineage tracing. Wild-type and *Akita* mice were crossed with *RIP-Cre:Rosa26-Yfp* reporter mice; (**c**) pancreatic sections of *Akita* mice were immunostained for insulin and somatostatin or glucagon. Lineage-traced *β*-cells (YFP^{+}) expressing somatostatin or glucagon is shown in squares and zoomed in; (**d**) quantification of insulin-expressing *β*-cells (percentage of insulin^{+}/YFP^{+} cells), insulin-degranulated *β*-cells (percentage of insulin^{-}/YFP^{+} cells) and of cells with misexpression of somatostatin or glucagon (percentage of somatostatin^{+} or glucagon^{+}/YFP^{+} cells) in WT and *Akita* mice is shown; *p<0.05.

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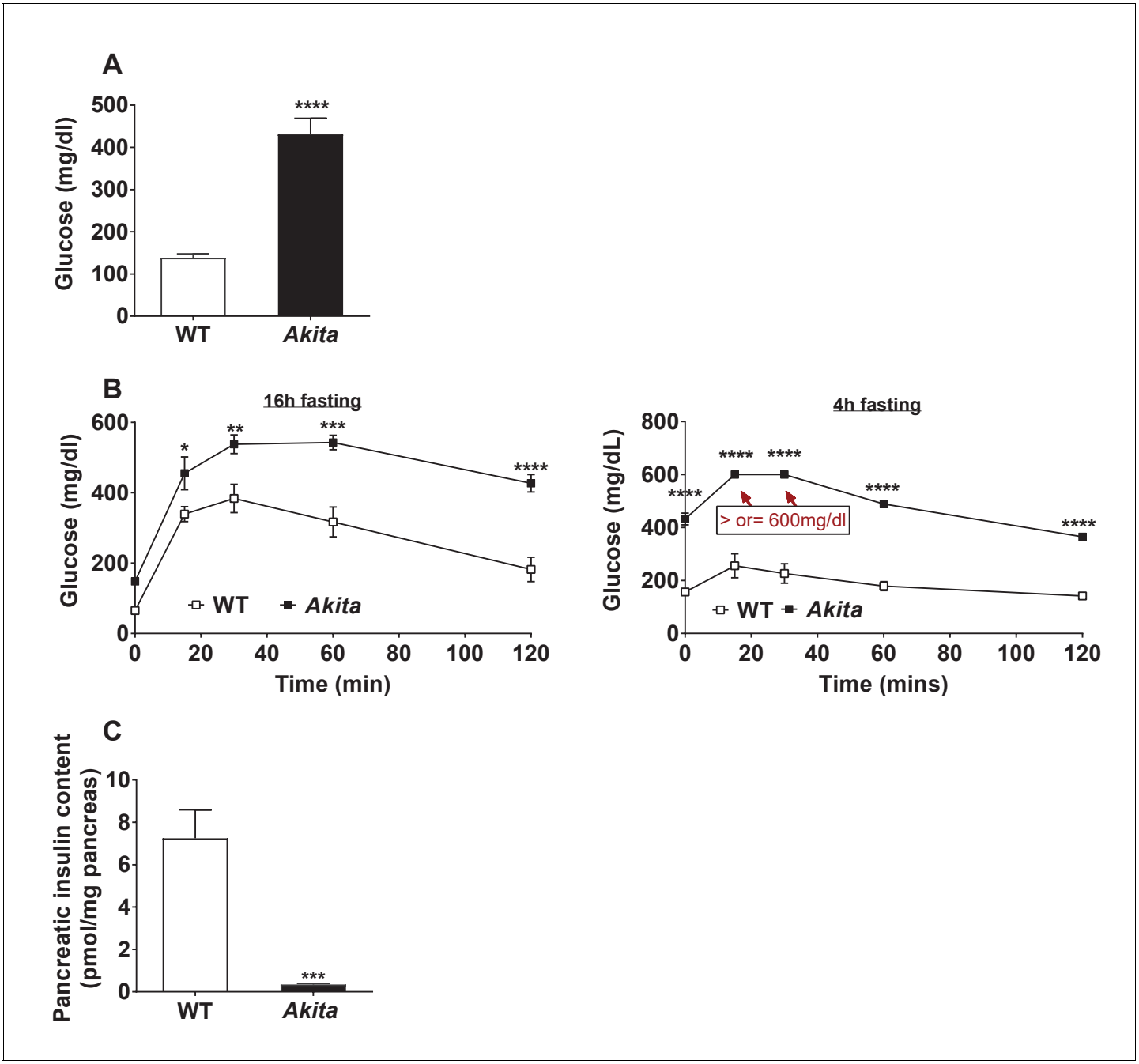

Figure 1—figure supplement 1. Glycemia and β-cell function in adult *Akita* and control mice. (**a**) Fed blood glucose (n = 11–15 in each group); (**b**) IPGTT- glucose (1.5 g/kg) was injected intraperitoneally after an overnight or 4 hr fast (n = 4–5 in each group); (**c**) pancreatic insulin content analyzed by ELISA on whole pancreas extracts (n = 4–5 in each group). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

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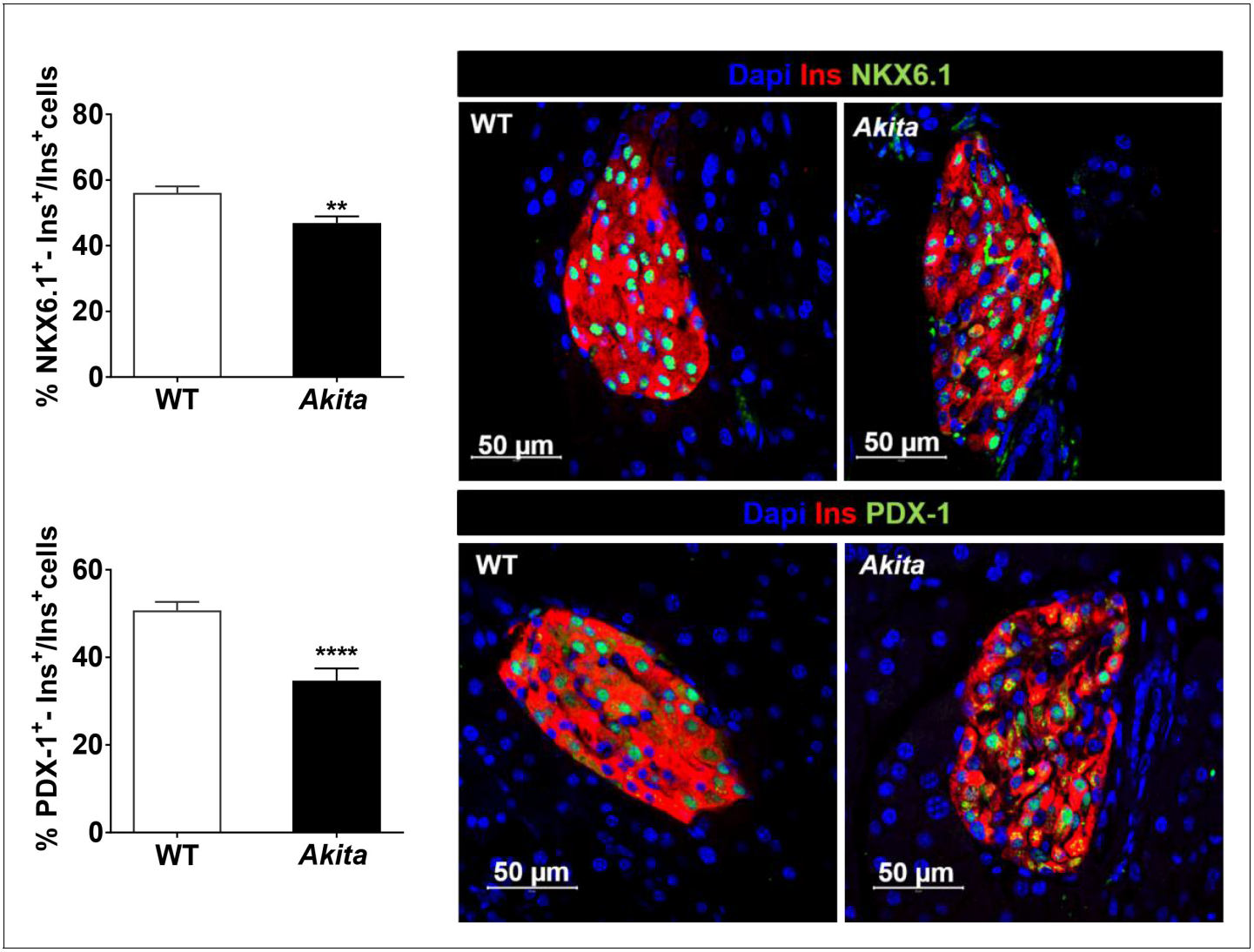

Figure 1—figure supplement 2. NKX6.1 and PDX-1 expression in adult *Akita*β-cells. Pancreatic sections of 2- to 3-month-old *Akita* and control mice were stained for NKX6.1 (n = 5 mice in each group; 3348 WT and 2370 *Akita* β-cells) or PDX-1 and insulin (n = 5–7 mice in each group; 4580 WT and 2320 *Akita* β-cells). Values are mean ± SE. **p<0.01, ****p<0.0001.

