

Title: Essential role of Notch/Hes1 signaling in postnatal pancreatic exocrine development

Short title: Notch in pancreas maturation

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Abstract

Background Notch/Hes1 signaling has been shown to play a role in determining the fate of pancreatic progenitor cells. However, its function in postnatal pancreatic maturation is not fully elucidated.

Methods We generated conditional *Hes1* knockout and/or Notch intracellular domain (NICD) overexpression mice in *Ptf1a*- or *Pdx1*-positive pancreatic progenitor cells and analyzed pancreatic tissues.

Results Both *Ptf1a^{cre/+};Hes1^{ff}* and *Ptf1a^{cre/+};Rosa26^{NICD}* mice showed normal pancreatic development at P0. However, exocrine tissue of the pancreatic tail in *Ptf1a^{cre/+};Hes1^{ff}* mice atrophied and was replaced by fat tissue by 4 weeks of age, with increased apoptotic cells and fewer centroacinar cells. This impaired exocrine development was completely rescued by NICD overexpression in *Ptf1a^{cre/+};Hes1^{ff};Rosa26^{NICD}* mice, suggesting compensation by a Notch signaling pathway other than Hes1. Conversely, *Pdx1-Cre;Hes1^{ff}* mice showed impaired postnatal exocrine development in both the pancreatic head and tail, revealing that the timing and distribution of embryonic Hes1 expression affects postnatal exocrine tissue development.

Conclusions Notch signaling has an essential role in pancreatic progenitor cells for the postnatal maturation of exocrine tissue, partly through the formation of centroacinar cells.

44 **Key words:**

45 Hes1, Notch signaling, pancreas, postnatal development, centroacinar cell

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Introduction

The pancreas consists of both exocrine and endocrine tissues. During embryonal development, these exocrine or endocrine cells are known to differentiate from common pancreatic progenitor cells that are positive for Pdx1 or Ptfla [1]. Ptfla is coexpressed with Pdx1 in the pancreatic epithelia at embryonic day 9.0. Pdx1 is expressed as early as embryonic day 8.5 not only in the pancreas but also in the duodenum, bile duct, and posterior part of the stomach [2]. Notch signaling plays an important role in the development and maintenance of these progenitor cells, in addition to determining their cell fate [3]. However, the detailed functions of Notch signaling after exocrine/endocrine determination or during postnatal pancreatic maturation have not been fully elucidated.

Notch signaling is triggered when ligands interact with Notch receptors. The Notch intracellular domain (NICD) then translocates to the nucleus and activates target genes such as *Hes1*, a main target of the signaling [3]. During pancreatic development, systemic *Hes1*-deficient mice have accelerated endocrine cell differentiation of pancreatic progenitor cells via upregulation of *Ngn3*, a master gene of endocrine differentiation. This leads to reduction of progenitor cells and pancreatic hypoplasia [4]. *Hes1*-deficient mice are also reported to have induced ectopic expression of Ptfla and subsequently form ectopic pancreatic tissue [5]. From these observations, Notch/Hes1 signaling is thought to play a critical role in the development, maintenance, and cell fate determination of pancreatic progenitors by regulating downstream molecules, including Ptfla and Ngn3. In contrast to these functions at embryonic day 8.5–9.0, Notch signaling also plays a role after Pdx1 or Ptfla expression. In mice that have conditional NICD expression in Pdx1-expressing cells, pancreatic progenitor cell differentiation was inhibited [6]. Conversely, mice that have *Hes1* conditionally knocked out in Ptfla-expressing cells revealed no apparent phenotype at birth, although pancreatic atrophy and fatty metaplasia were observed in adults [7]. Thus, the role

of Notch signaling in pancreatic cells after embryonic Pdx1 or Ptf1a expression is still controversial.

Rather than embryonic Pdx1-positive and Ptf1a-positive progenitor cells, centroacinar cells are considered one of the candidates for adult pancreatic tissue stem cells. Centroacinar cells are located at the junction between peripheral acinar cells and the adjacent ductal epithelium, and are characterized by high levels of ALDH1 enzymatic activity and marked Sca1, Sdf1, c-Met, Nestin, and Sox9 expression. These are markers that have been previously associated with progenitor populations in the embryonic pancreas and other tissues [8]. Cre-based lineage tracing showed that adult Sox9-positive duct and/or centroacinar cells continuously supplied acinar cells, suggesting that centroacinar cells have characteristics of stem cells [9]. Interestingly, Hes1 was reported to be expressed in centroacinar cells [10], and the abrogation of both Notch receptor ligands, Delta-like 1 and Jagged1, led to the loss of centroacinar cells [11]. These observations suggest that Notch signaling is highly related to centroacinar cell formation.

In the current study, to elucidate the role of Notch signaling in Pdx1-positive or Ptf1a-positive pancreatic progenitor cells, we analyzed the phenotype of mice with *Hes1* gene knockout and/or NICD transgenic expression in Pdx1- or Ptf1a-expressing cells. We found that in pancreatic progenitor cells, Notch signaling plays an essential role in postnatal exocrine tissue development, presumably in part via centroacinar cell formation.

Methods

Mice

Experimental animals were generated by crossing *Ptf1a^{cre/+}* [1] or *Pdx1-Cre* [12] (supplied by Y Kawaguchi, Kyoto University, Kyoto, Japan) with *Hes1^{ff}* [13] and/or *Rosa26^{NICD}* [6] (supplied by D Melton, Harvard University, MA, USA). To examine Cre expression in the pancreas, we crossed

these mice with *Rosa26^{LacZ}* mice. Each mouse strain is shown in Supplementary Figure 1. All mice were maintained on a mixed genetic background. To compare the phenotypes of genetically engineered mice and controls, we used 3 to 7 littermate mice from each group and age group. No selection for a specific sex was performed in this study except for body weight. All animal experiments were approved by the Kyoto University Graduate School and Faculty of Medicine Ethics Committee.

Histological study

For histological studies, tissue samples were excised from the mice, flushed with phosphate-buffered saline (PBS), fixed overnight in 10% neutral phosphate-buffered formalin, and embedded in paraffin. Sections of 5- μ m thickness were stained with H&E, and immunohistochemistry was carried out. Tissue samples were also frozen immediately in liquid nitrogen for X-gal staining and immunofluorescence studies. Bright-field and fluorescence images were captured with an Olympus BX53-33FL microscope (Olympus, Tokyo, Japan) or Keyence BZ-X710 microscope (Keyence, Osaka, Japan). The captured images were analyzed with Adobe Photoshop software.

Immunohistochemistry and immunofluorescence

Immunohistochemical study was performed using a standard method as previously reported [14]. Primary antibodies used in this study are listed in Supplementary Table 1. Staining signals were detected using a Vectorstain Elite ABC Kit (Vector Laboratories, Burlingame, CA) and a peroxidase substrate kit, DAB (Vector Laboratories). The peroxidase reaction was carried out with Liquid DAB+ Substrate Chromogen System (Dako). Finally, slides were counterstained with hematoxylin (Wako, Japan).

For immunofluorescence studies, the primary antibodies listed in Supplementary Table 1 were

used. The secondary antibodies were Alexa Fluor 594 anti-rabbit IgG 1:100 (A-21207, Thermo Fisher Scientific, Waltham, MA, USA), Alexa Fluor 488 anti-mouse IgG 1:100 (A-21200, Thermo Fisher Scientific), Dylight 488 anti-guinea pig IgG 1:100 (ab96959, Abcam plc, Cambridge, UK), and Alexa Fluor 488 anti-rabbit IgG 1:100 (A-11008, Thermo Fisher Scientific). To assess co-localization of Hes1 and Aldh1a1, frozen sections were stained with anti-Hes1 antibody with Alexa Fluor 594 (ab207048, Abcam) and anti-Aldh1a1 with Alexa Fluor 488 (ab195254, abcam).

X-gal staining

We performed X-gal staining as previously described [1]. Briefly, freshly isolated pancreas was incubated in ice-cold fixative solution (PBS containing 4% paraformaldehyde, 5 mmol/L EGTA, 2 mmol/L MgCl₂, 0.2% glutaraldehyde, and 0.02% NP-40) for 1 hour at 4 °C. After washing twice in PBS for 20 minutes, tissues were incubated with LacZ substrate (PBS containing 5 mmol/L K₃Fe(CN)₆, 5 mmol/L K₄Fe(CN)₆, 2 mmol/L MgCl₂, 0.02% NP-40, 0.1% sodium deoxycholate, and 1 mg/mL X-galactosidase) overnight at room temperature. After washing twice in PBS for 20 minutes, tissues were fixed overnight in 4% paraformaldehyde in PBS at 4 °C. Paraformaldehyde was removed, and the stained tissues were transferred to tissue cassettes.

Evaluation of Ki67-positive cells, cleaved caspase3-positive cells, and centroacinar cells

We compared the number of Ki67-positive cells and cleaved caspase3-positive cells between *Ptfla^{cre/+};Hes1^{ff}* mice and control mice at P0. To count Ki67-positive cells, we used two fields of 200-fold magnification randomly selected in both the head and tail of the pancreas. For cleaved caspase3-positive cells, we counted all positive cells in both the head and tail of the pancreas in a whole slice. We measured the area of the pancreas using ImageJ (<http://imagej.nih.gov/ij/>). To compare the ratio of acini without centroacinar cells, we counted the number of acini with vacant

space in the center of acini and the number of normal acini in *Ptfla^{cre/+};Hes1^{ff}* mice and control mice at P0.

Western blot analysis

To evaluate autophagy in the pancreatic tissue, we performed Western blot analysis for LC3-I and its lipidated form LC3-II, which are widely used to quantify autophagy [15]. Protein extracts from both pancreatic tissue of *Ptfla^{cre/+};Hes1^{ff}* mice and control mice at P4 were boiled in Laemmli sample buffer with 2.5% mercaptoethanol, fractionated on 4–15% sodium dodecyl sulfate polyacrylamide gels (456-1806, Bio-Rad, Tokyo, Japan), and transferred to nitrocellulose membranes according to standard protocols. After blocking with 5% dry skim milk, the blots were incubated with primary antibodies. The primary antibodies were anti-LC3 (1:1000; PM036, MBL, Japan) and anti- β actin (1:10000; ab6276, Abcam). The secondary antibodies were peroxidase-conjugated anti-mouse IgG (1:4000; NA931, GE Healthcare, Chicago, IL, USA) or anti-rabbit IgG (1:10 000; 31458, Thermo Fisher Scientific).

Microarray analysis

We sacrificed a *Ptfla^{cre/+};Hes1^{ff}* mouse and a control mouse (*Ptfla^{cre/+}; Hes1^{+/+}*) at postnatal day 7 and collected pancreatic head and tail tissues from each mouse. Total RNA was extracted from the pancreatic tissue collected in RNALater (Ambion) using RNeasy kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. RNA Integrity Number (RIN) of extracted RNA from pancreatic head of control mouse, tail of a control mouse, head of a *Ptfla^{cre/+};Hes1^{ff}* mouse, and tail of a *Ptfla^{cre/+};Hes1^{ff}* mouse were 9.2, 9.5, 9.2, and 9.1, respectively. RNA was analyzed by Agilent's microarray.

We determined genes associated with pancreatic development, which were specified in a review

article [16]. We then selected genes whose expression in the *Ptfla^{cre/+};Hes1^{ff}* mouse were more than twice or less than half of that of the control. We analyzed these genes and constructed heatmaps with R (R Core Team (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>.)

Quantitative RT-PCR

Total RNA was extracted from the pancreatic tissue collected in RNALater (Ambion) using RNeasy kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. For complementary DNA synthesis, 1 µg total RNA was reverse transcribed using ReverTra Ace[®] qPCR RT Master Mix (Toyobo, Osaka, Japan) and subjected to quantitative RT-PCR. Quantitative RT-PCR was performed by using the LightCycler[®] system (Roche, Switzerland). The mRNA expression of specific genes was measured using FastStart Universal SYBR Green Master (Roche, Switzerland). RNA levels were normalized to the level of the housekeeping gene GAPDH and calculated as delta-delta threshold cycle ($\Delta\Delta CT$). The primer sequences used for mouse ALDH1A1 and GAPDH were as follows: mouse ALDH1A1, 5'-GGGCTGACAAGATTCATGGT-3' (forward) and 5'-GGAAAATTCCAGGGGATGAT-3' (reverse); and GAPDH, 5'-AGGTCGGTGTGAACGGATTTG-3' (forward) and 5'-TGTAGACCATGTAGTTGAGGTCA-3' (reverse). All quantitative RT-PCR analyses were performed in triplicate.

