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

2

3 **Short title:** Notch in pancreas maturation

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5 Katsutoshi Kuriyama[1], Yuzo Kodama[1,2], Masahiro Shiokawa[1], Yoshihiro Nishikawa[1], Saiko

6 Marui[1], Takeshi Kuwada[1], Yuko Sogabe[1], Nobuyuki Kakiuchi[1], Teruko Tomono[1], Tomoaki

7 Matsumori[1], Atsushi Mima[1], Toshihiro Morita[1], Tatsuki Ueda[1], Motoyuki Tsuda[1], Yuki Yamauchi[1],

8 Yojiro Sakuma[1], Yuji Ota[1], Takahisa Maruno[1], Norimitsu Uza[1], Ryoichiro Kageyama[3], Tsutomu

9 Chiba[1,4], and Hiroshi Seno[1]

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11 [1]Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine,

12 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto, 606-8507, Japan.

13 [2]Department of Gastroenterology, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-

14 cho, Chuo-ku, Kobe, Hyogo, 650-0017, Japan.

15 [3]Institute for Frontier Life and Medical Sciences, Kyoto University, 53 Shogoin Kawahara-cho,

16 Sakyo-ku, Kyoto, 606-8507, Japan

17 [4]Kansai Electric Power Hospital, 2-1-7 Fukushima, Fukushima-ku, Osaka, 553-0003, Japan.

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19 Correspondence: Yuzo Kodama

20 Department of Gastroenterology, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-

21 cho, Chuo-ku, Kobe, Hyogo 650-0017, Japan.

22 E-mail: kodama@med.kobe-u.ac.jp

23 Phone: +81-78-382-6305

24 Fax: +81- 78-382-6309

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$^{f/f}$* and *Ptf1a$^{cre/+}$;Rosa26^{NICD}* mice showed normal pancreatic development at P0. However, exocrine tissue of the pancreatic tail in *Ptf1a$^{cre/+}$;Hes1$^{f/f}$* 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$^{f/f}$;Rosa26^{NICD}* mice, suggesting compensation by a Notch signaling pathway other than Hes1. Conversely, *Pdx1-Cre;Hes1$^{f/f}$* 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.

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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 Ptf1a [1]. Ptf1a 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 Ptf1a 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 Ptf1a and Ngn3. In contrast to these functions at embryonic day 8.5–9.0, Notch signaling also plays a role after Pdx1 or Ptf1a 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 Ptf1a-expresing cells revealed no apparent phenotype at birth, although pancreatic atrophy and fatty metaplasia were observed in adults [7]. Thus, the role

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

73 controversial.

74 Rather than embryonic Pdx1-positive and Ptf1a-positive progenitor cells, centroacinar cells are

75 considered one of the candidates for adult pancreatic tissue stem cells. Centroacinar cells are

76 located at the junction between peripheral acinar cells and the adjacent ductal epithelium, and are

77 characterized by high levels of ALDH1 enzymatic activity and marked Sca1, Sdf1, c-Met, Nestin,

78 and Sox9 expression. These are markers that have been previously associated with progenitor

79 populations in the embryonic pancreas and other tissues [8]. Cre-based lineage tracing showed that

80 adult Sox9-positive duct and/or centroacinar cells continuously supplied acinar cells, suggesting

81 that centroacinar cells have characteristics of stem cells [9]. Interestingly, Hes1 was reported to be

82 expressed in centroacinar cells [10], and the abrogation of both Notch receptor ligands, Delta-like

83 1 and Jagged1, led to the loss of centroacinar cells [11]. These observations suggest that Notch

84 signaling is highly related to centroacinar cell formation.

85 In the current study, to elucidate the role of Notch signaling in Pdx1-positive or Ptf1a-positive

86 pancreatic progenitor cells, we analyzed the phenotype of mice with *Hes1* gene knockout and/or

87 NICD transgenic expression in Pdx1- or Ptf1a-expressing cells. We found that in pancreatic

88 progenitor cells, Notch signaling plays an essential role in postnatal exocrine tissue development,

89 presumably in part via centroacinar cell formation.