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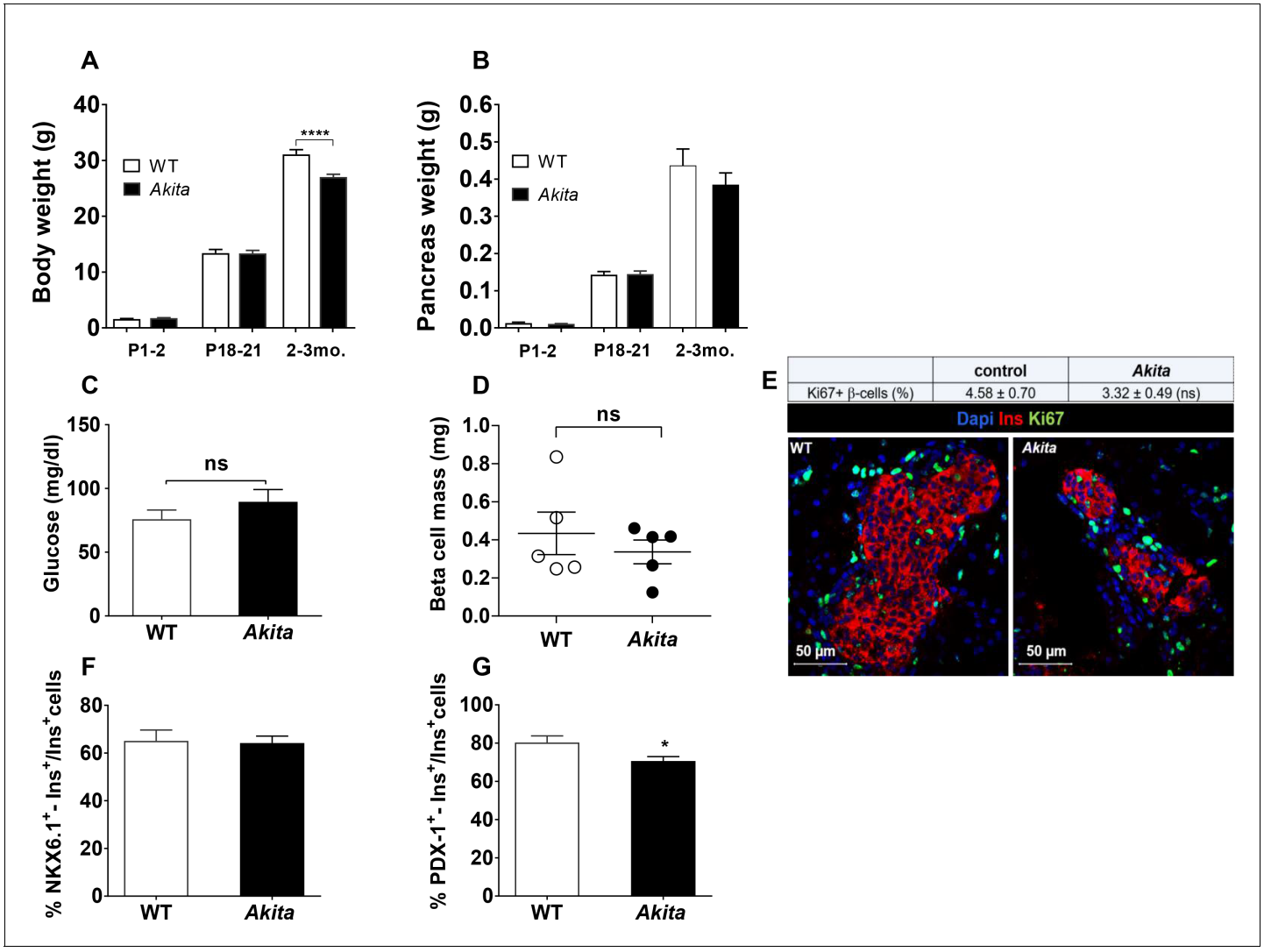

Figure 2. Dynamic changes of body and pancreas growth and glycemia, β-cell mass, proliferation and differentiation in *Akita* and control mice at P1-2. (**a**) body weight, (**b**) pancreas weight of wild-type and *Akita* mice at P1-2, P19-21 and at the age of 2–3 months. (**a**) P1-2: WT (n = 8); *Akita* mice (n = 4), P19-21: WT (n = 21); *Akita* mice (n = 23), 2–3 months: WT (n = 33); *Akita* mice (n = 39); (**b**) P1-2: WT (n = 8); *Akita* mice (n = 4), P19-21: n = 14 in each group, 2–3 months: n = 17 mice in each group). (**c**) fed blood glucose (n = 7–8 mice in each group); (**d**) β-cell mass (n = 5 mice in each group); (**e**) β-cell proliferation assessed by immunostaining for insulin and Ki67 (n = 4 mice in each group; 1886 WT and 1483 *Akita* β-cells). The percentage of Ki67$^+$ β-cells is shown in the table above; (**f–g**) quantification of β-cells (insulin$^+$) expressing NKX6.1 (n = 3–4 mice in each group; 1148 WT and 1808 *Akita* β-cells) and PDX-1 (n = 3–5 mice in each group; 1364 WT and 1507 *Akita* β-cells). *p<0.05, ****p<0.0001.

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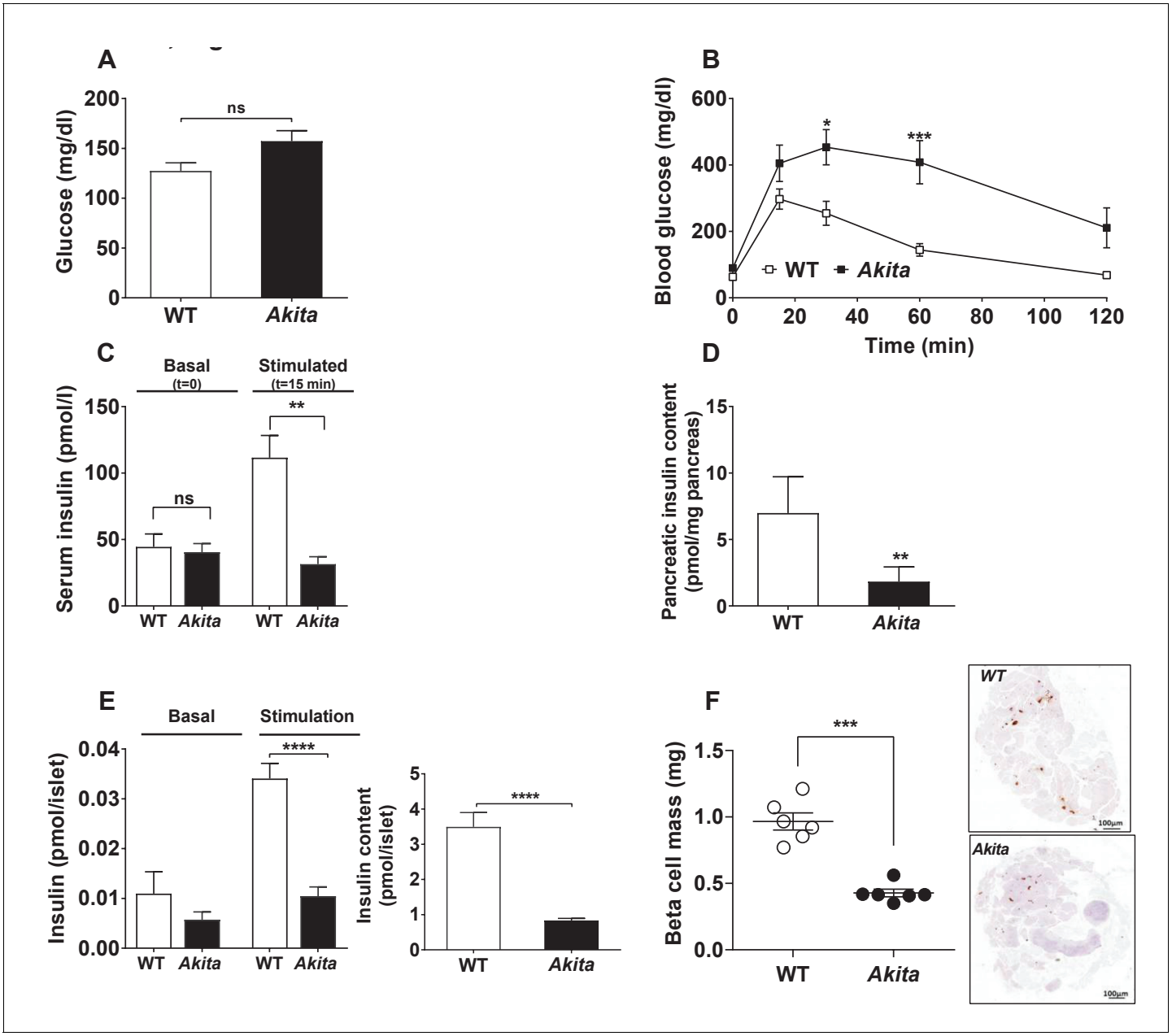