FACS isolation of Aldh-positive cells

FACS isolation of Aldh-positive cell was performed using just modified previously described method [17]. Pancreas of *Ptfla^{cre/+};Hes1^{ff}* mice and control mice were dissected at P7, washed in iced HBSS solution (Thermo Fisher Scientific, Waltham, MA, USA) and minced into small pieces. The minced pancreas was digested by 1 mg/ml collagenase D (Roche, Switzerland) dissolved in

DMEM (Gibco) and supplemented with 2 U/ml DNase I (Promega, Madison, WI, USA) and 1% BSA (Wako) for twenty minutes on a shaker at 37°C. Cells were pipetted through a 1 mL pipette tip every 5 minutes. The reaction was stopped by adding ice cold DMEM supplemented with 5% FBS. Cells were then passed through a 100, 70, and 40 µm cell strainer sequentially, centrifuged at 300 g, 4°C for 3 minutes, re-suspended with DMEM, and counted. Cell suspension were treated with Aldefluor reagent (Stem Cell Technologies, Vancouver, Canada) in the dark at 37 °C on a shaker for 30 minutes. Diethylaminobenzaldehyde (DEAB), an inhibitor of Aldh activity was also added for negative control. Following the Aldefluor reaction, cells were centrifuged and re-suspended with DMEM. FACS Aria II (BD, Franklin Lakes, NJ, USA) was used for FACS sorting. FSC and SSC were used for exclusion of cell debris. Aldh-positive cells were isolated by Aldefluor activity (FITC channel) and counted.

Three-dimensional organoid culture of adult pancreatic cells

Cells re-suspended with DMEM as described above were seeded in 25 µL of the growth factor-reduced Matrigel (BD) in a 48-well plate (Corning). In each well, 1000 cells were seeded. After the gelation of the Matrigel for 10 minutes at 37°C in the incubator, culture medium was added. Culture medium was based on AdDMEM/F12 (Thermo Fischer Scientific), supplemented with 2% B27 (Thermo Fischer Scientific), 1% penicillin/streptomycin (Thermo Fischer Scientific), 1 mM HEPES (Thermo Fischer Scientific), 1% Glutamax (Thermo Fischer Scientific), 1.25 mM N-Acetylcysteine (Sigma), 10 nM gastrin (Sigma), 50 ng/ml EGF (Peprotech), 250 ng/ml Rspodin1 (R&D Systems), 100 ng/ml Noggin (Peprotech), 100 ng/ml FGF10 (Peprotech), 10 mM Nicotinamide (Sigma), 500 nM A83-01 (Tocris), and 10 µM Y27632 (Tocris Bioscience). Culture medium was changed every 3 days. The number and size of organoids were counted 5 days and 9 days after seeding.

Statistical analysis

Data are expressed as the median and standard error of the mean. Statistical comparisons were calculated using an unpaired two-tailed Student's t-test for continuous data. A p-value of less than 0.05 was considered to indicate significance.

Results

Hes1 knockout in *Ptfla*-positive progenitor cells does not affect fetal pancreatic development

To elucidate the role of *Hes1* in *Ptfla*-positive progenitor cells during pancreatic development, we generated conditional *Hes1* knockout mice by crossing *Ptfla-Cre* mice (*Ptfla*^{cre/+}) [1], *Hes1* floxed mice (*Hes1*^{flf}) [13], and *Rosa26*^{LacZ} mice (Fig. S1a-c). Conditional *Hes1* knockout (*Hes1* *cKO*, *Ptfla*^{cre/+};*Hes1*^{flf};*Rosa26*^{LacZ}) mice were born at expected Mendelian ratio, and successful *Hes1* deletion was confirmed by immunohistochemistry (Fig. S2). First, we analyzed neonatal mice. As a result, there was no macroscopic difference in pancreatic size between *Hes1* *cKO* and control (*Ptfla*^{+/+};*Hes1*^{flf};*Rosa26*^{LacZ}, *Ptfla*^{cre/+};*Hes1*^{+/+};*Rosa26*^{LacZ} or *Ptfla*^{cre/+};*Hes1*^{flf};*Rosa26*^{LacZ}) mice at P0 (Fig. 1a). This was also clearly demonstrated by X-gal staining (Fig. 1a). There was also no difference in body weight and blood glucose levels between *Hes1* *cKO* and control mice at 1 week of age (Fig. S3). Microscopically, hematoxylin and eosin (H&E) staining revealed normal pancreatic acini, ducts, and islets of Langerhans in *Hes1* *cKO* mice (Fig. 1a). Furthermore, there was no difference between *Hes1* *cKO* and control mice when evaluating acinar cells, duct cells, β cells, and α cells via immunohistochemistry for amylase, cytokeratin, insulin, and glucagon, respectively (Fig. 1b). Thus, no obvious abnormality was detected in *Ptfla*^{cre/+};*Hes1*^{flf};*Rosa26*^{LacZ} mice at P0.

Transgenic Notch1 expression in Ptfla-positive progenitor cells does not affect fetal pancreatic development

To further analyze the role of Notch signaling in Ptfla-positive progenitor cells in pancreatic development, we next generated *Ptfla^{cre/+};Rosa26^{NICD}* mice (Fig. S1d) [6]. In these mice, transgenic expression of the Notch1 intracellular domain activates downstream molecules of Notch signaling, including Hes1. As a result, there were no macroscopic differences in pancreatic size between *Ptfla^{cre/+};Rosa26^{NICD}* and control (*Ptfla^{+/+}; Rosa26^{NICD}*) mice (Fig. 2a) at P0. Microscopically, there was no difference in the development of exocrine and endocrine tissues between *Ptfla^{cre/+};Rosa26^{NICD}* and control mice at P0 (Fig. 2a), whereas some acinar cells showed ductal change in part (Fig. 2b). Immunofluorescent staining showed normal number of amylase-positive acinar cells, cytokeratin-positive duct cells (Fig. 2b), insulin-positive β cells, and glucagon-positive α cells (Fig. 2c) in *Ptfla^{cre/+};Rosa26^{NICD}* mice as compared to control mice. These results indicated that both *Hes1* knockout and *Notch1* overexpression in Ptfla-positive progenitor cells do not affect the fetal development of pancreatic tissue.

Hes1 knockout in Ptfla-positive progenitor cells results in exocrine atrophy of the adult pancreatic tail

As shown above, *Hes1 cKO* (*Ptfla^{cre/+};Hes1^{flf};Rosa26^{LacZ}*) mice showed normal pancreatic development at birth. After birth, there was no difference in body weight and glucose levels between *Hes1 cKO* and control mice (Fig. S3). However, detailed time-course analysis by X-gal staining revealed that the pancreatic tail gradually showed atrophic changes, and was replaced by fat tissue by 4 weeks of age (Fig. 3a, Fig. S4). Meanwhile, the pancreas head was not affected by fat replacement. Histological analysis by H&E staining and immunohistochemistry was performed

at 4 weeks of age for amylase and cytokeratin. This confirmed remarkable loss of acinar cells, abnormal alignment of duct cells, and prominent deposition of fat tissue in the pancreatic tail of *Hes1 cKO* mice compared to that of control mice or the pancreatic head of *Hes1 cKO* mice (Fig. 3b). In contrast to the marked impairment of exocrine cells, normal endocrine cell development was observed in both the pancreatic head and tail of *Hes1 cKO* mice when analyzed via immunohistochemistry for insulin and glucagon (Fig. 3b).

To investigate the cause of atrophic changes in the pancreatic tail of *Hes1 cKO* mice, apoptosis and cell growth levels were evaluated by immunohistochemistry for cleaved caspase3 and Ki67, respectively, at age P0 (Fig. 4a). Although there was no difference in histology (Fig. 1) and the Ki67 index (Fig. 4b) between *Hes1 cKO* and control mice in both the pancreatic head and tail, significantly more apoptotic cells were detected in *Hes1 cKO* than in control mice, especially in the pancreatic tail (Fig 4c). Western blot analysis revealed expression of LC3- I , but failed to detect its lipidated form LC3- II in both pancreatic head and tail of *Hes1 cKO* mice and control mice (Fig. S5), suggesting a limited involvement of autophagy. These results showed that conditional *Hes1* knockout in *Ptf1a*-positive progenitor cells led to atrophic changes in the pancreatic tail during the postnatal growth and maturation process, partly due to exocrine cell apoptosis.

***Hes1* knockout in *Ptf1a*-positive progenitor cells induces centroacinar cell reduction**

To further investigate the cause of atrophic changes in the pancreatic tail of *Hes1 cKO* mice, we focused on centroacinar cells located in the center of the pancreatic acinus. These are considered to be one of the adult tissue stem cell candidates of pancreatic exocrine tissue. As previously reported, centroacinar cells with *Hes1* and *Aldh1* expression were observed in the pancreatic tail

of control (*Ptfla^{cre/+};Hes1^{f/+}*) mice at P0 (Fig 5a). Co-localization of Hes1 and Aldh1a1 was confirmed by immunofluorescence at P3 (Fig. S6a). In contrast, in *Hes1 cKO* (*Ptfla^{cre/+};Hes1^{f/f}*) mice, there were a substantial number of acini with vacant space in the center and without Hes1- and Aldh1-positive centroacinar cells (Fig 5a). The ratio of acini without centroacinar cells was significantly higher in the pancreatic head and tail of *Hes1 cKO* mice than in control mice at P0 (Fig 5b). At P3, this trend of centroacinar cell reduction was prolonged in the pancreatic tail, whereas this trend was disappeared in pancreatic head (Fig 5c). At P7, microarray analysis using tissue from the pancreatic tail of *Hes1 cKO* mice revealed distinct gene expression patterns from that of the pancreatic head of *Hes1 cKO* mice or the pancreatic head/tail of control mice (Fig. S6b). Notably, the pancreatic tail of *Hes1 cKO* mice showed decreased exocrine-related gene expression (Fig. 5d) and increased endocrine-related gene expression (Fig. 5e). Lower *Aldh1*-related gene expression and significantly reduced *Aldh1a1* gene expression were demonstrated by microarray (Fig. 5f) and qPCR analysis (Fig. 5g), respectively, in the pancreatic tail of *Hes1 cKO* mice compared to that of control mice. This confirmed the previously observed reduction in centroacinar cells in the pancreatic tail of *Hes1 cKO* mice (Fig. 5a-c). The presence of fewer centroacinar cells in the pancreatic tail of *Hes1 cKO* mice in the neonatal state may explain pancreatic atrophy being observed only in the pancreatic tail.

To further assess the mechanisms for fewer centroacinar cells and subsequent impaired development in *Hes1 cKO* mice, we isolated pancreatic cells from *Hes1 cKO* and control mice and evaluated their stem cell features by FACS analysis and organoid culture. As a result, confirming the altered *Aldh1*-related gene expression (Fig. 5f), FACS analysis revealed that pancreatic tail of *Hes1 cKO* mice had significantly lower number of Aldh-positive cells than control mice at P7 (Fig. S7a, b). Furthermore, pancreatic cells from *Hes1 cKO* mice formed significantly lower number of organoids than those from control mice (Fig. S7c, d). These observations suggest that loss of Hes1

may affect the maintenance of tissue stem cells required for postnatal pancreatic development.