90

91 **Methods**

92 **Mice**

93 Experimental animals were generated by crossing *Ptf1a$^{cre/+}$* [1] or *Pdx1-Cre* [12] (supplied by Y

94 Kawaguchi, Kyoto University, Kyoto, Japan) with *Hes1$^{f/f}$* [13] and/or *Rosa26^{NICD}* [6] (supplied by

95 D Melton, Harvard University, MA, USA). To examine Cre expression in the pancreas, we crossed

96   these mice with *Rosa26^LacZ* mice. Each mouse strain is shown in Supplementary Figure 1. All mice

97   were maintained on a mixed genetic background. To compare the phenotypes of genetically

98   engineered mice and controls, we used 3 to 7 littermate mice from each group and age group. No

99   selection for a specific sex was performed in this study except for body weight. All animal

100   experiments were approved by the Kyoto University Graduate School and Faculty of Medicine

101   Ethics Committee.

102

103   **Histological study**

104   For histological studies, tissue samples were excised from the mice, flushed with phosphate-

105   buffered saline (PBS), fixed overnight in 10% neutral phosphate-buffered formalin, and embedded

106   in paraffin. Sections of 5-μm thickness were stained with H&E, and immunohistochemistry was

107   carried out. Tissue samples were also frozen immediately in liquid nitrogen for X-gal staining and

108   immunofluorescence studies. Bright-field and fluorescence images were captured with an

109   Olympus BX53-33FL microscope (Olympus, Tokyo, Japan) or Keyence BZ-X710 microscope

110   (Keyence, Osaka, Japan). The captured images were analyzed with Adobe Photoshop software.

111

112   **Immunohistochemistry and immunofluorescence**

113   Immunohistochemical study was performed using a standard method as previously reported [14].

114   Primary antibodies used in this study are listed in Supplementary Table 1. Staining signals were

115   detected using a Vectorstain Elite ABC Kit (Vector Laboratories, Burlingame, CA) and a

116   peroxidase substrate kit, DAB (Vector Laboratories). The peroxidase reaction was carried out with

117   Liquid DAB+ Substrate Chromogen System (Dako). Finally, slides were counterstained with

118   hematoxylin (Wako, Japan).

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

120 used. The secondary antibodies were Alexa Fluor 594 anti-rabbit IgG 1:100 (A-21207, Thermo

121 Fisher Scientific, Waltham, MA, USA), Alexa Fluor 488 anti-mouse IgG 1:100 (A-21200, Thermo

122 Fisher Scientific), Dylight 488 anti-guinea pig IgG 1:100 (ab96959, Abcam plc, Cambridge, UK),

123 and Alexa Fluor 488 anti-rabbit IgG 1:100 (A-11008, Thermo Fisher Scientific). To assess co-

124 localization of Hes1 and Aldh1a1, frozen sections were stained with anti-Hes1 antibody with Alexa

125 Fluor 594 (ab207048, Abcam) and anti-Aldh1a1 with Alexa Fluor 488 (ab195254, abcam).

126

127 **X-gal staining**

128 We performed X-gal staining as previously described [1]. Briefly, freshly isolated pancreas was

129 incubated in ice-cold fixative solution (PBS containing 4% paraformaldehyde, 5 mmol/L EGTA,

130 2 mmol/L MgCl2, 0.2% glutaraldehyde, and 0.02% NP-40) for 1 hour at 4 °C. After washing twice

131 in PBS for 20 minutes, tissues were incubated with LacZ substrate (PBS containing 5 mmol/L

132 $K_3Fe(CN)_6$, 5 mmol/L $K_4Fe(CN)_6$, 2 mmol/L $MgCl_2$, 0.02% NP-40, 0.1% sodium deoxycholate,

133 and 1 mg/mL X-galactosidase) overnight at room temperature. After washing twice in PBS for 20

134 minutes, tissues were fixed overnight in 4% paraformaldehyde in PBS at 4 °C. Paraformaldehyde

135 was removed, and the stained tissues were transferred to tissue cassettes.