Figure 3. Metabolic state, β-cell function and mass in pre-weaning (P19-21) *Akita* mice and age-matched controls. (**a**) fed blood glucose (n = 7 in each group); (**b**) IPGTT- glucose (1.5 g/kg) was injected intraperitoneally after an overnight fast (n = 5 in each group); (**c**) glucose-stimulated insulin secretion in vivo. Insulin was measured before and 15 min following IP glucose injection (1.5 g/kg); (**d**) pancreatic insulin content (n = 4–5 in each group); (**e**) basal (3.3 mmol/l glucose) and stimulated (16.7 mmol/l glucose) insulin secretion and insulin content of *Akita* and control islets analyzed by static incubations. Islets were divided into 4 batches of 25 islets per group (n = 3); (**f**) β-cell mass (n = 6 mice in each group). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
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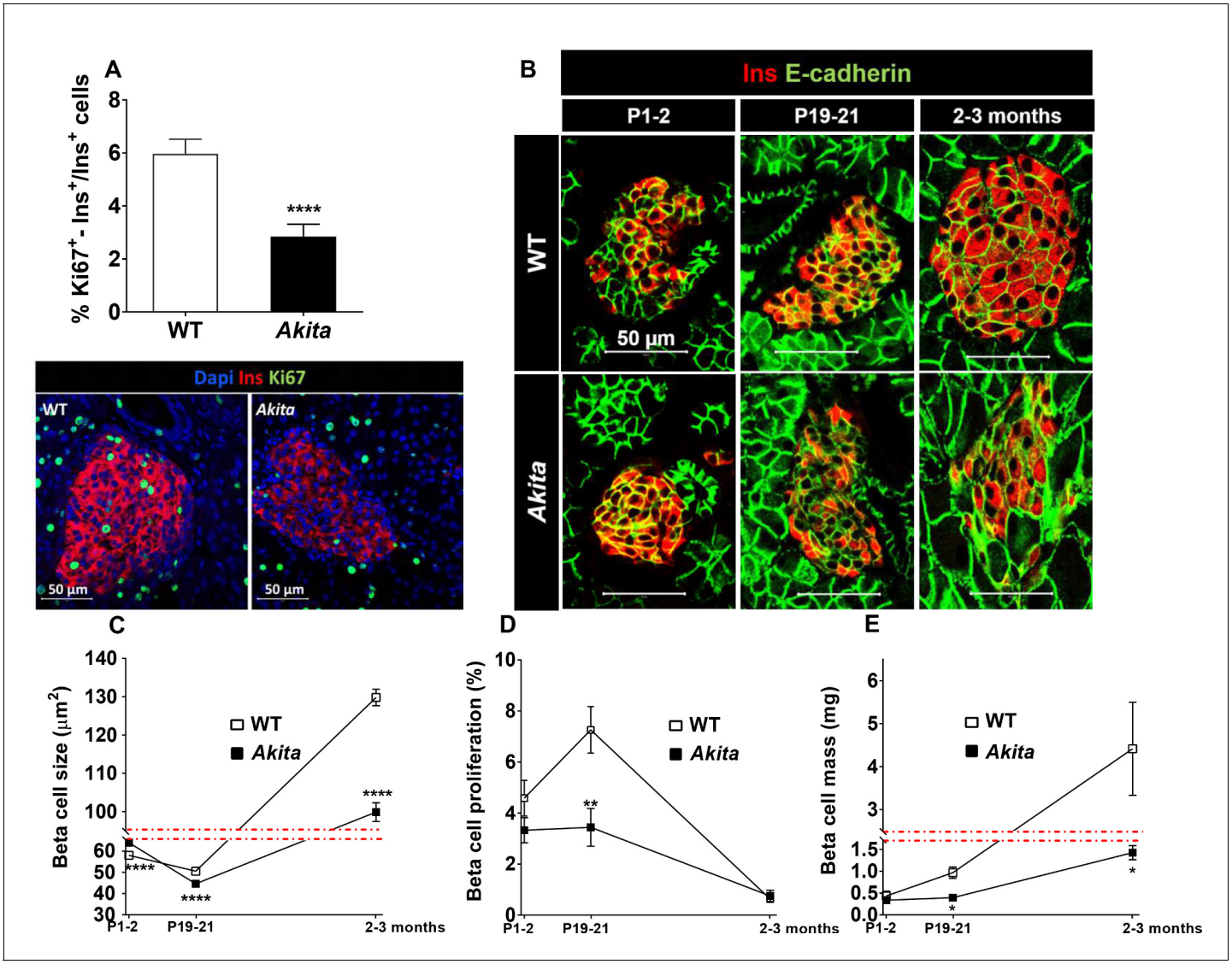

Figure 4. Dynamic changes in β-cell expansion in *Akita* and control mice. (**a**) β-cell proliferation assessed by immunostaining for insulin and Ki67 (n = 6 mice in each group; 2541 WT and 3391 *Akita* β−cells); (**b**) β-cell size at P1-2 (newborn, n = 4–5 mice in each group; 334 WT and 435 *Akita* β-cells), P19-21 (pre-weaning, n = 3 mice in each group; 330 WT and 364 *Akita* β−cells) and in adult mice (2–3 month-old, n = 3 mice in each group; 266 WT and 417 *Akita* β−cells) assessed by immunostaining for E-cadherin and insulin. Quantifications of β-cell size (**c**), proliferation (**d**), and mass (**e**) are shown. *p<0.05, **p<0.01, ****p<0.0001.

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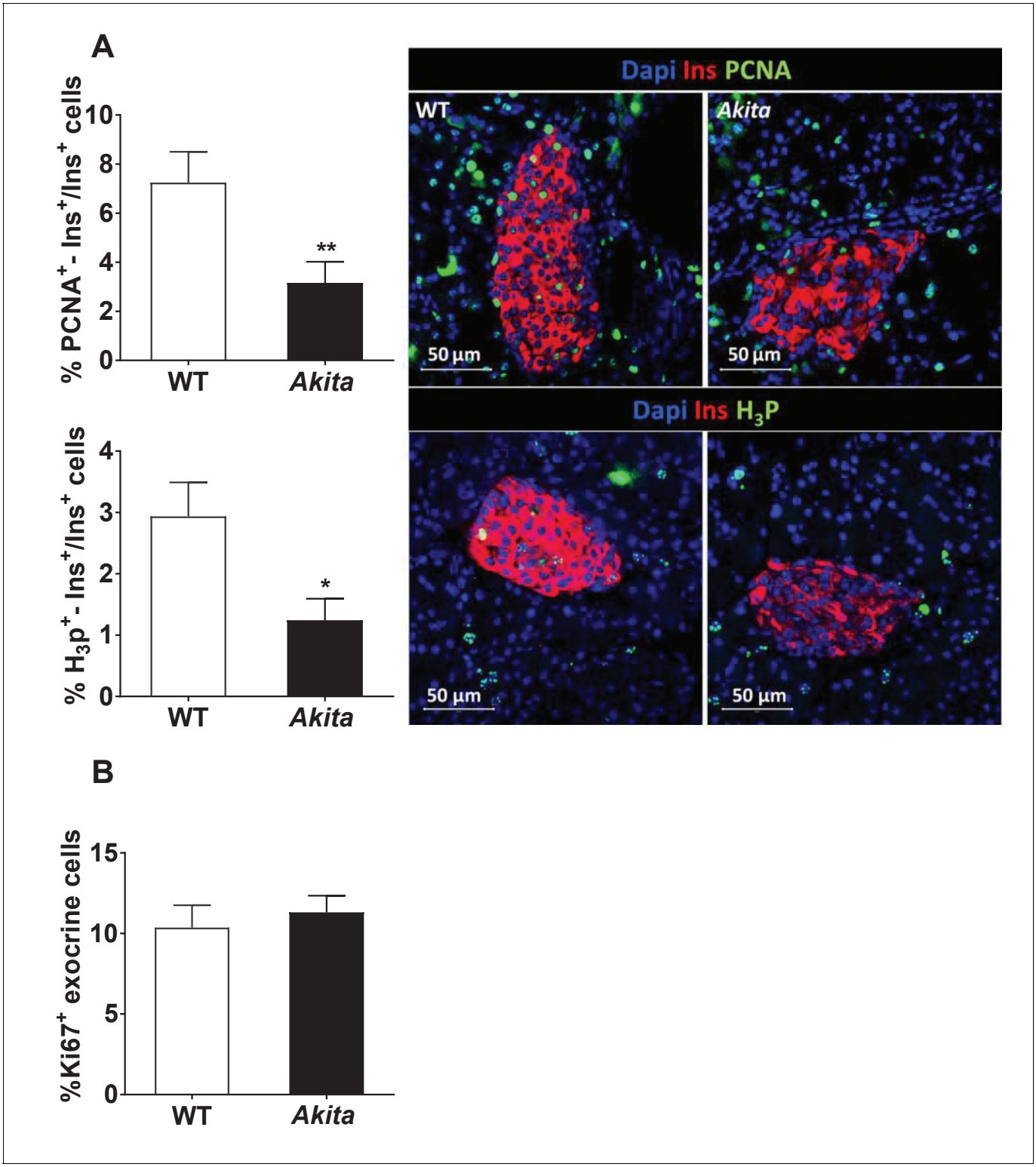

Figure 4—figure supplement 1. Proliferation of β-cells and exocrine cells in pre-weaning *Akita* and control mice. Analyses were performed on pancreatic sections of *Akita* mice and age-matched controls at 19–21 days stained for proliferation markers. (**a**) β-Cell proliferation was assessed by staining for insulin and PCNA (n = 3 mice in each group; 1241 WT and 1944 *Akita* β cells), or phospho-Histone H_3 (H_3P n = 3 mice in each group; 1176 WT and 1982 *Akita* β−cells). (**b**) Proliferation of pancreatic exocrine cells in pre-weaning *Akita* and control mice. Pancreatic sections of pre-weaning *Akita* and control mice were stained for insulin and Ki67 and exocrine cells surrounding the islets were used for quantification. The percentage of proliferating exocrine cells ($Ki67^+/INS^-$) is shown (n = 6 mice in each group; 2486 WT and 2670 *Akita* cells). *p<0.05, **p<0.01.