Transgenic *Notch1* expression compensates for the *Hes1* knockout phenotype in *Ptf1a*-positive progenitor cells

To assess the role of the Notch signaling pathway in postnatal pancreatic tail development, we analyzed *Ptf1a^{cre/+};Hes1^{ff};Rosa26^{NICD}* mice. In these mice, downstream molecules of Notch signaling, other than *Hes1*, were activated in *Ptf1a*-positive progenitor cells. As a result, some acini showed a vacant space without centroacinar cells in *Ptf1a^{cre/+};Hes1^{ff};Rosa26^{NICD}* mice at P0 (Fig. 6a), as observed in *Ptf1a^{cre/+};Hes1^{ff}* mice (Fig. 5a). However, even with *Hes1* gene deletion, atrophic changes in the pancreatic tail were not observed in *Ptf1a^{cre/+};Hes1^{ff};Rosa26^{NICD}* mice at 16 weeks of age. H&E staining, immunohistochemistry for amylase and cytokeratin, and immunofluorescence staining for insulin and glucagon showed normal development of acinar cells, duct cells, α cells, and β cells in *Ptf1a^{cre/+};Hes1^{ff};Rosa26^{NICD}* mice at 16 weeks (Fig. 6b,c). These results suggested that activation of the Notch signaling pathway, other than *Hes1*, could compensate for defective *Hes1* in pancreatic tail development in the postnatal to adult state. To further analyze this compensatory mechanism, we assessed the expressions of *Hes5* and *Hey1*, which are major downstream molecules of Notch signaling other than *Hes1*, in pancreatic tissues of *Ptf1a^{cre/+};Hes1^{ff};Rosa26^{NICD}* mice, *Ptf1a^{cre/+};Rosa26^{NICD}* mice, *Ptf1a^{cre/+};Hes1^{ff}* (*cKO*) mice, and control mice at P0 by immunohistochemistry. As a result, a marked increase of *Hes5* expression was observed in *Ptf1a^{cre/+};Hes1^{ff};Rosa26^{NICD}* mice compared to *Ptf1a^{cre/+};Rosa26^{NICD}* mice, *Ptf1a^{cre/+};Hes1^{ff}* (*cKO*) mice and control mice, whereas *Hey1* expression was similar in all the strains of mice (Fig. S8). These results suggested that *Hes5* could be one of the factors contributing the compensation for *Hes1* deficiency in pancreatic development in *Ptf1a^{cre/+};Hes1^{ff};Rosa26^{NICD}* mice.

***Hes1* knockout in Pdx1-positive progenitor cells induces impaired exocrine development in both the pancreatic head and tail**

We sought to investigate why the pancreatic head did not have impaired exocrine development in mice with conditional *Hes1* knockout in Ptf1a-positive progenitor cells (*Ptf1a^{cre/+};Hes1^{ff};Rosa26^{LacZ}*). To this end, we generated and analyzed *Pdx1-Cre;Hes1^{ff};Rosa26^{LacZ}* mice, in which *Hes1* gene is expected to be knocked out in pancreatic progenitor cells by Pdx1-Cre [12] (Fig. S1e) about 0.5 day prior to that by Ptf1a-Cre. Similar to *Ptf1a^{cre/+};Hes1^{ff}* mice, normal exocrine/endocrine cells and a substantial number of acini without centroacinar cells were observed in *Pdx1-Cre;Hes1^{ff};Rosa26^{LacZ}* mice via H&E staining and immunofluorescent staining for insulin and glucagon at P0 (Fig. 7a,b). X-gal staining of *Pdx1-Cre;Hes1^{ff};Rosa26^{LacZ}* mice stained almost all pancreatic cells blue at P0, suggesting that *Hes1* was successfully knocked out in these cells (Fig. 7c). In contrast, at 2 weeks and 54 weeks after birth, although the size of the pancreas was normal, the number of blue cells stained by X-gal staining was markedly reduced in both the pancreatic head and tail in *Pdx1-Cre;Hes1^{ff};Rosa26^{LacZ}* mice as compared to that in control mice (Fig. 7c). In the microscopic analysis of X-gal staining, while all the pancreatic cells were stained blue at P0, only endocrine cells and a few exocrine cells were stained blue in *Pdx1-Cre;Hes1^{ff};Rosa26^{LacZ}* mice at 8 weeks of age. Further, pancreatic tissue replaced non-stained exocrine cells in both the pancreatic head and tail at 8 weeks of age (Fig. 7d). These results indicated that *Hes1*-knocked out Pdx1-positive progenitor cells could not mature into exocrine cells in the pancreatic head or tail. This is different from the observations found in *Hes1*-knocked out Ptf1a-positive progenitor cells. The results suggest that the timing of *Hes1* expression in the embryonic stage critically affects the postnatal development of exocrine tissue.

359

360 **Discussion**

361 The importance of Notch signaling in pancreatic embryonic development has been thoroughly
362 studied [3, 4]. However, since systemic *Hes1* deficient mice or *Pdx1-Cre;Rosa26^{NICD}* mice are
363 reported to be embryonic lethal mutants [4, 6] the detailed functions of Notch signaling in Pdx1-
364 or Ptfla-positive progenitor cells has been mostly unknown. In this study, we analyzed mice with
365 conditional *Hes1* knockout or transgenic NICD expression in Ptfla/Pdx1-positive progenitor cells.
366 We found that Notch signaling in pancreatic progenitor cells plays an essential role in postnatal
367 exocrine tissue development.

368 Previous reports have shown that systemic *Hes1* knockout induced accelerated differentiation of
369 pancreatic progenitor cells to endocrine cells, resulting in a reduction in progenitor cells. This
370 suggests the crucial role of Notch/Hes1 signaling in exocrine-endocrine cell fate determination [4].
371 This function of Notch signaling is still observed in Pdx1-positive progenitor cells, in which
372 exocrine-endocrine differentiation was inhibited by conditional NICD expression [6]. However, in
373 our analysis, both *Ptfla^{cre/+};Hes1^{flf}* mice and *Ptfla^{cre/+};Rosa26^{NICD}* mice showed normal
374 embryonic development, and no abnormality was observed in exocrine-endocrine differentiation
375 at birth. Considering that gene recombination is induced in pancreatic progenitor cells by Pdx1-
376 Cre at about 0.5 to 1 day prior to that by Ptfla-Cre [2], cell fate determination by Notch signaling
377 may have already been completed in Ptfla-positive pancreatic progenitor cells.

378 In contrast, our analysis showed that *Ptfla^{cre/+};Hes1^{flf}* mice had atrophic changes in the pancreatic
379 tail by 4 weeks of age due to impaired postnatal development exocrine tissue. This is similar to the
380 observations details in the report by Hidalgo-Sastre et al. [7], From these observations, it is clear
381 that *Hes1* plays a critical role in exocrine tissue maturation after exocrine cell fate determination
382 in Ptfla-positive progenitor cells. To elucidate the mechanism of impaired postnatal pancreatic

exocrine tissue development in *Ptfla^{cre/+};Hes1^{ff}* mice, we focused on centroacinar cells. These cells are one of the candidates for adult pancreatic tissue stem cells, and have been reported to express Hes1 [10]. As a result, we found sustained loss of centroacinar cells during the neonatal period in the pancreatic tail, but not in the pancreatic head, in *Ptfla^{cre/+};Hes1^{ff}* mice. This phenotype of centroacinar cell loss and acini with vacant space in the center is markedly analogous to that of Notch ligands *Dll1/Jag1* double knockout mice [11], suggesting the role of Notch signaling in centroacinar cell development. Together with our results of increased apoptotic cells in the pancreatic tail of *Ptfla^{cre/+};Hes1^{ff}* mice, Notch signaling in pancreatic progenitor cells may contribute to postnatal centroacinar cell development and subsequent exocrine maturation. To confirm this hypothesis, we analyzed *Ptfla^{cre/+};Hes1^{ff};Rosa26^{NICD}* mice in which Notch signaling, save for Hes1, is activated. As expected, the impaired postnatal pancreatic exocrine tissue development in *Ptfla^{cre/+};Hes1^{ff}* mice was completely rescued by the activation of Notch signaling in *Ptfla^{cre/+};Hes1^{ff};Rosa26^{NICD}* mice, suggesting that *Hes/Hey* family members other than *Hes1* may have compensated for the function of *Hes1*. In fact, expression of Hes5 was strongly enhanced in the pancreatic tissue of *Ptfla^{cre/+};Hes1^{ff};Rosa26^{NICD}* mice, suggesting that Hes5 may contribute to this compensatory mechanism.

We sought to determine when Notch/Hes1 signaling carries out its critical function in postnatal pancreatic progenitor cell maturation after performing its role in exocrine/endocrine cell fate determination at the embryonic stage. We, therefore, analyzed *Pdx1-Cre;Hes1^{ff}* mice in comparison with *Ptfla^{cre/+};Hes1^{ff}* mice. As a result, impaired postnatal exocrine maturation was observed only in the pancreatic tail in *Ptfla^{cre/+};Hes1^{ff}* mice. However, this was observed in both the pancreatic head and tail in *Pdx1-Cre;Hes1^{ff}* mice. We speculate that this phenotypic difference in the pancreatic head between *Ptfla^{cre/+}* and *Pdx1-Cre* is due to their difference in timing of genetic recombination, as mentioned above [2]. The appearance of this phenotype in *Pdx1-Cre* but

not in *Ptfla*^{cre/+} may indicate the importance of Notch signaling activation between Pdx1 and *Ptfla* expression in progenitor cells, at least in the pancreatic head. The greater effect by earlier *Hes1* deletion is further confirmed by our previous observation that adult pancreatic tissue is resistant to *Hes1* deletion [14]. Interestingly, unlike *Ptfla*^{cre/+};*Hes1*^{ff} mice, pancreatic atrophy was not observed in *Pdx1-Cre*;*Hes1*^{ff} mice. This is probably because the loss of X-gal positive / *Hes1* knockout cells was replaced by X-gal negative exocrine cells that escaped the recombination of *Hes1* gene.

In conclusion, in addition to the previously reported role of exocrine/endocrine cell fate determination, Notch signaling in pancreatic progenitor cells plays an essential role in the postnatal maturation of exocrine tissue, partly through centroacinar cell formation.

419 **Acknowledgements**

420 We thank Y Kawaguchi (Kyoto University, Kyoto, Japan) for supplying *Ptfla*^{cre/+} mice. We thank
421 D Melton (Harvard University, MA, USA) for supplying *Rosa26*^{NICD} mice. We thank Yuta
422 Kawamata and Taichi Ito for the excellent technical support.

423

424 **Funding**

425 This work was supported by the Japan Society for the Promotion of Science (JSPS) KAKENHI
426 Grant Numbers JP16K09395.

427

428 **Competing interests**

429 The authors declare that they have no conflict of interest.

430

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Figure Captions

Fig. 1

***Hes1* knockout in *Ptf1a*-positive progenitor cells does not affect fetal pancreatic development.**

Analysis at P0 in conditional *Hes1* knockout (*Hes1 cKO*) (*Ptf1a^{cre/+}; Hes1^{ff}; Rosa26^{LacZ}*) and control (Ctrl) (*Ptf1a^{+/+}; Hes1^{ff}; Rosa26^{LacZ}*, *Ptf1a^{cre/+}; Hes1^{+/+}; Rosa26^{LacZ}* or *Ptf1a^{cre/+}; Hes1^{ff/+}; Rosa26^{LacZ}*) mice. **a** Stereomicroscopic images, stereomicroscopic image of X-gal staining, and H&E staining of the pancreas. **b** Immunohistochemical staining for amylase, cytokeratin, insulin, and glucagon. There is no obvious difference between *Hes1 cKO* and control mice. Scale bars: 50 μ m.

Fig. 2

Transgenic *Notch1* expression in *Ptf1a*-positive progenitor cells does not affect fetal pancreatic development.

Analysis of *Ptf1a^{cre/+}; Rosa26^{NICD}* and control mice (*Ptf1a^{+/+}; Rosa26^{NICD}*) at P0 (**a**) and P3 (**b** and **c**). Stereomicroscopic images, H&E staining, immunofluorescent staining for amylase, cytokeratin, insulin, and glucagon showed no obvious difference between *Ptf1a^{cre/+}; Rosa26^{NICD}* and control mice. Scale bars: 1000 μ m (**a**), 50 μ m (**a-c**).

Fig. 3

***Hes1* knockout in *Ptf1a*-positive progenitor cells resulted in exocrine atrophy of the adult pancreatic tail.**

a Time-course analysis by X-gal staining in conditional *Hes1 cKO* (*Ptf1a^{cre/+}; Hes1^{ff}; Rosa26^{LacZ}*) and control (*Ptf1a^{cre/+}; Hes1^{ff/+}; Rosa26^{LacZ}*) mice at indicated weeks of age. *Hes1 cKO* mice showed

atrophic changes in the pancreatic tail compared to control mice. **b** H&E staining and immunohistochemical staining for amylase, cytokeratin, insulin, and glucagon in *Hes1 cKO* mice and control mice at 4 weeks of age. Impaired development of exocrine cells and fat replacement was found in *Hes1 cKO* mice, whereas no change was observed in endocrine cells. Scale bars: 50 μ m (**b**).