136

137 **Evaluation of Ki67-positive cells, cleaved casepase3-positive cells, and centroacinar cells**

138 We compared the number of Ki67-positive cells and cleaved caspase3-positive cells between

139 $Ptf1a^{cre/+};Hes1^{f/f}$ mice and control mice at P0. To count Ki67-positive cells, we used two fields of

140 200-fold magnification randomly selected in both the head and tail of the pancreas. For cleaved

141 caspase3-positive cells, we counted all positive cells in both the head and tail of the pancreas in a

142 whole slice. We measured the area of the pancreas using ImageJ (http://imagej.nih.gov/ij/). To

143 compare the ratio of acini without centroacinar cells, we counted the number of acini with vacant

144　space in the center of acini and the number of normal acini in *Ptf1a*$^{cre/+}$*;Hes1*$^{f/f}$ mice and control

145　mice at P0.

146

147　**Western blot analysis**

148　To evaluate autophagy in the pancreatic tissue, we performed Western blot analysis for LC3-I and

149　its lapidated form LC3-II, which are widely used to quantify autophagy [15]. Protein extracts from

150　both pancreatic tissue of *Ptf1a*$^{cre/+}$*;Hes1*$^{f/f}$ mice and control mice at P4 were boiled in Laemmli

151　sample buffer with 2.5% mercaptoethanol, fractionated on 4–15% sodium dodecyl sulfate

152　polyacrylamide gels (456-1806, Bio-Rad, Tokyo, Japan), and transferred to nitrocellulose

153　membranes according to standard protocols. After blocking with 5% dry skim milk, the blots were

154　incubated with primary antibodies. The primary antibodies were anti-LC3 (1:1000; PM036, MBL,

155　Japan) and anti-β actin (1:10000; ab6276, Abcam). The secondary antibodies were peroxidase-

156　conjugated anti-mouse IgG (1:4000; NA931, GE Healthcare, Chicago, IL, USA) or anti-rabbit IgG

157　(1:10 000; 31458, Thermo Fisher Scientific).

158

159　**Microarray analysis**

160　We sacrificed a *Ptf1a*$^{cre/+}$*;Hes1*$^{f/f}$ mouse and a control mouse (*Ptf1a*$^{cre/+}$*; Hes1*$^{+/+}$) at postnatal day

161　7 and collected pancreatic head and tail tissues from each mouse. Total RNA was extracted from

162　the pancreatic tissue collected in RNALater (Ambion) using RNeasy kit (QIAGEN, Hilden,

163　Germany) according to the manufacturer's instructions. RNA Integrity Number (RIN) of extracted

164　RNA form pancreatic head of control mouse, tail of a control mouse, head of a *Ptf1a*$^{cre/+}$*;Hes1*$^{f/f}$

165　mouse, and tail of a *Ptf1a*$^{cre/+}$*;Hes1*$^{f/f}$ mouse were 9.2, 9.5, 9.2, and 9.1, respectively. RNA was

166　analyzed by Agilent's microarray.

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

168   article [16]. We then selected genes whose expression in the *Ptf1a^{cre/+};Hes1^{f/f}* mouse were more

169   than twice or less than half of that of the control. We analyzed these genes and constructed

170   heatmaps with R (R Core Team (2016). R: A language and environment for statistical computing.

171   R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.)

172

173   **Quantitative RT-PCR**

174   Total RNA was extracted from the pancreatic tissue collected in RNALater (Ambion) using

175   RNeasy kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. For

176   complementary DNA synthesis, 1 µg total RNA was reverse transcribed using ReverTra Ace®

177   qPCR RT Master Mix (Toyobo, Osaka, Japan) and subjected to quantitative RT-PCR. Quantitative

178   RT-PCR was performed by using the LightCycler® system (Roche, Switzerland). The mRNA

179   expression of specific genes was measured using FastStart Universal SYBR Green Master (Roche,

180   Switzerland). RNA levels were normalized to the level of the housekeeping gene GAPDH and

181   calculated as delta-delta threshold cycle (ΔCT). The primer sequences used for mouse ALDH1A1

182   and GAPDH were as follows: mouse ALDH1A1, 5′-GGGCTGACAAGATTCATGGT-3′

183   (forward) and 5′-GGAAAATTCCAGGGGATGAT-3′ (reverse); and GAPDH, 5′-

184   AGGTCGGTGTGAACGGATTTG-3′ (forward) and 5′- TGTAGACCATGTAGTTGAGGTCA -

185   3′ (reverse). All quantitative RT-PCR analyses were performed in triplicate.