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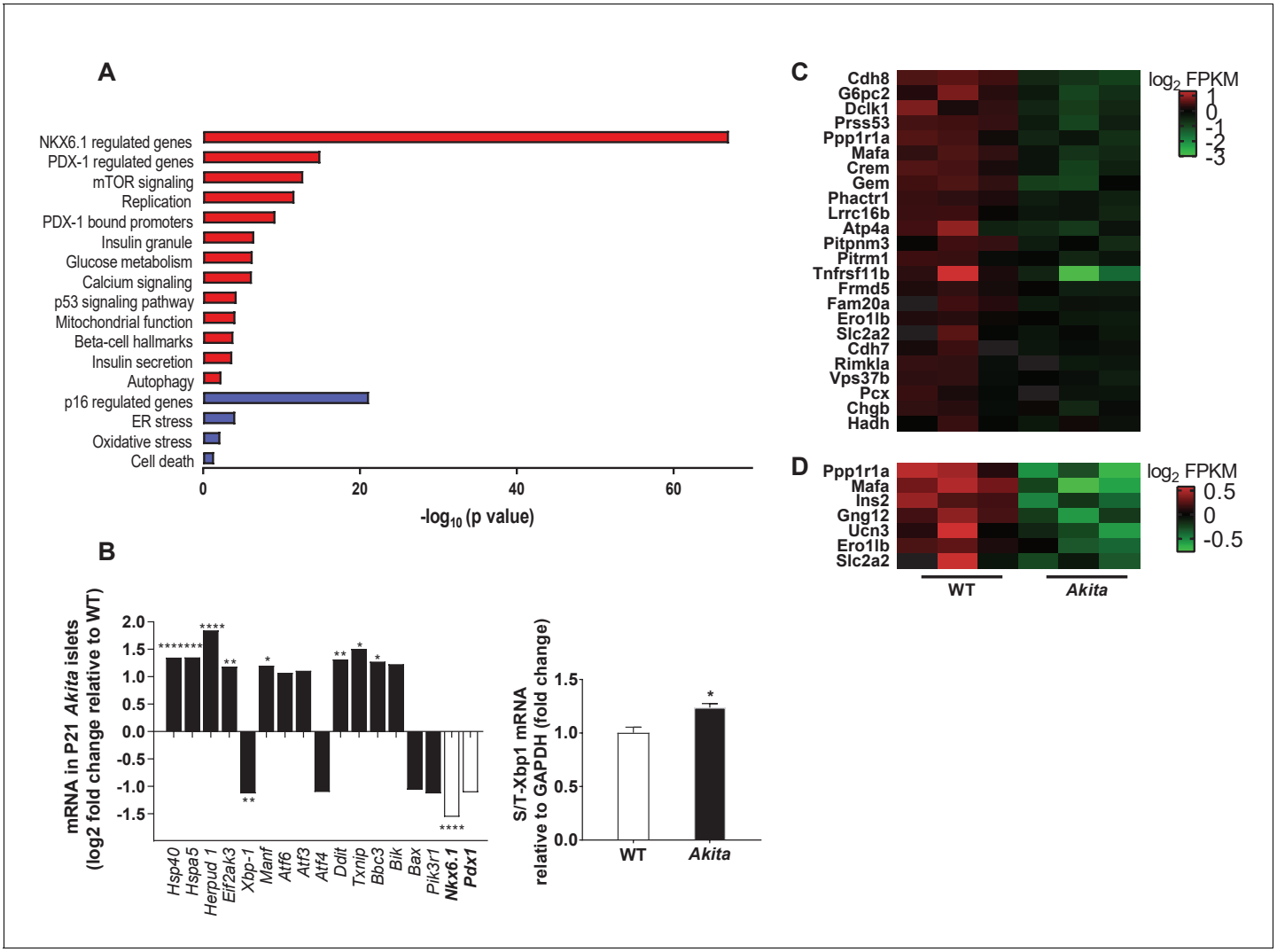

Figure 5. Transcriptomic analysis of ER stress markers and β-cell gene signature in neonate *Akita* islets. (**a**) RNA-seq comparing the transcriptome of islets from P19-21 *Akita* and age-matched control mice (n = 3 samples in each group, each sample is a pool of islets from three mice). Columns represent pathways that are differentially regulated in *Akita* mice; (**b**) expression of UPR and apoptosis genes and of *Nkx6.1* and *Pdx1* in islets of *Akita* compared to control mice at P19-21. Spliced and total *Xbp1* were also quantified by qPCR. The spliced/total *Xbp1* ratio is shown beside (n = 3); (**c–d**) heat map of genes regulated by NKX6.1 (**c**) and PDX-1 (**d**) in *Akita* islets and controls. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

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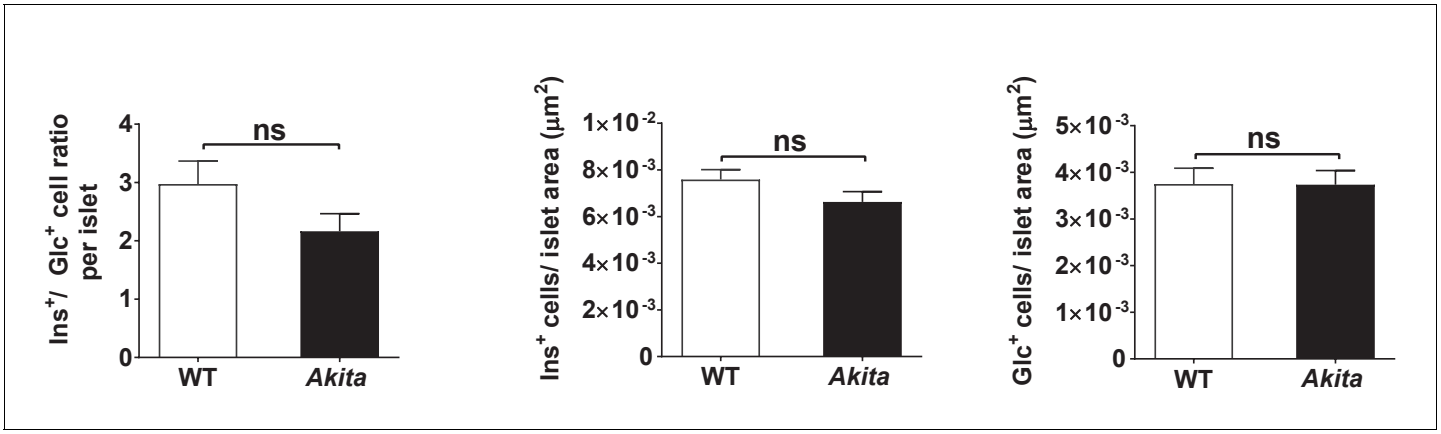

Figure 5—figure supplement 1. Islet composition of wild-type and *Akita* mice. β/α cell ratio and number of β and α cells per islet area in P19-21 wild-type and *Akita* mice (n = 44 and 35 islets isolated from three to four mice in each group).

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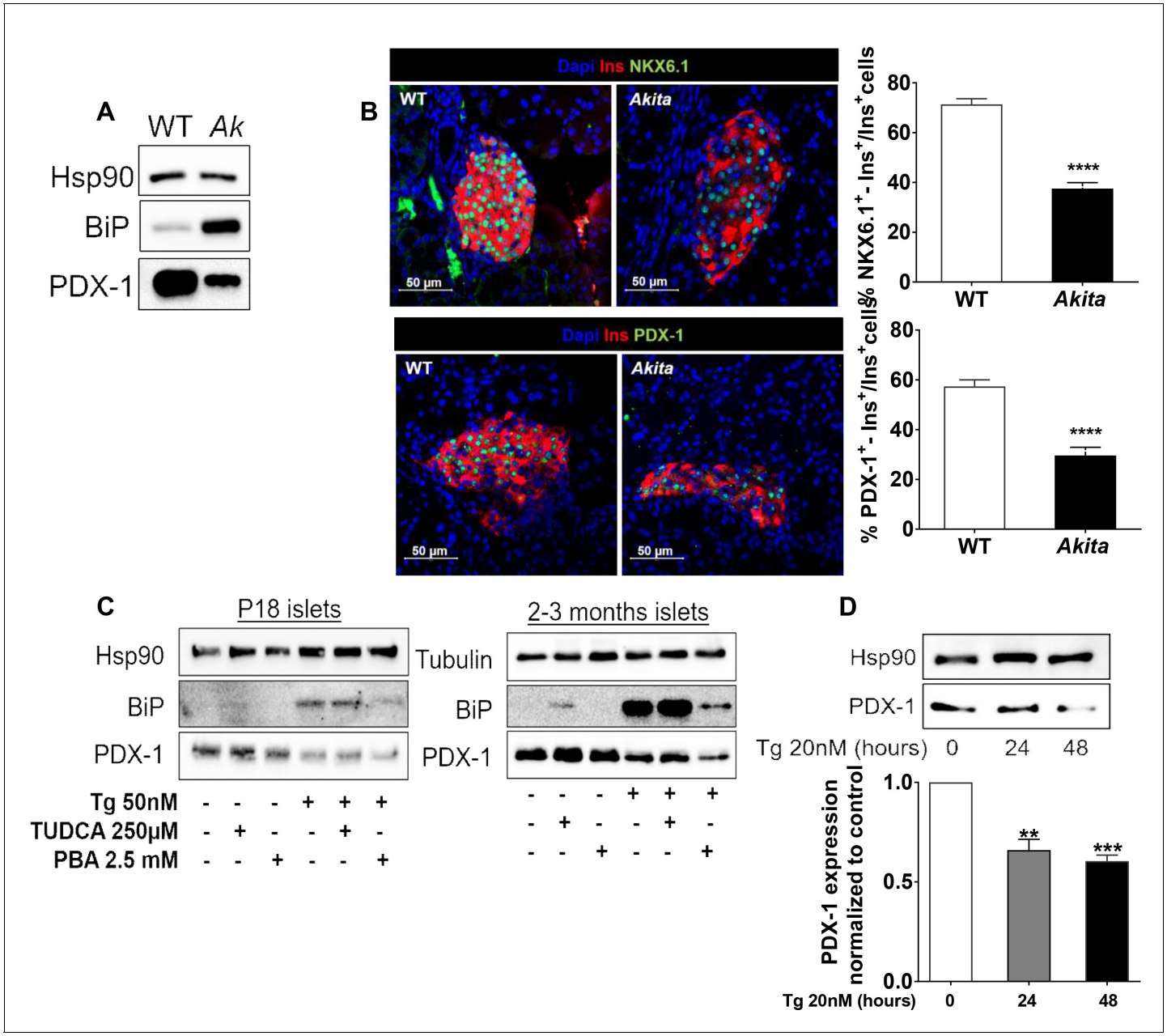