Fig. 4

***Hes1* knockout in *Ptf1a*-positive progenitor cells induced more apoptotic cells in the pancreatic tail.**

a Immunohistochemical analysis of Ki67 and cleaved caspase3 in the pancreatic head and tail in *Hes1 cKO* (*Ptf1a^{cre/+};Hes1^{ff}*) and control (*Ptf1a^{cre/+};Hes1^{ff/+}*) mice at age P0. Representative immunohistochemical images (**a**) and their graphic representation (**b**, **c**). Arrow heads show apoptotic cells in pancreas. Arrows show apoptotic cells in duodenum (positive control). Significantly more apoptotic cells were detected in the pancreatic tail in *Hes1 cKO* mice than in control mice (**a**, **c**), whereas there was no difference in the number of Ki67-positive cells (**a**, **b**). Scale bars: 50 μ m (**a**, Ki67 and Cleaved Caspase3 of pancreatic tail), 100 μ m (**a**, Cleaved Caspase3 of pancreatic head). *P < 0.05.

Fig. 5

***Hes1* knockout in *Ptf1a*-positive progenitor cells induces reduction of centroacinar cells.**

a H&E staining and immunohistochemical images of *Hes1* and ALDH1A1 in *Hes1 cKO* (*Ptf1a^{cre/+};Hes1^{ff}*) and control (Ctrl, *Ptf1a^{cre/+};Hes1^{ff/+}*) mice at P0. Magnifications of the broken line are shown. Centroacinar cells expressed *Hes1* and ALDH1A1 in control mice, whereas there was a vacant space in the center of acini of *Hes1 cKO* mice. **b**, **c** The ratio of acini without

centroacinar cells at P0 (**b**) and P3 (**c**) in the pancreatic head and tail of *Hes1 cKO* and control mice are shown. The ratio of acini without centroacinar cells was significantly higher in *Hes1 cKO* than in control mice at P0 (**b**). Centroacinar cell reduction was still observed in the pancreatic tail of *Hes1 cKO* mice at P3 (**c**). **d-f**: Microarray analysis of pancreatic head and tail tissue in *Hes1 cKO* and control mice at P7. Expression of exocrine-related genes was reduced in the pancreatic tail of *Hes1 cKO* mice (**d**), whereas expression of endocrine-related genes was elevated (**e**) compared to in the control group or head of *Hes1 cKO* mice. Expression of *Aldh1*, a centroacinar cell marker, was lower in the pancreatic tail of *Hes1 cKO* mice (**f**). **g** Quantitative analysis of *Aldh1a1* mRNA expression in the pancreatic head and tail of *Hes1 cKO* and control mice. Scale bars: 50 μ m (**a**, H&E), 15 μ m (**a**, ALDH1A1). *P < 0.05.

Fig. 6

Transgenic *Notch1* expression compensates for the *Hes1* knockout phenotype in *Ptf1a*-positive progenitor cells.

Analysis of *Ptf1a^{cre/+}; Hes1^{ff}; Rosa26^{NICD}* mice and control (*Ptf1a^{+/+}; Hes1^{ff}; Rosa26^{NICD}*) at P0 (**a**) and at 16 weeks of age (**b**, **c**). **a** H&E staining showed almost normal pancreatic development in *Ptf1a^{cre/+}; Hes1^{ff}; Rosa26^{NICD}* mice at P0, although some acini showed a vacant space in the center without centroacinar cells. **b**, **c** At 16 weeks, H&E staining, immunohistochemistry of amylase and cytokeratin, and immunofluorescent staining for insulin and glucagon showed normal pancreatic tissue development in *Ptf1a^{cre/+}; Hes1^{ff}; Rosa26^{NICD}* mice compared to *Ptf1a^{+/+}; Hes1^{ff}; Rosa26^{NICD}* mice. Scale bars: 50 μ m (**a-c**).

Fig. 7

Hes1 knockout in Pdx1-positive progenitor cells induces impaired exocrine development in

the pancreatic head and tail.

a Analysis of *Pdx1-Cre;Hes1^{ff}* mice and control (*Hes1^{ff}*) mice at P0. Hematoxylin and eosin (H&E) staining showed normal exocrine/endocrine cells and some acini without centroacinar cells.

b The ratio of acini without centroacinar cells was higher in *Pdx1-Cre;Hes1^{ff}* than control mice at P0. **c** Time-course analysis of *Pdx1-Cre;Hes1^{ff};Rosa26^{LacZ}* and control (*Pdx1-*

Cre;Hes1^{ff/+};Rosa26^{LacZ}) mice by X-gal staining at indicated weeks of age. Broken lines show the pancreas. X-gal staining showed gradual reduction of exocrine cells in *Pdx1-cre;Hes1^{ff};Rosa26^{LacZ}*

mice. **d** Microscopic image of X-gal staining of pancreatic tissues in *Pdx1-Cre;Hes1^{ff};Rosa26^{LacZ}*

and control (*Pdx1-Cre;Hes1^{ff/+};Rosa26^{LacZ}*) mice at P0 and 8 weeks of age. Only endocrine cells and a few exocrine cells were stained blue, and pancreatic tissue was replaced by non-stained

exocrine cells in both the pancreatic head and tail at 8 weeks of age. Scale bars: 50 μ m (**a, d**), 1000 μ m (**a**). An arrow head shows an islet (**d**).

Supplementary Figure S1

Mouse strains

a The protein-coding region of *Ptfla* was precisely replaced with that of *Cre*, encoding the

recombinase Cre. **b** *Hes1* is floxed by *loxP* and conditionally knocked out in the Cre recombinase

promoter site. **c** *Rosa-26*, *Lox-STOP-Lox*, and *LacZ* construct. LacZ expresses in the Cre

recombinase promoter site. **d** *Rosa-26*, *Lox-STOP-Lox*, and *NICD* construct. NICD expresses in

the Cre recombinase promoter site. **e** Cre recombinase is under the transcriptional control of the

mouse *Pdx1* promoter.

Supplementary Figure S2

Hes1 immunohistochemistry

Hes1 expression in pancreatic tissue was analyzed by immunohistochemistry in *Hes1 cKO* (*Ptfla^{cre/+};Hes1^{ff};Rosa26^{LacZ}*) and control (Ctrl, *Ptfla^{+/+};Hes1^{ff};Rosa26^{LacZ}*) mice at P0. Hes1 expression was deleted in *Hes1 cKO* mice (**b**), while it was positive in the ducts and centroacinar cells of control mice (**a**).

Supplementary Figure S3

Changes in body weight and blood glucose level

Changes in body weight (**a**) and blood glucose level (**b**) after birth in *Hes1 cKO* (*Ptfla^{cre/+};Hes1^{ff};Rosa26^{LacZ}*) and control (Ctrl, *Ptfla^{+/+};Hes1^{ff};Rosa26^{LacZ}*) mice. The median and the standard error of mean are shown.

Supplementary Figure S4

***Hes1* knockout in Ptfla-positive progenitor cells.**

X-gal staining of *Hes1* conditional knockout mice (*Ptfla^{cre/+};Hes1^{ff};Rosa26^{LacZ}*) and control mice (*Ptfla^{cre/+};Hes1^{ff/+};Rosa26^{LacZ}*) at 4 weeks of age. **b** and **d** are a magnification of the broken line in **a** and **c**. Arrow head: islet, arrow: duct. Scale bars: 50 μ m (**b**, **d**).

Supplementary Figure S5

a Western blot analysis for LC3-I and its lipidated form LC3-II. We found expression of LC3-I but not its lipidated form LC3-II in both pancreatic head and tail of *Hes1 cKO* mice and control mice at P4.

Supplementary Figure S6

a Immunofluorescence images of Hes1 and Aldh1a1 in *Hes1 cKO* (*Ptfla^{cre/+};Hes1^{flf}*) and control (Ctrl, *Ptfla^{+/+};Hes1^{flf}*) mice at P3. Scale bars: 50 μ m. **b** Microarray analysis of pancreatic head and tail tissues of *Hes1 cKO* and control mice at P7. A heatmap of genes related to pancreatic development is shown.

Supplementary Figure S7

a FACS isolation of Aldh-positive cells with Aldefluor reagent. FACS gating was established by negative control. The number of Aldh-positive cells were decreased in *Hes1 cKO* (*Ptfla^{cre/+};Hes1^{flf}*) mice compared with that in control mice at P7 (**b**). The number of organoid formation from pancreatic cells (**c, d**). *P < 0.05.

Supplementary Figure S8

a H&E staining and immunohistochemical staining for Hes5 and Hey1 of control mice, *Ptfla^{cre/+};Hes1^{flf}* mice (*cKO*), *Ptfla^{cre/+};Rosa26^{NICD}* mice, and *Ptfla^{cre/+};Hes1^{flf};Rosa26^{NICD}* mice at P0. Marked increase of Hes5 expression was observed in *Ptfla^{cre/+};Hes1^{flf};Rosa26^{NICD}* mice. Scale bars: 50 μ m.

Supplementary Table 1.

Primary antibodies used in this study.