186

187   **FACS isolation of Aldh-positive cells**

188   FACS isolation of Aldh-positive cell was performed using just modified previously described

189   method [17]. Pancreas of *Ptf1a^{cre/+};Hes1^{f/f}* mice and control mice were dissected at P7, washed in

190   iced HBSS solution (Thermo Fisher Scientific, Waltham, MA, USA) and minced into small pieces.

191   The minced pancreas was digested by 1 mg/ml collagenase D (Roche, Switzerland) dissolved in

192   DMEM (Gibco) and supplemented with 2 U/ml DNase I (Promega, Madison, WI, USA) and 1%

193   BSA (Wako) for twenty minutes on a shaker at 37°C. Cells were pipetted through a 1 mL pipette

194   tip every 5 minutes. The reaction was stopped by adding ice cold DMEM supplemented with 5%

195   FBS. Cells were then passed through a 100, 70, and 40 μm cell strainer sequentially, centrifuged

196   at 300 g, 4°C for 3 minutes, re-suspended with DMEM, and counted. Cell suspension were treated

197   with Aldefluor reagent (Stem Cell Technologies, Vancouver, Canada) in the dark at 37 °C on a

198   shaker for 30 minutes. Diethylaminobenzaldehyde (DEAB), an inhibitor of Aldh activity was also

199   added for negative control. Following the Aldefluor reaction, cells were centrifuged and re-

200   suspended with DMEM. FACS Aria II (BD, Franklin Lakes, NJ, USA) was used for FACS sorting.

201   FSC and SSC were used for exclusion of cell debris. Aldh-positive cells were isolated by Aldefluor

202   activity (FITC channel) and counted.

203

204   **Three-dimensional organoid culture of adult pancreatic cells**

205   Cells re-suspended with DMEM as described above were seeded in 25 μL of the growth factor-

206   reduced Matrigel (BD) in a 48-well plate (Corning). In each well, 1000 cells were seeded. After

207   the gelation of the Matrigel for 10 minutes at 37°C in the incubator, culture medium was added.

208   Culture medium was based on AdDMEM/F12 (Thermo Fischer Scientific), supplemented with 2%

209   B27 (Thermo Fischer Scientific), 1% penicillin/streptomycin (Thermo Fischer Scientific), 1 mM

210   HEPES (Thermo Fischer Scientific), 1% Glutamax (Thermo Fischer Scientific), 1.25 mM N-

211   Acetylcysteine (Sigma), 10 nM gastrin (Sigma), 50 ng/ml EGF (Peprotech), 250 ng/ml Rspondin1

212   (R&D Systems), 100 ng/ml Noggin (Peprotech), 100 ng/ml FGF10 (Peprotech), 10 mM

213   Nicotinamide (Sigma), 500 nM A83-01 (Tocris), and 10 μM Y27632 (Tocris Bioscience). Culture

214   medium was changed every 3 days. The number and size of organoids were counted 5 days and 9

215   days after seeding.

216

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.

221

222

Results

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

To elucidate the role of *Hes1* in Ptf1a-positive progenitor cells during pancreatic development,

we generated conditional *Hes1* knockout mice by crossing *Ptf1a-Cre* mice (*Ptf1a$^{cre/+}$*) [1], *Hes1*

floxed mice (*Hes1$^{f/f}$*) [13], and *Rosa26^{LacZ}* mice (Fig. S1a-c). Conditional *Hes1* knockout (*Hes1*

cKO, Ptf1a$^{cre/+}$;Hes1$^{f/f}$;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   (*Ptf1a$^{+/+}$;Hes1$^{f/f}$;Rosa26^{LacZ}*,   *Ptf1a$^{cre/+}$;Hes1$^{+/+}$;Rosa26^{LacZ}*   or

Ptf1a$^{cre/+}$;Hes1$^{f/+}$;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 *Ptf1a$^{cre/+}$;Hes1$^{f/f}$;Rosa26^{LacZ}* mice at P0.