Figure 6. Effects of ER stress on the expression of β-cell transcription factors in neonate *Akita* islets (P19-21) and islets treated with thapsigargin. (**a**) PDX-1 and BiP protein level analyzed by Western blotting (n = 3, each sample is a pool of islets from four to six mice); (**b**) quantification of NKX6.1 (n = 3 mice in each group; 1646 WT and 728 *Akita* β−cells), and PDX-1 (n = 3 mice in each group; 1534 WT and 844 *Akita* β−cells) expressing β-cells. Pancreatic sections were immunostained for NKX6.1 or PDX-1 and insulin. The percentage of NKX6.1- and PDX-1-positive β-cells is shown. (**c**) Islets from young (P19-21) and adult wild-type mice were treated with low-dose thapsigargin (50 nmol/l) and TUDCA (250 µmol/l) or PBA (2.5 mmol/l) for 48 hr with daily media changes and further analyzed by western blotting for PDX-1 and BiP (n = 3, each sample is a pool of islets from six to nine mice); (**d**) INS-1E cells were treated with 20 nmol/l thapsigargin for 24 and 48 hr followed by western blotting for PDX-1. **p<0.01, ***p<0.001, ****p<0.0001.
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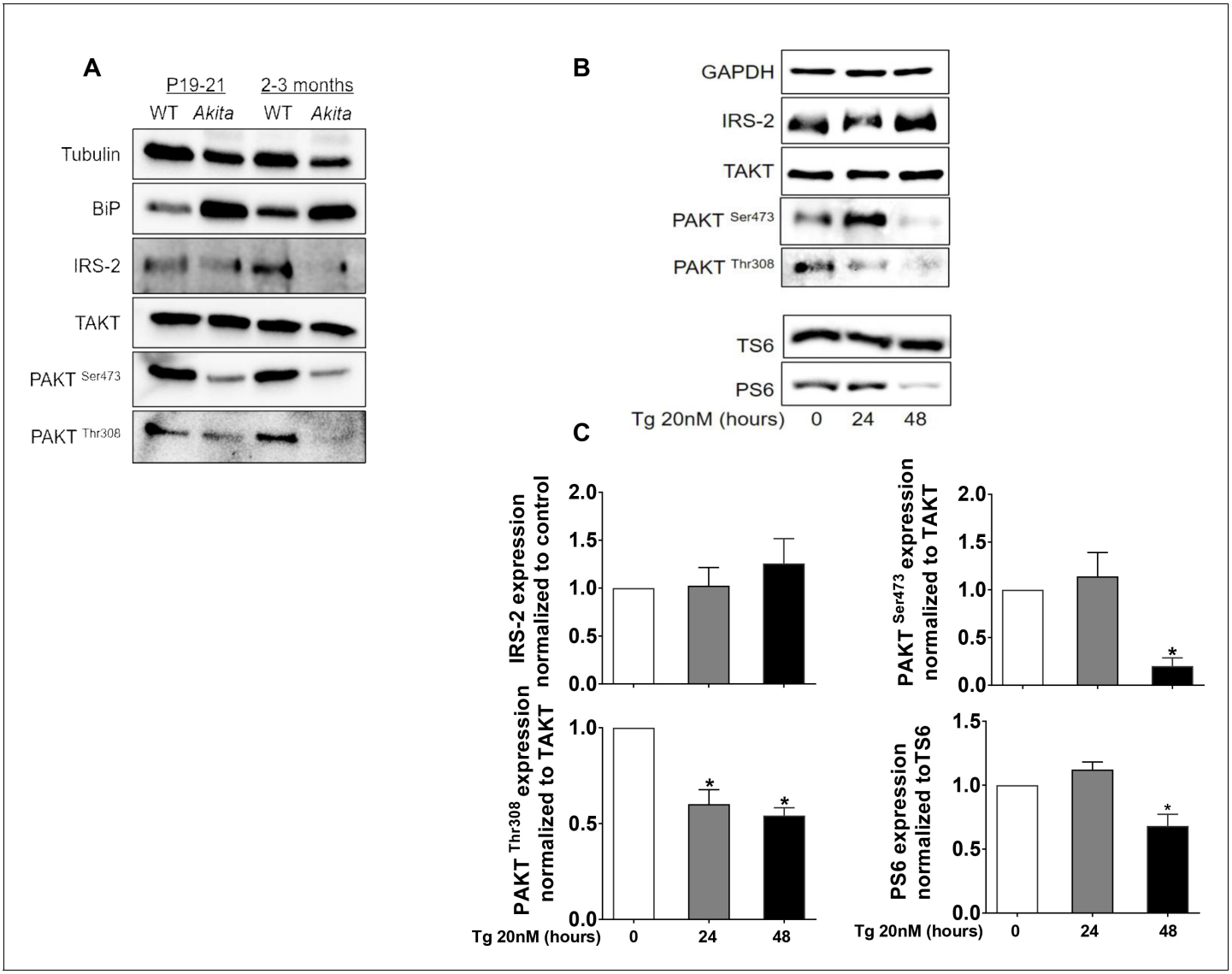

Figure 7. Effects of ER stress on IRS2/Akt signaling in *Akita* islets and in INS-1E treated with low-dose thapsigargin. (**a**) IRS2/Akt signaling in islets from neonate (P19-21) and adult wild-type and *Akita* mice. Each sample is a pool of islets from 4 to 15 mice (n = 4 for neonate islets and n = 2 for adult islets). (**b–c**) INS-1E cells were treated with 20 nmol/l thapsigargin for 24 and 48 hr followed by western blotting for IRS2, total and phosphorylated Akt (Ser473 and Thr308) and S6 (Ser240/244). A representative experiment (**b**) and quantification (**c**) are shown (n = 4–6). *p<0.05.

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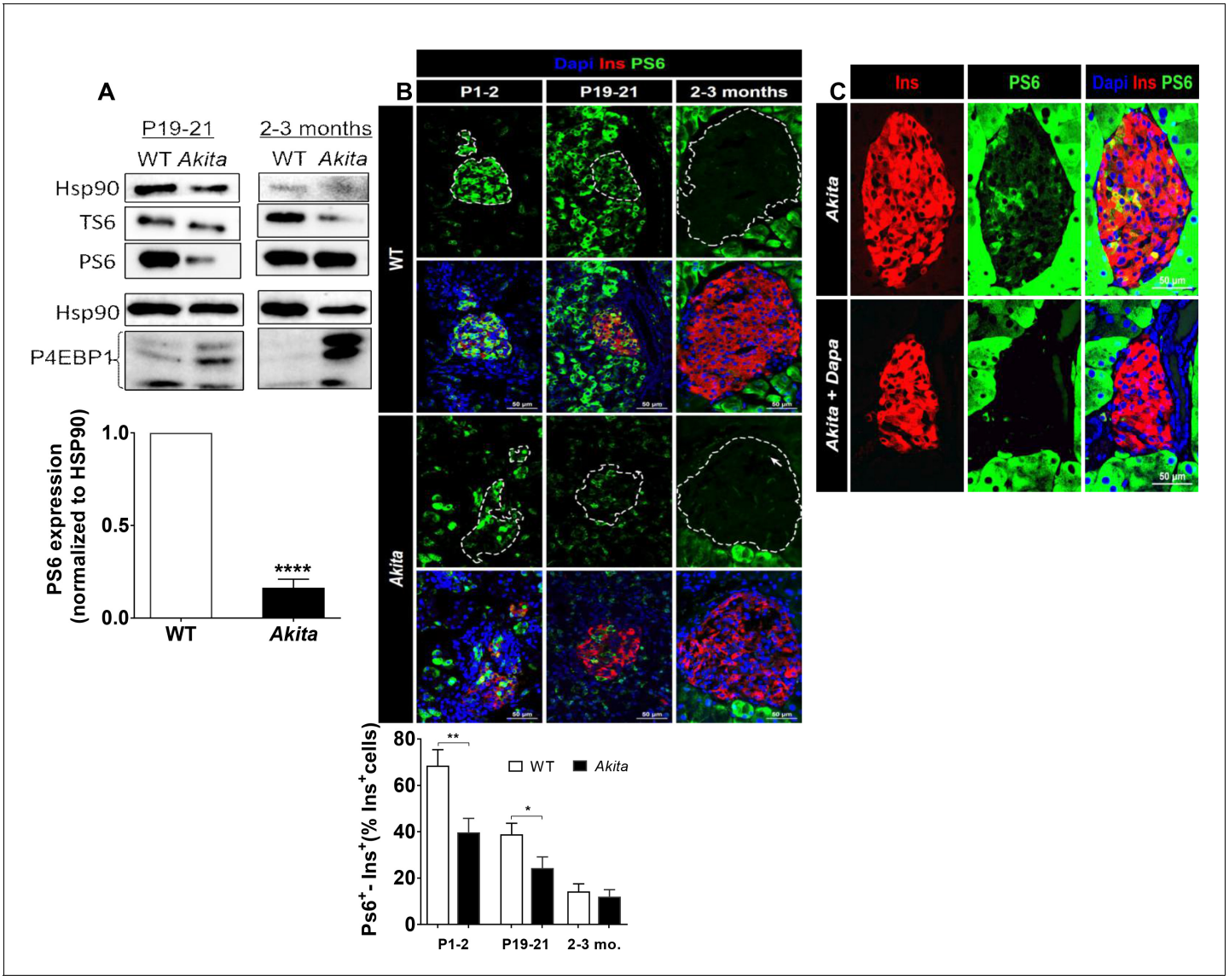