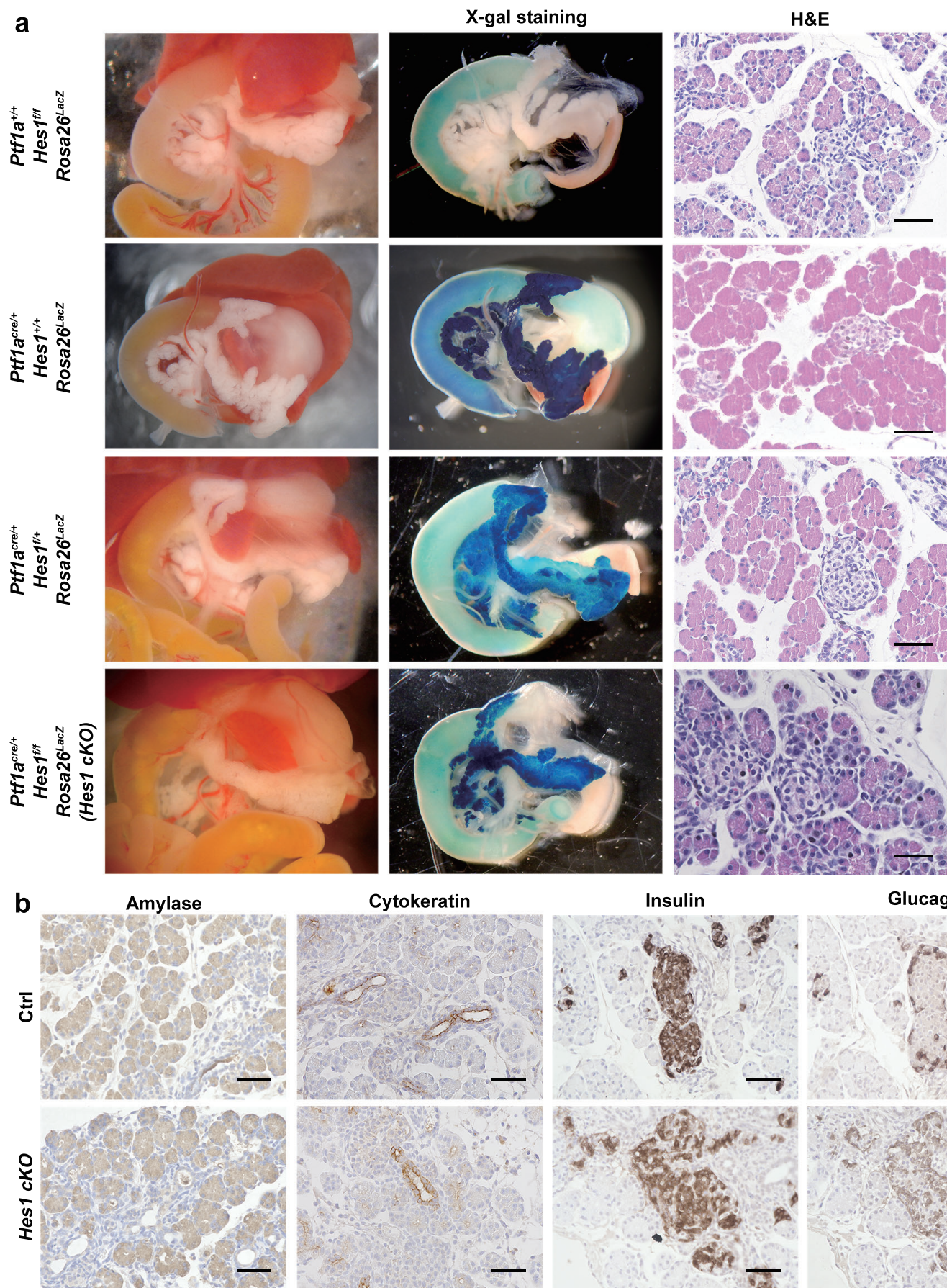


Fig. 1

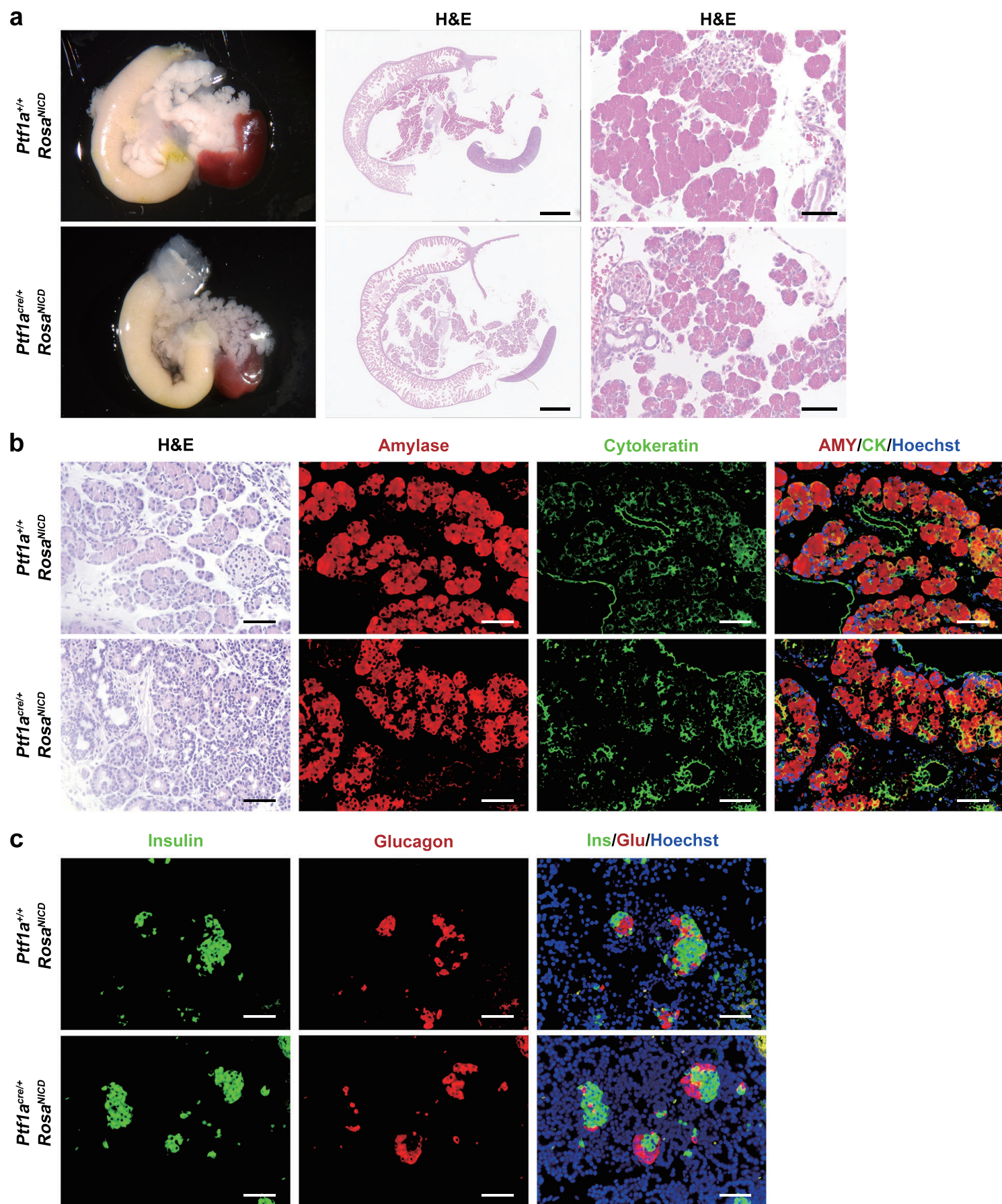


Fig. 2

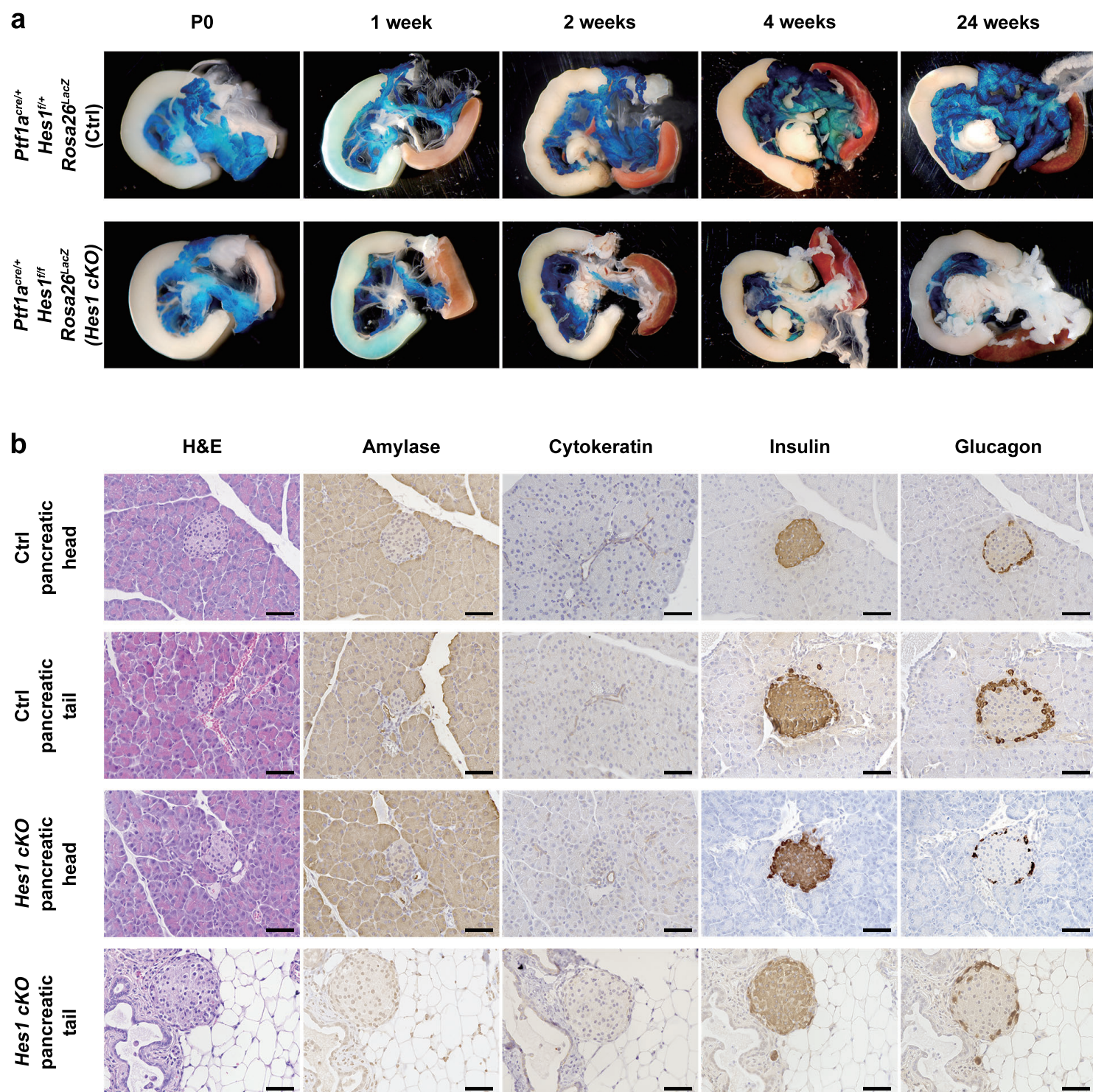


Fig. 3

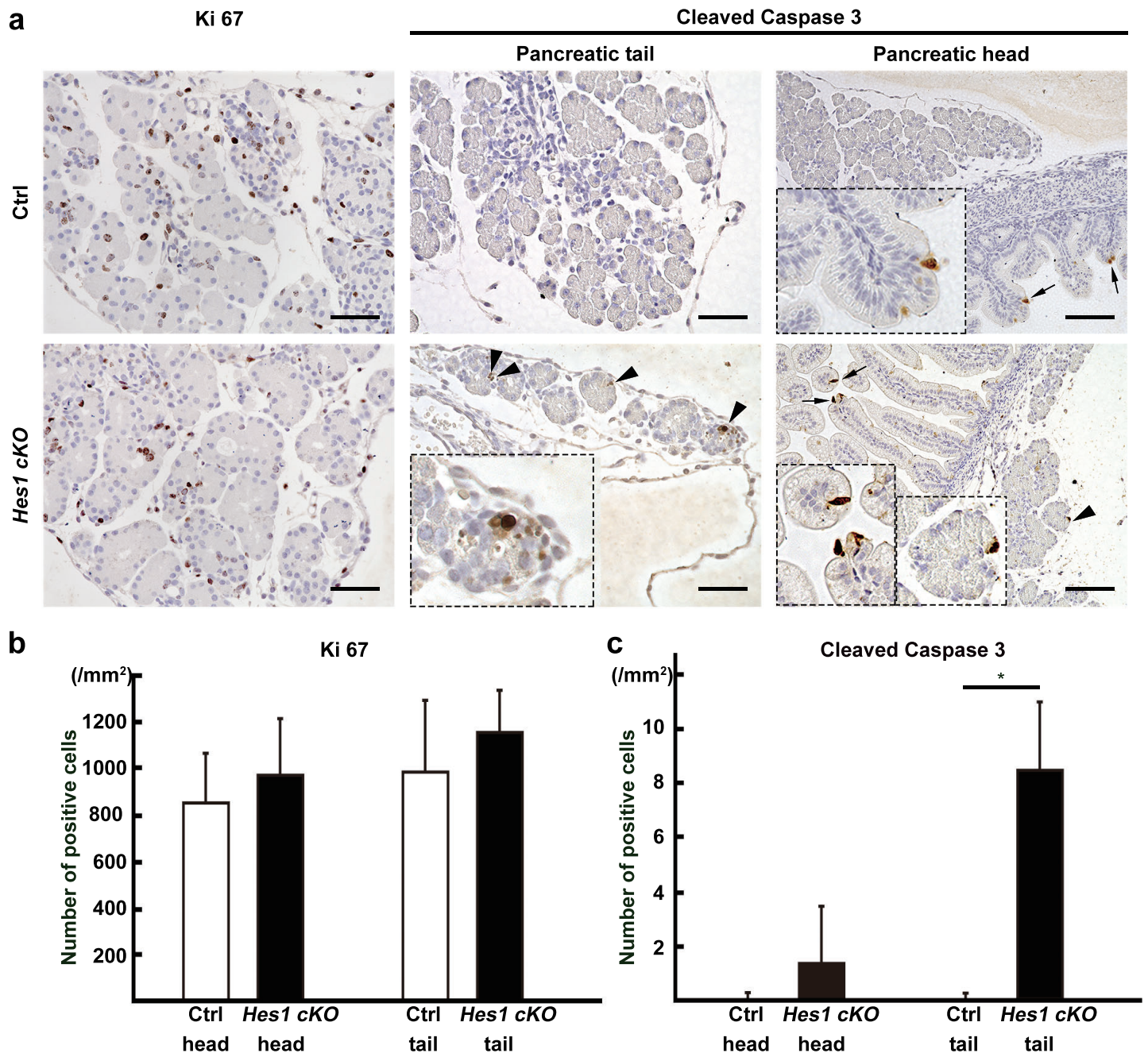


Fig. 4

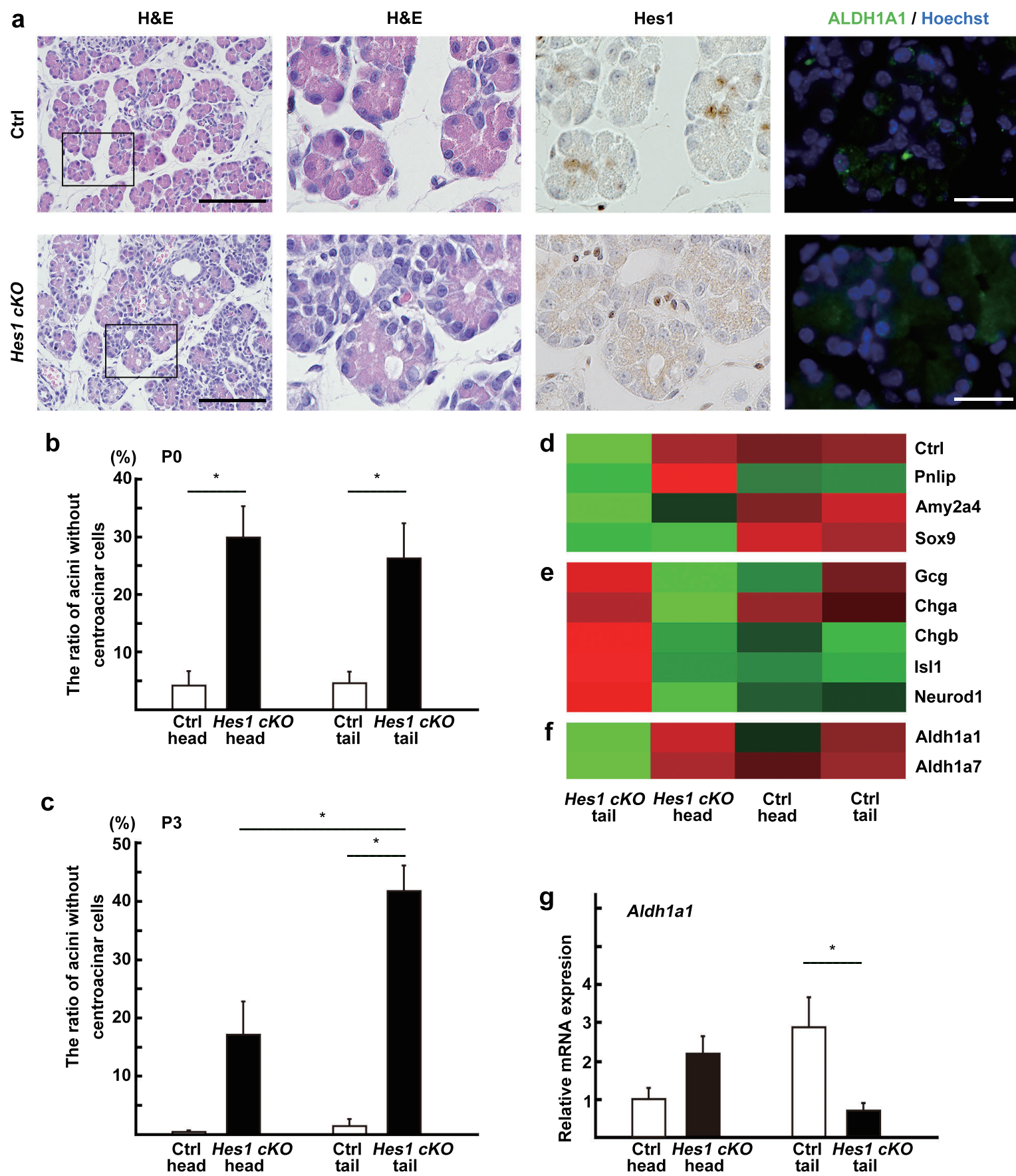