240

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

To further analyze the role of Notch signaling in Ptf1a-positive progenitor cells in pancreatic development, we next generated *Ptf1a$^{cre/+}$;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 *Ptf1a$^{cre/+}$;Rosa26^{NICD}* and control (*Ptf1a$^{+/+}$; Rosa26^{NICD}*) mice (Fig. 2a) at P0. Microscopically, there was no difference in the development of exocrine and endocrine tissues between *Ptf1a$^{cre/+}$;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 *Ptf1a$^{cre/+}$;Rosa26^{NICD}* mice as compared to control mice. These results indicated that both *Hes1* knockout and *Notch1* overexpression in Ptf1a-positive progenitor cells do not affect the fetal development of pancreatic tissue.

Hes1 knockout in Ptf1a-positive progenitor cells results in exocrine atrophy of the adult pancreatic tail

As shown above, *Hes1 cKO* (*Ptf1a$^{cre/+}$;Hes1$^{f/f}$;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

264   at 4 weeks of age for amylase and cytokeratin. This confirmed remarkable loss of acinar cells,

265   abnormal alignment of duct cells, and prominent deposition of fat tissue in the pancreatic tail of

266   *Hes1 cKO* mice compared to that of control mice or the pancreatic head of *Hes1 cKO* mice (Fig.

267   3b). In contrast to the marked impairment of exocrine cells, normal endocrine cell development

268   was observed in both the pancreatic head and tail of *Hes1 cKO* mice when analyzed via

269   immunohistochemistry for insulin and glucagon (Fig. 3b).

270   To investigate the cause of atrophic changes in the pancreatic tail of *Hes1 cKO* mice, apoptosis

271   and cell growth levels were evaluated by immunohistochemistry for cleaved caspase3 and Ki67,

272   respectively, at age P0 (Fig. 4a). Although there was no difference in histology (Fig. 1) and the

273   Ki67 index (Fig. 4b) between *Hes1 cKO* and control mice in both the pancreatic head and tail,

274   significantly more apoptotic cells were detected in *Hes1 cKO* than in control mice, especially in

275   the pancreatic tail (Fig 4c). Western blot analysis revealed expression of LC3-Ⅰ, but failed to

276   detect its lipidated form LC3-Ⅱ in both pancreatic head and tail of *Hes1 cKO* mice and control

277   mice (Fig. S5), suggesting a limited involvement of autophagy. These results showed that

278   conditional *Hes1* knockout in Ptf1a-positive progenitor cells led to atrophic changes in the

279   pancreatic tail during the postnatal growth and maturation process, partly due to exocrine cell

280   apoptosis.

281

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

283   To further investigate the cause of atrophic changes in the pancreatic tail of *Hes1 cKO* mice, we

284   focused on centroacinar cells located in the center of the pancreatic acinus. These are considered

285   to be one of the adult tissue stem cell candidates of pancreatic exocrine tissue. As previously

286   reported, centroacinar cells with Hes1 and Aldh1 expression were observed in the pancreatic tail

287   of control *(Ptf1a^{cre/+};Hes1^{f/+})* mice at P0 (Fig 5a). Co-localization of Hes1 and Aldh1a1 was

288   confirmed by immunofluorescence at P3 (Fig. S6a). In contrast, in *Hes1 cKO (Ptf1a^{cre/+};Hes1^{f/f})*

289   mice, there were a substantial number of acini with vacant space in the center and without Hes1-

290   and Aldh1-positive centroacinar cells (Fig 5a). The ratio of acini without centroacinar cells was

291   significantly higher in the pancreatic head and tail of *Hes1 cKO* mice than in control mice at P0

292   (Fig 5b). At P3, this trend of centroacinar cell reduction was prolonged in the pancreatic tail,

293   whereas this trend was disappeared in pancreatic head (Fig 5c). At P7, microarray analysis using

294   tissue from the pancreatic tail of *Hes1 cKO* mice revealed distinct gene expression patterns from

295   that of the pancreatic head of *Hes1 cKO* mice or the pancreatic head/tail of control mice (Fig. S6b).