Figure 8. mTORC1 signaling in neonate and adult *Akita* islets. (**a**) Western blot analysis of S6 and 4EBP1 phosphorylation in islets of neonate (P19-21) and adult wild-type and *Akita* mice. Quantification of phosphorylated S6 in neonate *Akita* compared to control islets is shown (n = 3, each sample is a pool of islets from 4 to 7 mice); (**b**) immunostaining for phospho-S6 on pancreatic sections of P1-2, P19-21 and adult *Akita* mice and age-matched controls and quantifications of the percentage of $S6^{+}$ β-cells (P1-2: n = 4 mice in each group; 1159 WT and 1655 *Akita* β−cells; P19-21: n = 6 mice in each group; 2259 WT and 1567 *Akita* β−cells; adult: n = 4–5 mice in each group; 2391 WT and 1383 *Akita* β-cells). Islet boundaries are marked by dotted line; (**c**) adult *Akita* mice were treated with 25 mg/kg dapagliflozin in drinking water for 72 hr. Blood glucose in dapagliflozin-treated *Akita* mice was ~ 200 mg/dl compared to ~ 500 mg/dl in control *Akita* mice. Pancreatic sections were immunostained for insulin and phospho-S6 (n = 3 mice in each group). *p<0.05, **p<0.01, ****p<0.0001.

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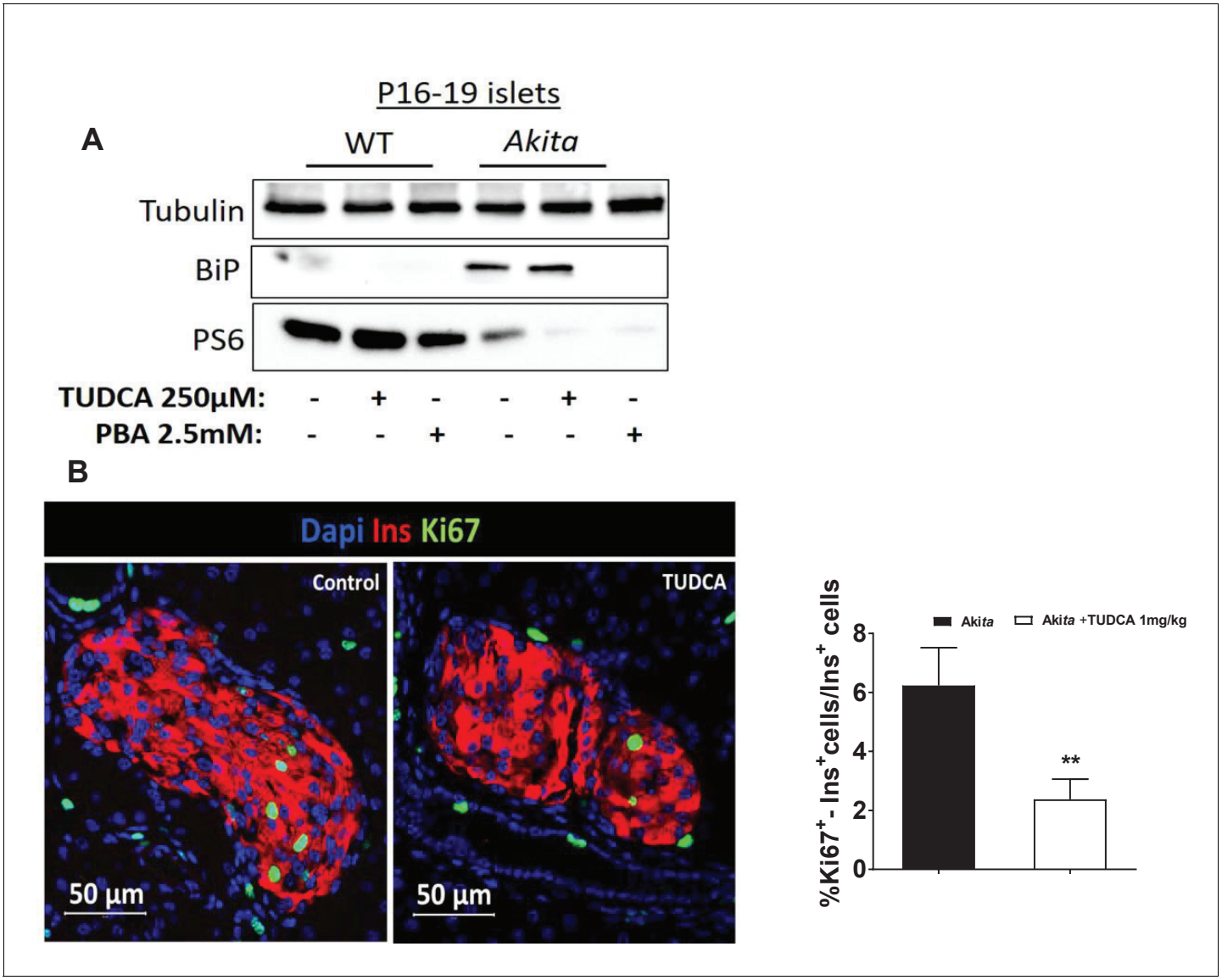

Figure 8—figure supplement 1. Effects of chemical chaperones on mTORC1 activity in neonate *Akita* islets and controls. (**a**) islets of P16-19 *Akita* and wild-type (WT) mice were treated with 250 µmol/l TUDCA or 2.5 mmol/l PBA for 48 hr followed by western blotting for BiP and phosphorylated S6 (n = 3, each sample is a pool of islets from 4 to 9 mice); (**b**) effects of TUDCA on β-cell proliferation in neonate *Akita* mice (P18-20). TUDCA (1 mg/kg) was injected IP twice daily for 48 hr followed by immunostaining of pancreatic sections for Ki67 and insulin (n = 4–6 mice in each group; 1403 control *Akita* and 2183 TUDCA-treated *Akita*β-cells). **p<0.01.

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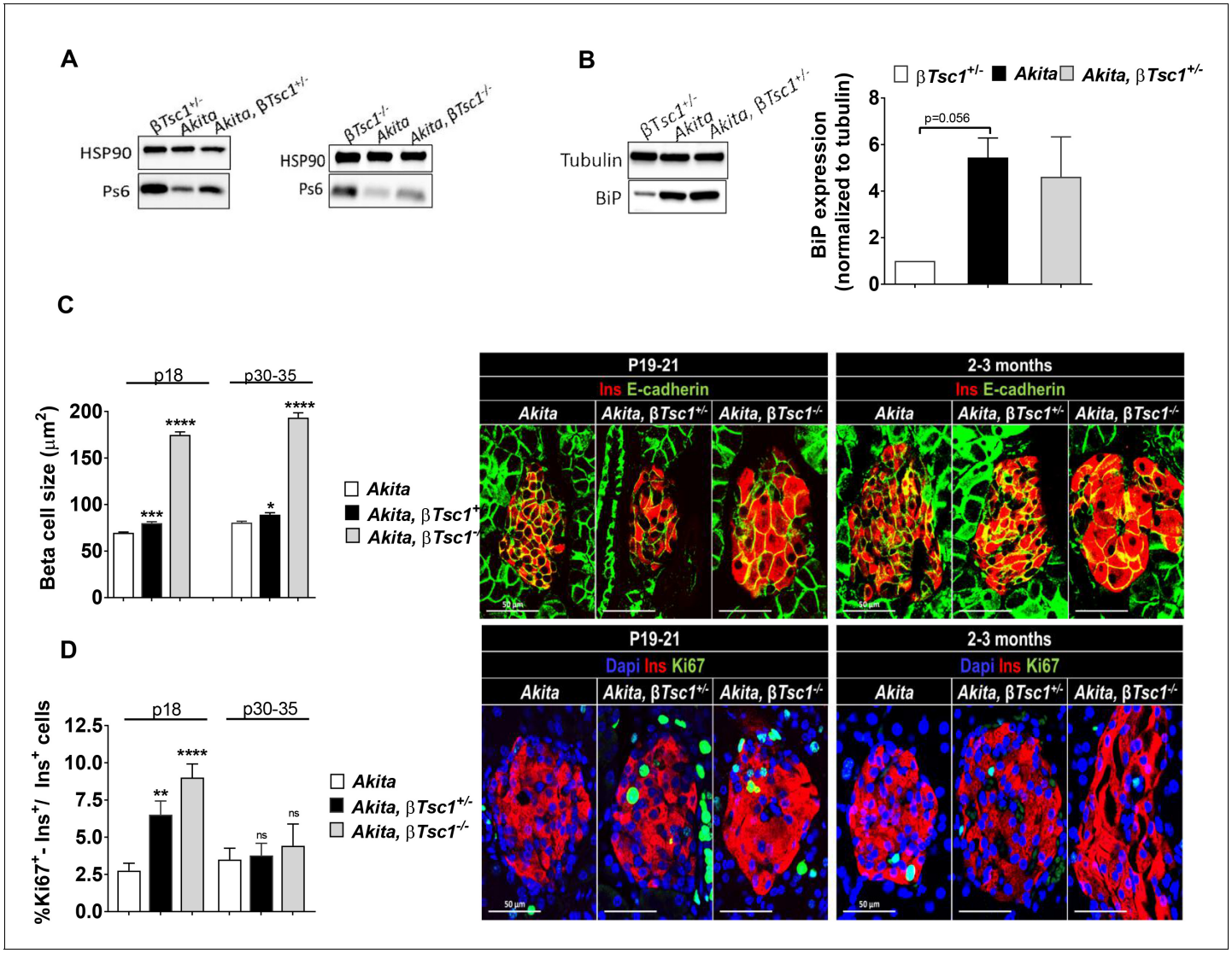