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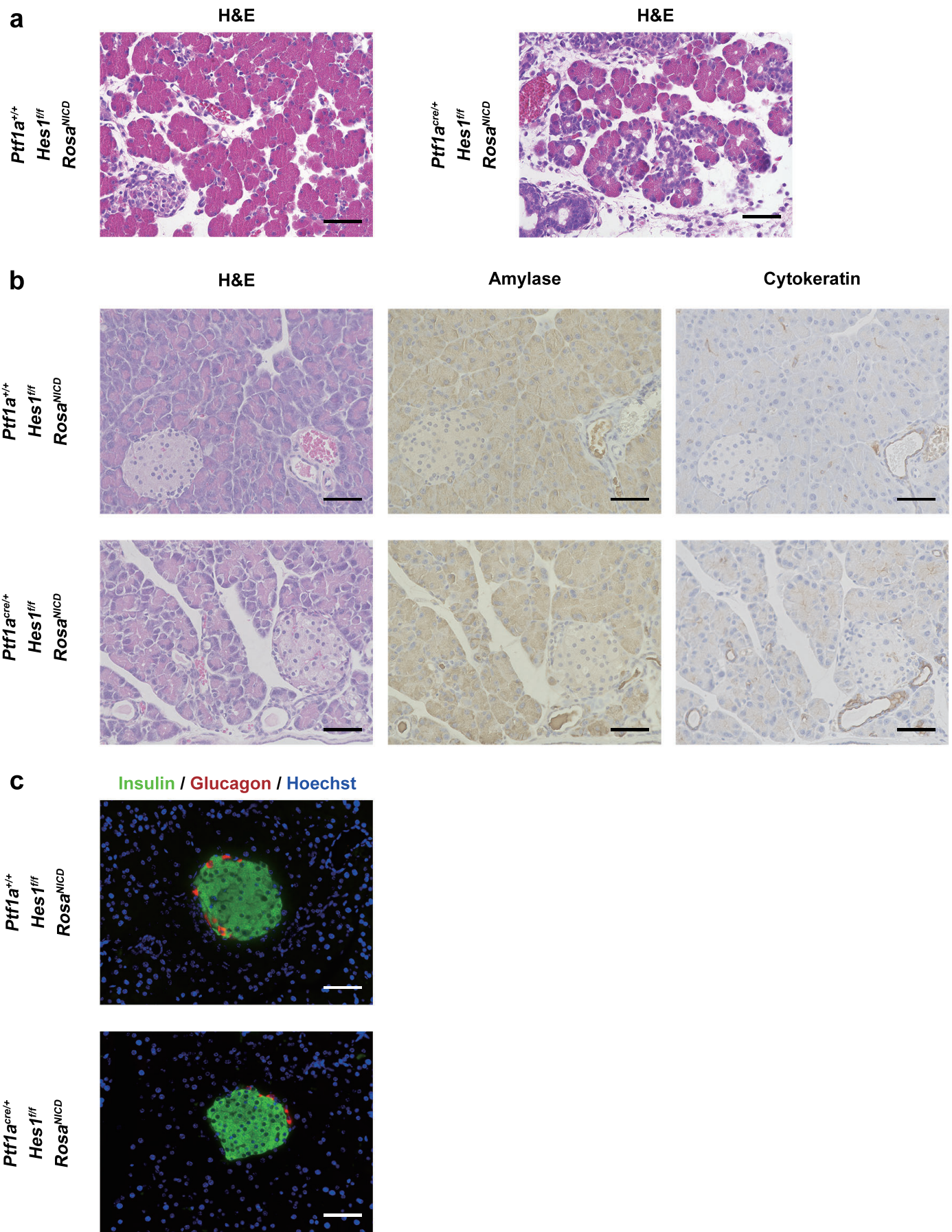


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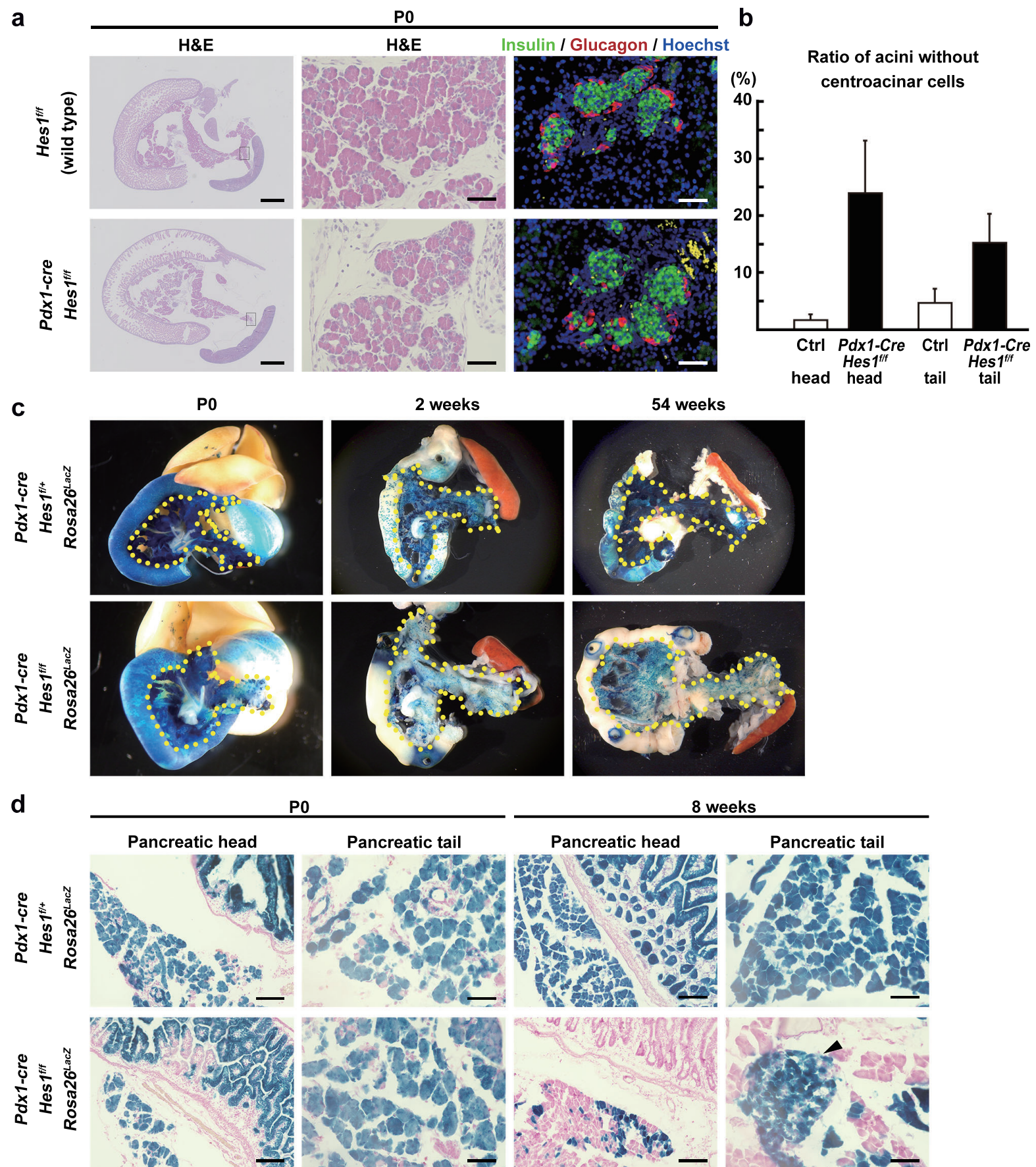
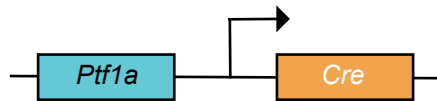
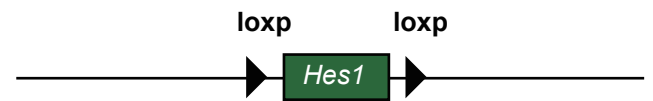