296   Notably, the pancreatic tail of *Hes1 cKO* mice showed decreased exocrine-related gene expression

297   (Fig. 5d) and increased endocrine-related gene expression (Fig. 5e). Lower *Aldh1*-related gene

298   expression and significantly reduced *Aldh1a1* gene expression were demonstrated by microarray

299   (Fig. 5f) and qPCR analysis (Fig. 5g), respectively, in the pancreatic tail of *Hes1 cKO* mice

300   compared to that of control mice. This confirmed the previously observed reduction in centroacinar

301   cells in the pancreatic tail of *Hes1 cKO* mice (Fig. 5a-c). The presence of fewer centroacinar cells

302   in the pancreatic tail of *Hes1 cKO* mice in the neonatal state may explain pancreatic atrophy being

303   observed only in the pancreatic tail.

304   To further assess the mechanisms for fewer centroacinar cells and subsequent impaired

305   development in *Hes1 cKO* mice, we isolated pancreatic cells from *Hes1 cKO* and control mice and

306   evaluated their stem cell features by FACS analysis and organoid culture. As a result, confirming

307   the altered *Aldh1*-related gene expression (Fig. 5f), FACS analysis revealed that pancreatic tail of

308   *Hes1 cKO* mice had significantly lower number of Aldh-positive cells than control mice at P7 (Fig.

309   S7a, b). Furthermore, pancreatic cells from *Hes1 cKO* mice formed significantly lower number of

310   organoids than those from control mice (Fig. S7c, d). These observations suggest that loss of Hes1

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

312

313   **Transgenic *Notch1* expression compensates for the *Hes1* knockout phenotype in Ptf1a-**

314   **positive progenitor cells**

315   To assess the role of the Notch signaling pathway in postnatal pancreatic tail development, we

316   analyzed *Ptf1a^{cre/+};Hes1^{f/f};Rosa26^{NICD}* mice. In these mice, downstream molecules of Notch

317   signaling, other than Hes1, were activated in Ptf1a-positive progenitor cells. As a result, some acini

318   showed a vacant space without centroacinar cells in *Ptf1a^{cre/+};Hes1^{f/f};Rosa26^{NICD}* mice at P0 (Fig.

319   6a), as observed in *Ptf1a^{cre/+};Hes1^{f/f}* mice (Fig. 5a). However, even with *Hes1* gene deletion,

320   atrophic changes in the pancreatic tail were not observed in *Ptf1a^{cre/+};Hes1^{f/f};Rosa26^{NICD}* mice at

321   16 weeks of age. H&E staining, immunohistochemistry for amylase and cytokeratin, and

322   immunofluorescence staining for insulin and glucagon showed normal development of acinar cells,

323   duct cells, α cells, and β cells in *Ptf1a^{cre/+};Hes1^{f/f};Rosa26^{NICD}* mice at 16 weeks (Fig. 6b,c).

324   These results suggested that activation of the Notch signaling pathway, other than Hes1, could

325   compensate for defective *Hes1* in pancreatic tail development in the postnatal to adult state. To

326   further analyze this compensatory mechanism, we assessed the expressions of Hes5 and Hey1,

327   which are major downstream molecules of Notch signaling other than Hes1, in pancreatic tissues

328   of *Ptf1a^{cre/+};Hes1^{f/f};Rosa26^{NICD}* mice, *Ptf1a^{cre/+};Rosa26^{NICD}* mice, *Ptf1a^{cre/+};Hes1^{f/f}* (*cKO*) mice,

329   and control mice at P0 by immunohistochemistry. As a result, a marked increase of Hes5

330   expression was observed in *Ptf1a^{cre/+};Hes1^{f/f};Rosa26^{NICD}* mice compared to *Ptf1a^{cre/+};Rosa26^{NICD}*

331   mice, *Ptf1a^{cre/+};Hes1^{f/f}* (*cKO*) mice and control mice, whereas Hey1 expression was similar in all

332   the strains of mice (Fig. S8). These results suggested that Hes5 could be one of the factors

333   contributing the compensation for Hes1 deficiency in pancreatic development in

334   *Ptf1a^{cre/+};Hes1^{f/f};Rosa26^{NICD}* mice.

335

Hes1 knockout in Pdx1-positve 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$^{f/f}$;Rosa26^{LacZ}_). To this end, we generated and analyzed _Pdx1-Cre;Hes1$^{f/f}$;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$^{f/f}$_ mice, normal exocrine/endocrine cells and a substantial number of acini without centroacinar cells were observed in _Pdx1-Cre;Hes1$^{f/f}$;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$^{f/f}$;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$^{f/f}$;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$^{f/f}$;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.