Figure 9. Effects of mTORC1 activation in neonate *Akita* β-cells on β-cell size and proliferation. Studies were performed on heterozygous and homozygous β*Tsc1* knockout *Akita* mice (*RIP-Cre:Tsc1*$^{flox/+}$*:Akita* (*Akita, βTsc1*$^{+/-}$) and *RIP-Cre:Tsc1*$^{flox/flox}$*:Akita* (*Akita, βTsc1*$^{-/-}$). *Tsc1*$^{flox/+}$*:Akita* and *Tsc1*$^{flox/flox}$*:Akita* were used as *Akita* controls. *RIP-Cre:Tsc1*$^{flox/+}$ mice (*βTsc1*$^{+/-}$) and *RIP-Cre:Tsc1*$^{flox/flox}$ mice (*βTsc1*$^{-/-}$) were used as WT controls (**a, b**). (**a**) Western blotting for phospho-S6 on islets from homozygous and heterozygous knockout mice and matched controls (n = 4, each sample is a pool of islets from two to four mice); (**b**) Western blotting and quantification of BiP expression in wild-type, *Akita* and *Akita, βTsc1* $^{+/-}$ mice (n = 4, each sample is a pool of islets from two to four mice); (**c**) β-cell size was assessed by immunostaining for insulin and E-cadherin (n = 400–500 β-cells per group), (**d**) β-cell proliferation was assessed by immunostaining for insulin and Ki67 (n = 1200–1400 β−cells per group). Quantifications and representative images are shown. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

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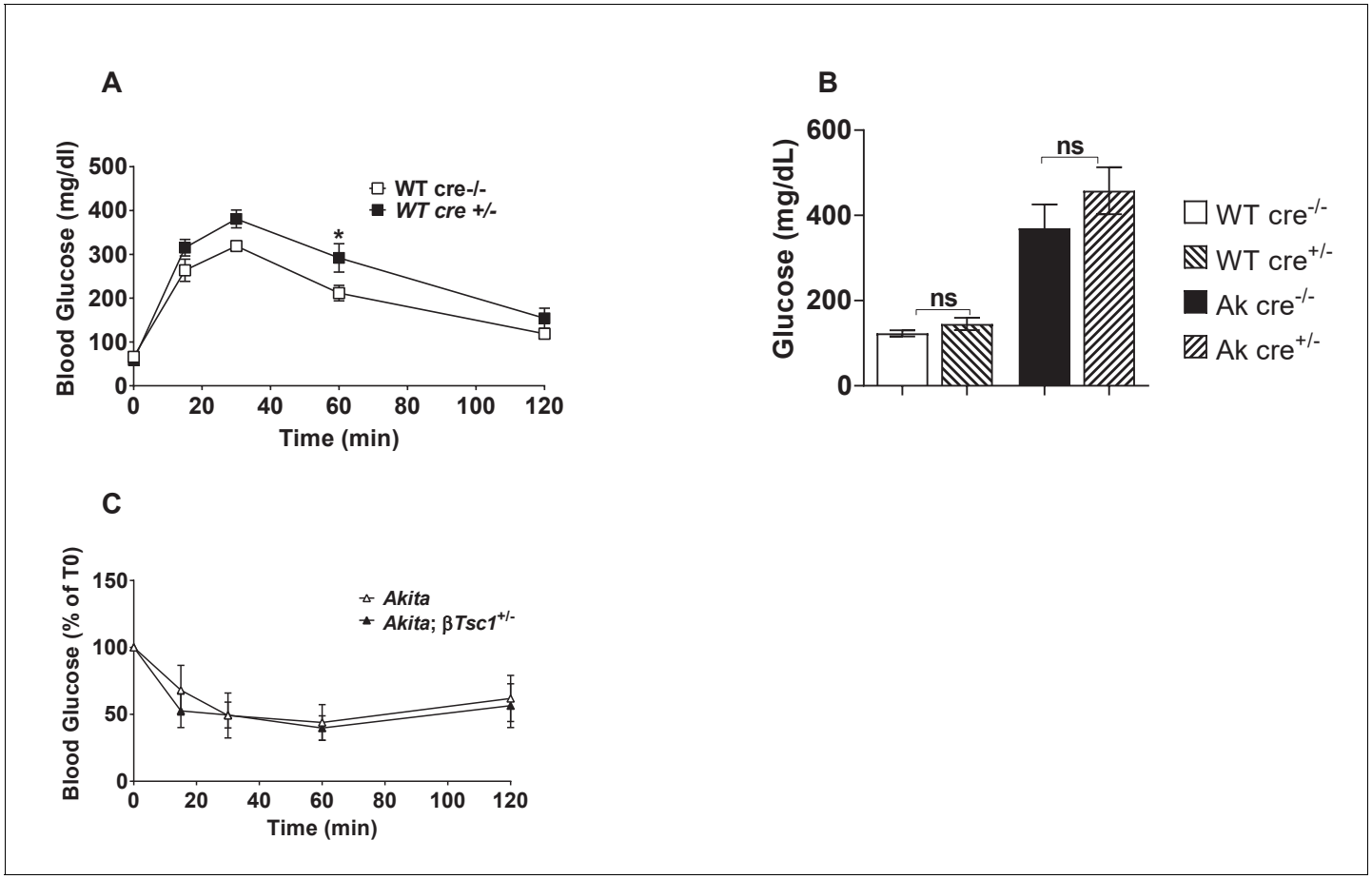

Figure 9—figure supplement 1. Metabolic characterization of *RIP-Cre* mouse. (**a**) IPGTT- glucose (1.5 gr/kg) was injected after an overnight fast to adult *RIP-Cre* and non-transgenic control mice (n = 3); (**b**) fed blood glucose of adult *RIP-Cre* mice and *RIP-Cre:Akita* mice compared to non-transgenic controls (n = 6–8); (**c**) insulin tolerance test on *Akita* and *RIP-Cre:Akita* mice (n = 3). *p<0.05.

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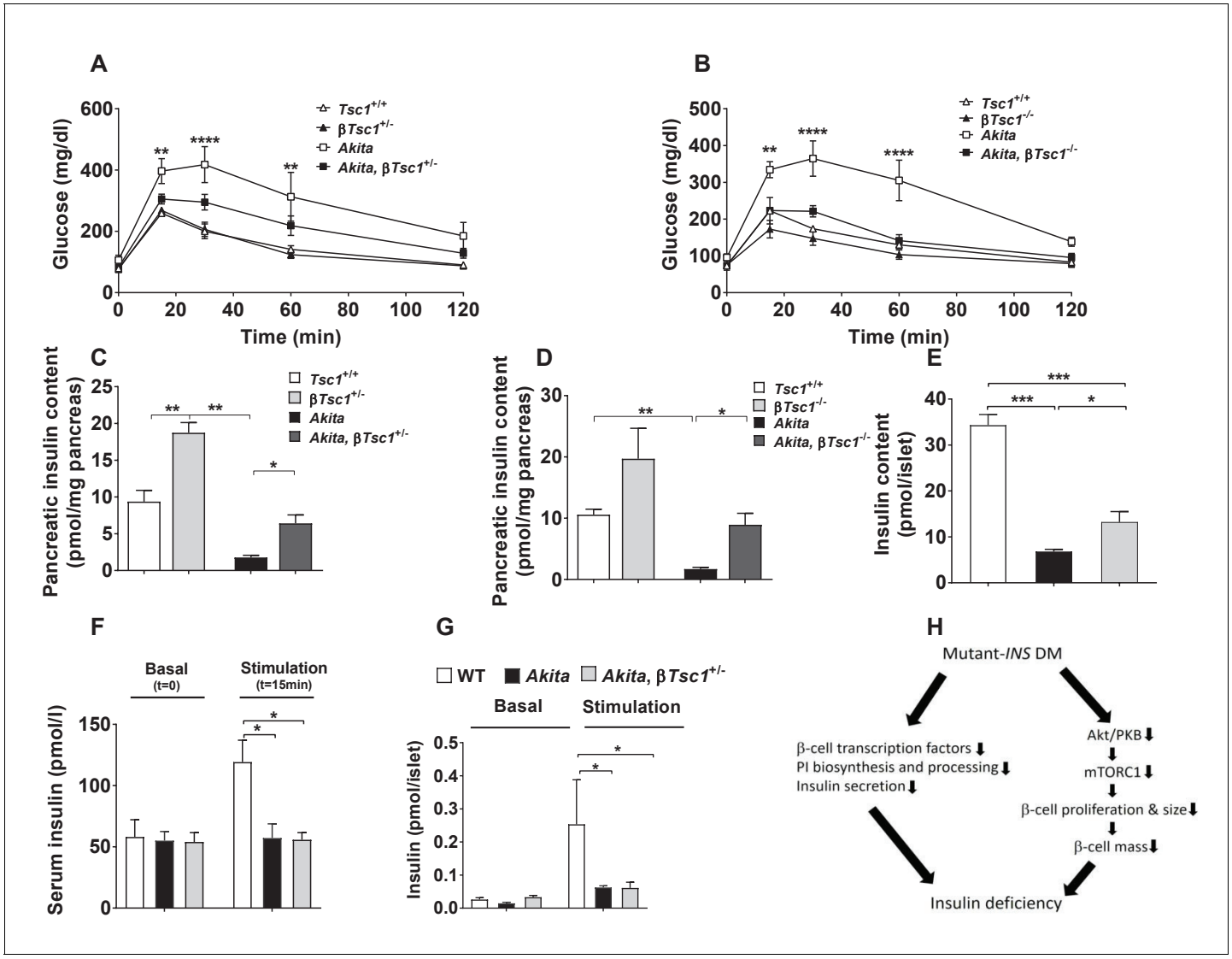