Fig. 7

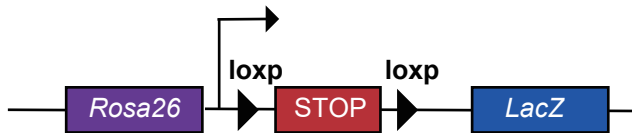
a *Ptf1a*^{cre/+}



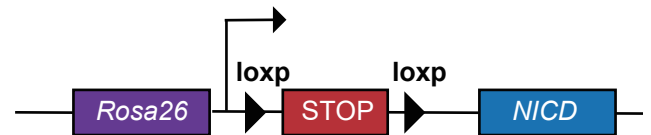
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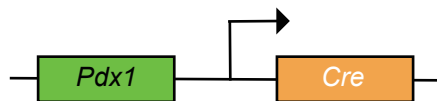
c *Rosa26*^{LacZ}



d *Rosa26*^{NICD}



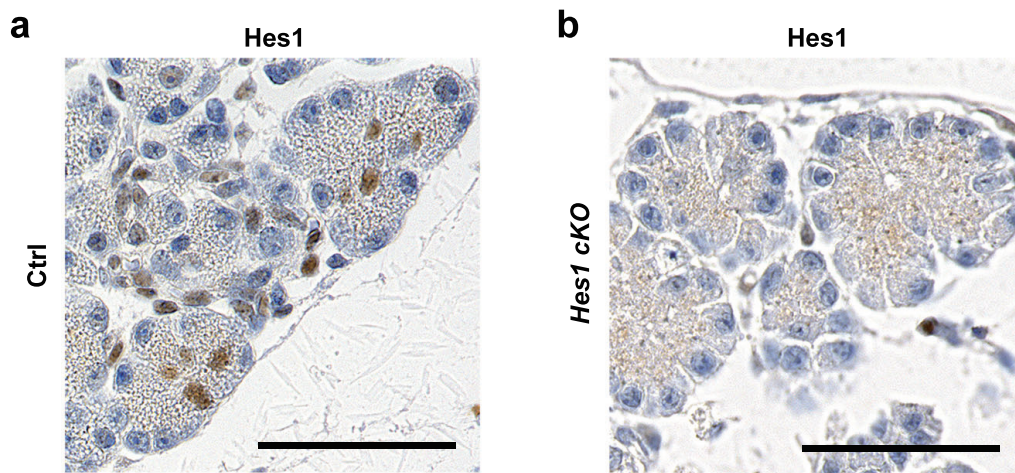
e *Pdx1*-Cre



Supplementary Figure S1

Mouse strains

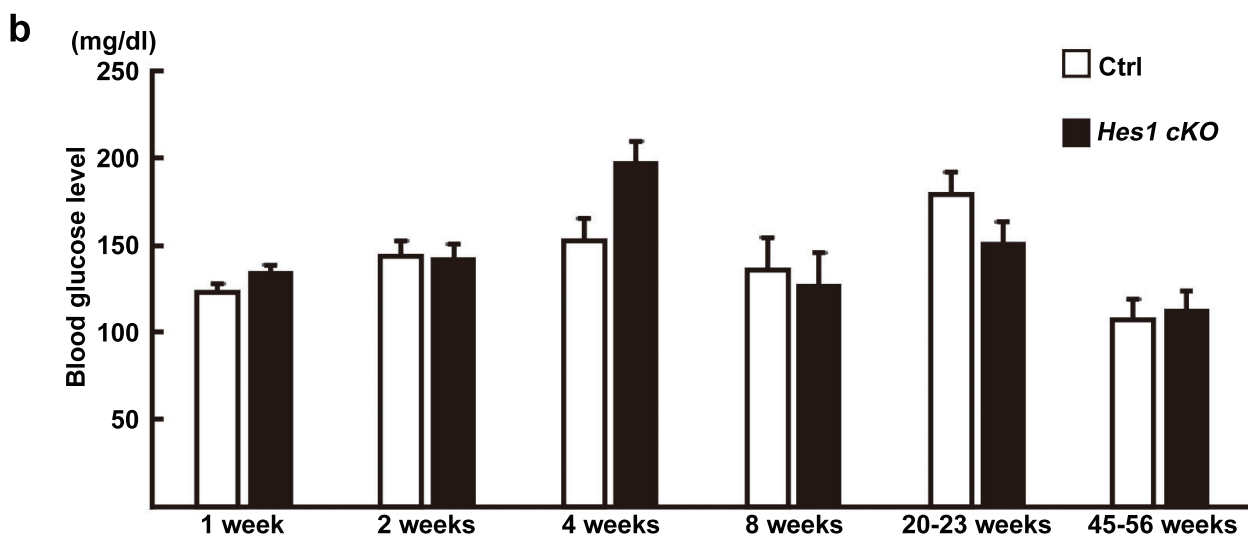
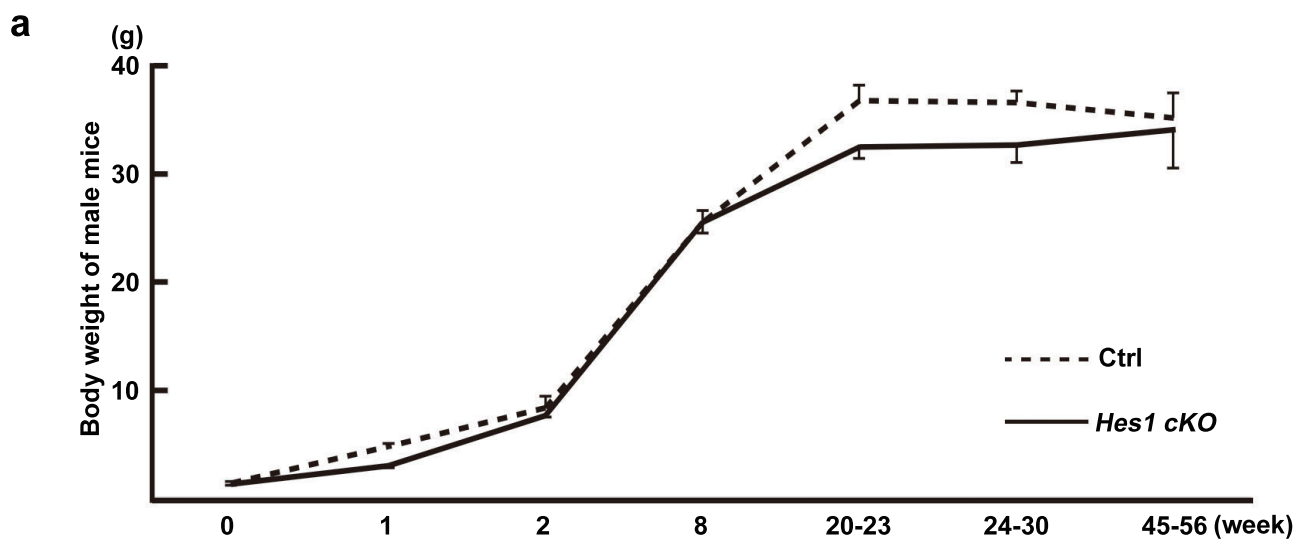
a The protein-coding region of *Ptf1a* was precisely replaced with that of *Cre*, encoding the recombinase Cre. **b** *Hes1* is floxed by *loxP* and conditionally knocked out in the Cre recombinase promoter site. **c** *Rosa-26*, *Lox-STOP-Lox*, and *LacZ* construct. *LacZ* expresses in the Cre recombinase promoter site. **d** *Rosa-26*, *Lox-STOP-Lox*, and *NICD* construct. *NICD* expresses in the Cre recombinase promoter site. **e** Cre recombinase is under the transcriptional control of the mouse *Pdx1* promoter.



Supplementary Figure S2

Hes1 immunohistochemistry

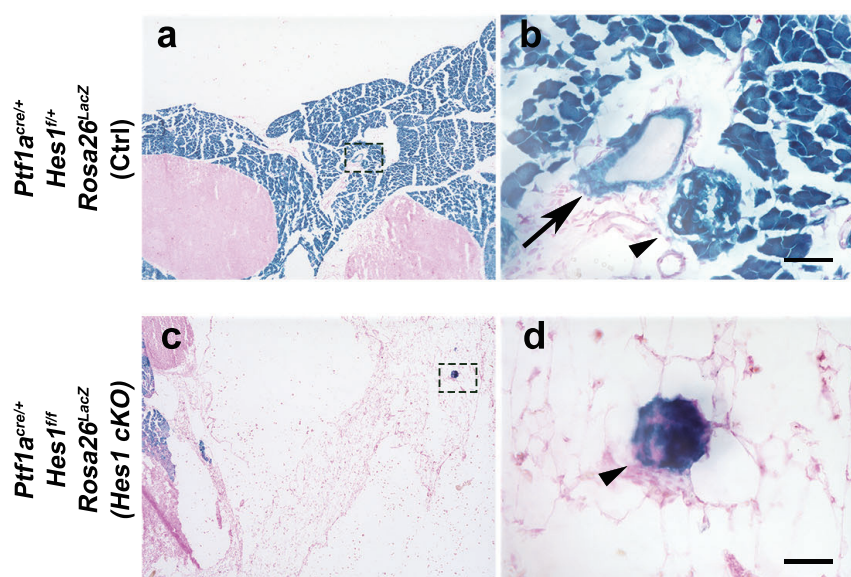
Hes1 expression in pancreatic tissue was analyzed by immunohistochemistry in *Hes1 cKO* (*Ptf1a^{cre/+};Hes1^{ff};Rosa26^{LacZ}*) and control (Ctrl, *Ptf1a^{+/+};Hes1^{ff};Rosa26^{LacZ}*) mice at P0. Hes1 expression was deleted in *Hes1 cKO* mice (b), while it was positive in the ducts and centroacinar cells of control mice (a).



Supplementary Figure S3

Changes in body weight and blood glucose level

Changes in body weight (**a**) and blood glucose level (**b**) after birth in *Hes1 cKO* (*Ptfla^{cre/+};Hes1^{flf};Rosa26^{LacZ}*) and control (Ctrl, *Ptfla^{+/+};Hes1^{flf};Rosa26^{LacZ}*) mice. The median and the standard error of mean are shown.