Figure 10. Effects of mTORC1 activation in neonate *Akita* β-cells on diabetes. (**a–b**) IPGTT at P30-35: glucose (1 g/kg) was injected IP after an overnight fast; (**a**) heterozygous *Tsc1* knockout *Akita* mice (*RIP-Cre:Tsc1*$^{flox/+}$*:Akita* (*Akita, βTsc1*$^{+/-}$) and matched controls: *Tsc1*$^{flox/+}$ mice (*Tsc1*$^{+/+}$), *RIP-Cre:Tsc1*$^{flox/+}$ mice (*βTsc1*$^{+/-}$), and *Tsc1*$^{flox/+}$*:Akita* (*Akita*) (n = 3–5 mice in each group); (**b**) homozygous *Tsc1* knockout *Akita* mice (*RIP-Cre:Tsc1*$^{flox/flox}$*:Akita* (*Akita, βTsc1*$^{-/-}$) and matched controls: *Tsc1*$^{flox/flox}$ mice (*Tsc*$^{+/+}$), *RIP-Cre:Tsc1*$^{flox/flox}$ mice (*βTsc1*$^{-/-}$), and *Tsc1*$^{flox/flox}$*:Akita* (*Akita*) (n = 3–5 in each group); (**c–d**) pancreatic insulin content of heterozygous and homozygous *Tsc1* knockout *Akita* mice and matched controls at P30-35 (WT (n = 7), *Akita* (n = 11), *Akita, βTsc1*$^{+/-}$ (n = 3) and *Akita, βTsc1*$^{-/-}$ (n = 4); (**e**) islet insulin content. (**f–g**) Effects of mTORC1 activation in neonate *Akita* β-cells on insulin secretion in vivo and ex vivo. (**f**) insulin secretion in response to IP glucose injection (n = 6 mice in each group); (**g**) islets were isolated from *Tsc1*$^{flox/+}$ WT mice (WT), *Tsc1*$^{flox/+}$*:Akita* (*Akita*) and *RIP-Cre:Tsc1*$^{flox/+}$*:Akita* (*Akita, βTsc1*$^{+/-}$) mice and insulin secretion assessed following static incubations at basal (3.3 mmol/l) and stimulated (16.7) mmol/l glucose. (**h**) a model of the pathophysiology of permanent neonatal diabetes. *p<0.05 **, p<0.01, ***, p<0.001****, p<0.0001.

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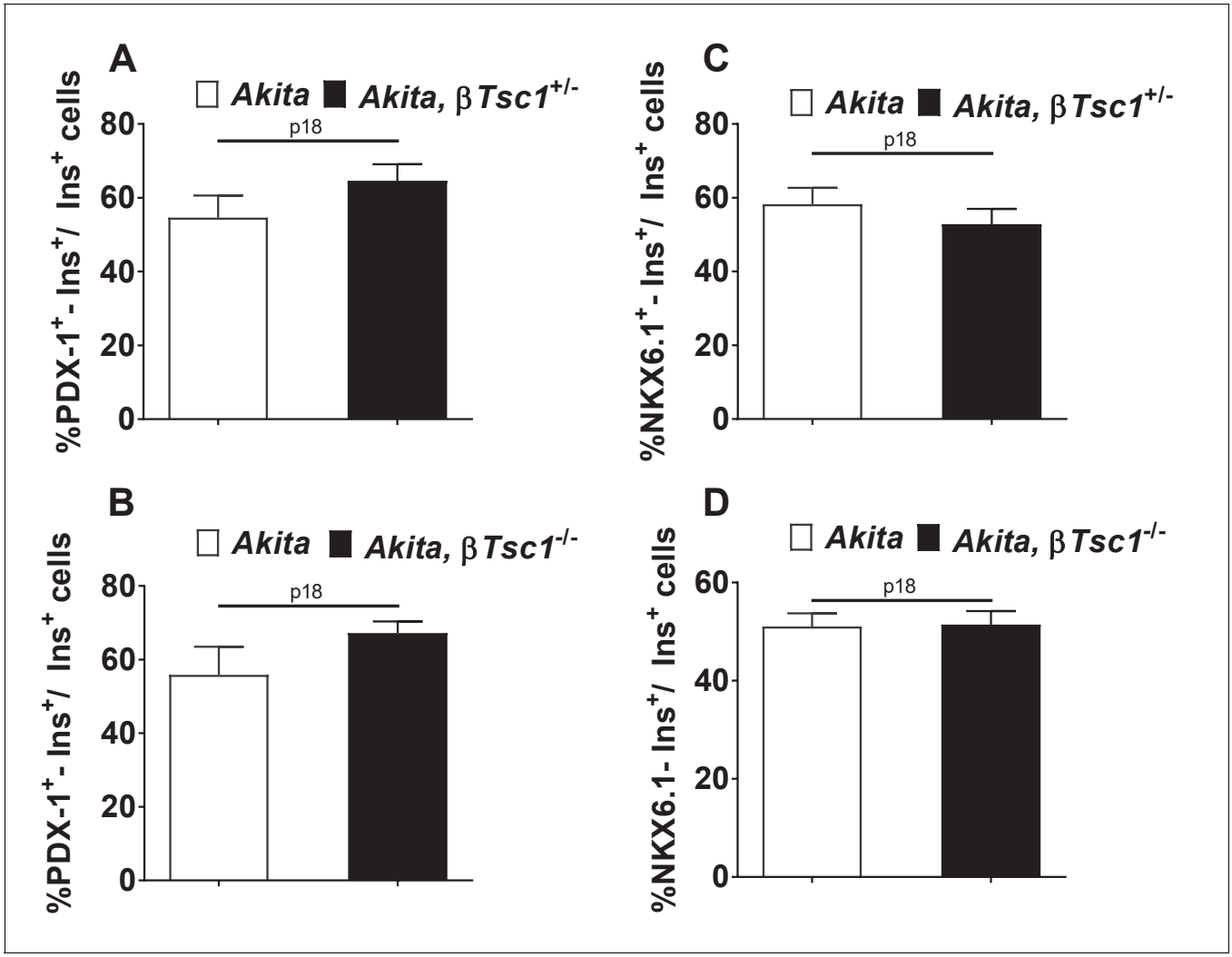

Figure 10—figure supplement 1. Effects of mTORC1 activation in *Akita*β-cells on PDX-1 (**a, b**) and NKX6.1 expression (**c, d**). Heterozygous (**a, c**) and homozygous (**b, d**) *βTsc1* knockout *Akita* and age-matched controls were sacrificed at P18. Pancreatic sections were stained for insulin and PDX-1 (*Akita* (n = 1760 β-cells); *Akita*, $\beta Tsc1^{+/-}$ (n = 814 β-cells); *Akita*, $\beta Tsc1^{-/-}$ (n = 1458 β−cells) or NKX6.1 (*Akita* (n = 1500 β-cells); *Akita*, $\beta Tsc1^{+/-}$ (n = 1438 β-cells); *Akita*, $\beta Tsc1^{-/-}$ (n = 1584 β-cells).

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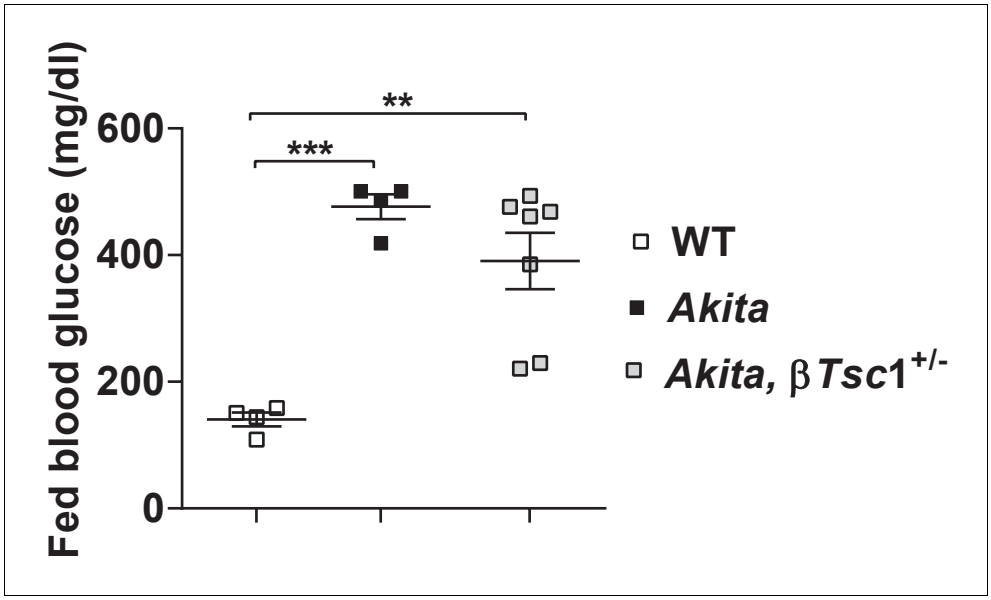

Figure 10—figure supplement 2. Fed blood glucose of *Tsc1*$^{flox/+}$ mice (WT), *Tsc1*$^{flox/+}$*:Akita* (*Akita*) and heterozygous *Tsc1* knockout *RIP-Cre:Tsc1*$^{flox/+}$*:Akita* (*Akita,βTsc1*$^{+/-}$) mice at the age of 2–3 months. Blood glucose levels are the mean of the last three consecutive glucose measurements.

**p<0.01, ***p<0.001.

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