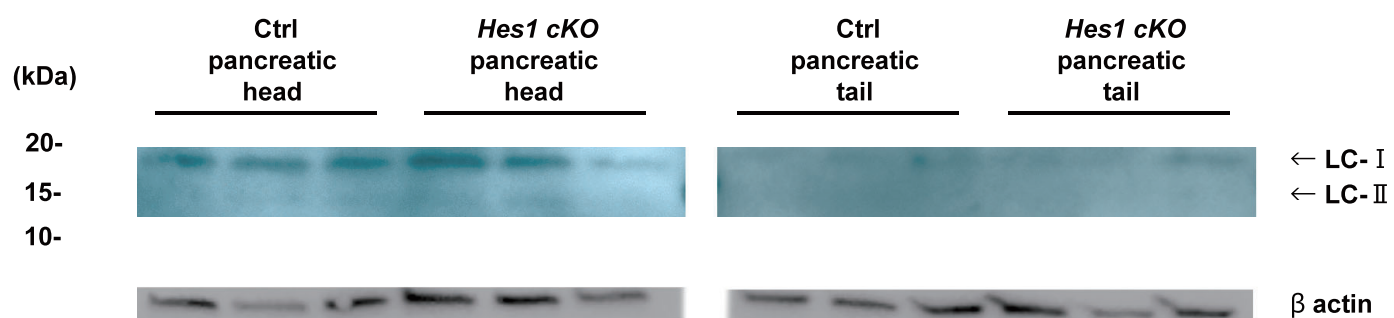


Supplementary Figure S4

***Hes1* knockout in *Ptf1a*-positive progenitor cells.**

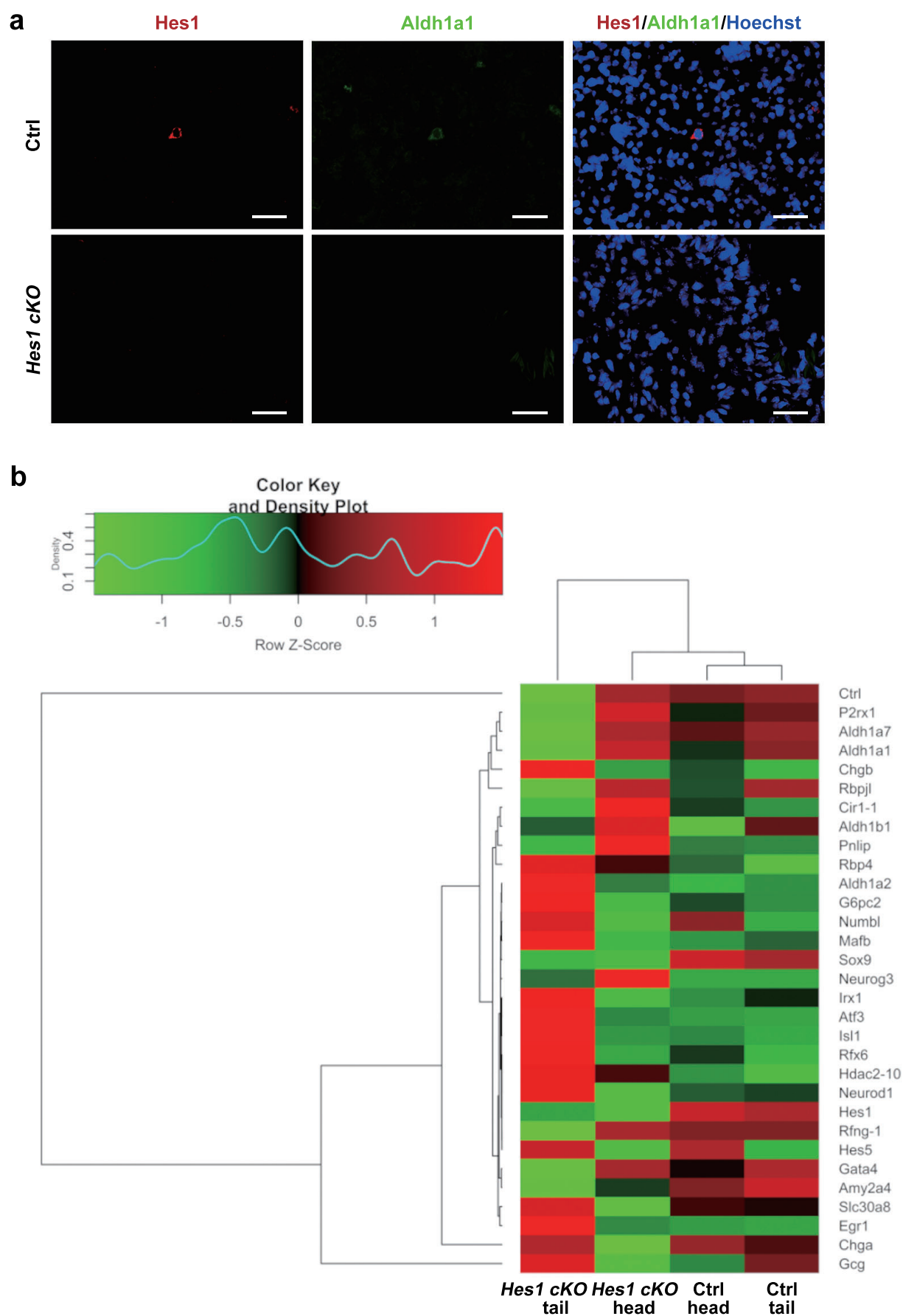
X-gal staining of *Hes1* conditional knockout mice ($Ptf1a^{cre/+}; Hes1^{fl/fl}; Rosa26^{LacZ}$) and control mice ($Ptf1a^{cre/+}; Hes1^{fl/+}; Rosa26^{LacZ}$) at 4 weeks of age. **b** and **d** are a magnification of the broken line in **a** and **c**. Arrow head: islet, arrow: duct. Scale bars: 50 μ m (**b**, **d**).

a



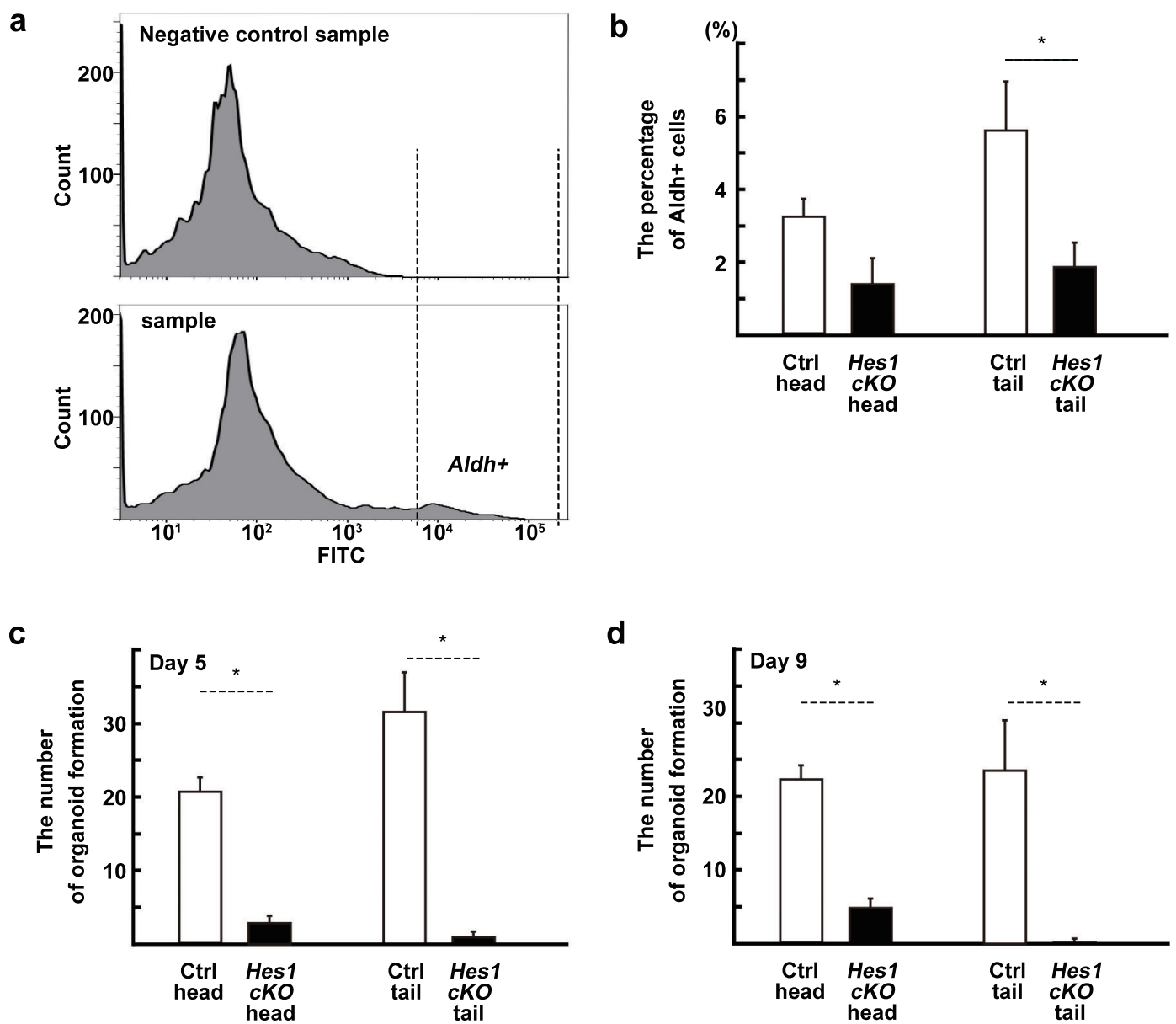
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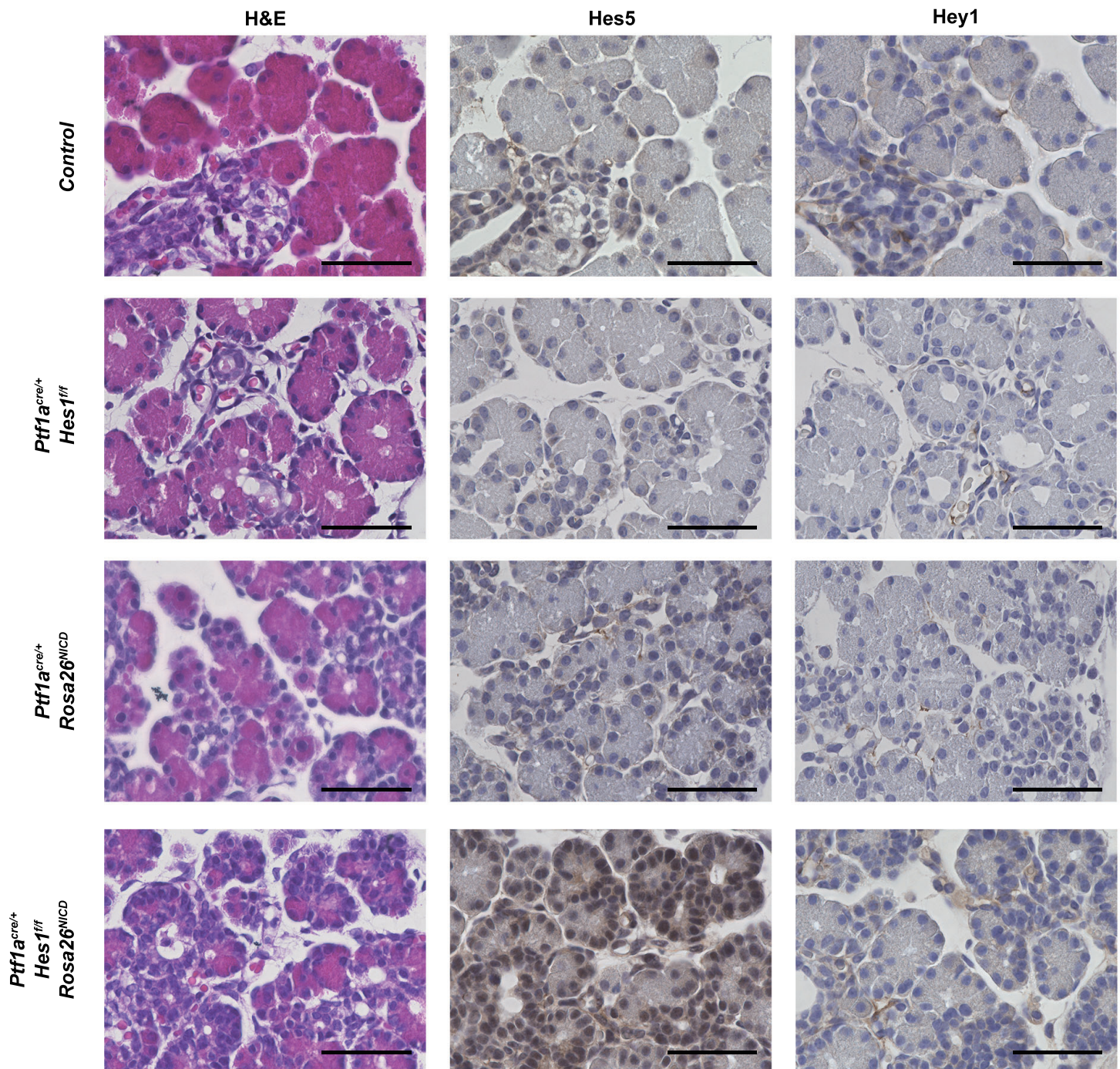
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Supplementary Figure S7

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a**Supplementary Figure S8**

a H&E staining and immunohistochemical staining for Hes5 and Hey1 of control mice, *Ptfla*^{cre/+}; *Hes1*^{f/f} mice (*cKO*), *Ptfla*^{cre/+}; *Rosa26*^{NICD} mice, and *Ptfla*^{cre/+}; *Hes1*^{f/f}; *Rosa26*^{NICD} mice at P0. Marked increase of Hes5 expression was observed in *Ptfla*^{cre/+}; *Hes1*^{f/f}; *Rosa26*^{NICD} mice. Scale bars: 50 μ m.

Antigen	Species	Source	Catalog number	Dilution	Antigen retrieval
ALDH1A1	Rabbit	Abcam	ab52492	1:100	autoclave with ph6.0 citrate buffer
Amylase	Rabbit	Abcam	ab21156	1:300	microwave with ph6.0 citrate buffer
Cleaved caspase-3	Rabbit	Cell signaling	9664S	1:400	microwave with ph6.0 citrate buffer
Cytokeratin	Mouse	Dako	M3515	1:100	microwave with ph6.0 citrate buffer
Glucagon	Rabbit	Dako	A0565	1:300	none
Hes1	Rabbit	Gifted from Mr. Sudo (Toray, Japan)		1:5000	autoclave with ph6.0 citrate buffer
Hes1	Rabbit	Santa Cruz	SC-25392	1:400	autoclave with ph6.0 citrate buffer
Hes5	Rabbit	Abcam	ab194111	1:500	microwave with ph9.0 Tris / EDTA buffer
Heyl	Rabbit	Abcam	ab22614	1:200	microwave with ph9.0 Tris / EDTA buffer
Insulin	Guinea pig	Dako	A0564	1:400	none
Ki-67	Rabbit	Dako	M7249	1:400	microwave with ph6.0 citrate buffer

Supplementary Table 1.

Primary antibodies used in this study.