358

359

Discussion

  The importance of Notch signaling in pancreatic embryonic development has been thoroughly studied [3, 4]. However, since systemic *Hes1* deficient mice or *Pdx1-Cre;Rosa26^{NICD}* mice are reported to be embryonic lethal mutants [4, 6] the detailed functions of Notch signaling in Pdx1- or Ptf1a-positive progenitor cells has been mostly unknown. In this study, we analyzed mice with conditional *Hes1* knockout or transgenic NICD expression in Ptf1a/Pdx1-positive progenitor cells. We found that Notch signaling in pancreatic progenitor cells plays an essential role in postnatal exocrine tissue development.

Previous reports have shown that systemic *Hes1* knockout induced accelerated differentiation of pancreatic progenitor cells to endocrine cells, resulting in a reduction in progenitor cells. This suggests the crucial role of Notch/Hes1 signaling in exocrine-endocrine cell fate determination [4]. This function of Notch signaling is still observed in Pdx1-positive progenitor cells, in which exocrine-endocrine differentiation was inhibited by conditional NICD expression [6]. However, in our analysis, both *Ptf1a$^{cre/+}$;Hes1$^{f/f}$* mice and *Ptf1a$^{cre/+}$;Rosa26^{NICD}* mice showed normal embryonic development, and no abnormality was observed in exocrine-endocrine differentiation at birth. Considering that gene recombination is induced in pancreatic progenitor cells by Pdx1-Cre at about 0.5 to 1 day prior to that by Ptf1a-Cre [2], cell fate determination by Notch signaling may have already been completed in Ptf1a-positeve pancreatic progenitor cells.

  In contrast, our analysis showed that *Ptf1a$^{cre/+}$;Hes1$^{f/f}$* mice had atrophic changes in the pancreatic tail by 4 weeks of age due to impaired postnatal development exocrine tissue. This is similar to the observations details in the report by Hidalgo-Sastre et al. [7], From these observations, it is clear that *Hes1* plays a critical role in exocrine tissue maturation after exocrine cell fate determination in Ptf1a-positive progenitor cells. To elucidate the mechanism of impaired postnatal pancreatic

383   exocrine tissue development in *Ptf1a^{cre/+};Hes1^{f/f}* mice, we focused on centroacinar cells. These

384   cells are one of the candidates for adult pancreatic tissue stem cells, and have been reported to

385   express Hes1 [10]. As a result, we found sustained loss of centroacinar cells during the neonatal

386   period in the pancreatic tail, but not in the pancreatic head, in *Ptf1a^{cre/+};Hes1^{f/f}* mice. This

387   phenotype of centroacinar cell loss and acini with vacant space in the center is markedly analogous

388   to that of Notch ligands *Dll1/Jag1* double knockout mice [11], suggesting the role of Notch

389   signaling in centroacinar cell development. Together with our results of increased apoptotic cells

390   in the pancreatic tail of *Ptf1a^{cre/+};Hes1^{f/f}* mice, Notch signaling in pancreatic progenitor cells may

391   contribute to postnatal centroacinar cell development and subsequent exocrine maturation. To

392   confirm this hypothesis, we analyzed *Ptf1a^{cre/+};Hes1^{f/f};Rosa26^{NICD}* mice in which Notch signaling,

393   save for Hes1, is activated. As expected, the impaired postnatal pancreatic exocrine tissue

394   development in *Ptf1a^{cre/+};Hes1^{f/f}* mice was completely rescued by the activation of Notch signaling

395   in *Ptf1a^{cre/+};Hes1^{f/f};Rosa26^{NICD}* mice, suggesting that *Hes/Hey* family members other than *Hes1*

396   may have compensated for the function of *Hes1*. In fact, expression of Hes5 was strongly enhanced

397   in the pancreatic tissue of *Ptf1a^{cre/+};Hes1^{f/f};Rosa26^{NICD}* mice, suggesting that Hes5 may contribute

398   to this compensatory mechanism.

399   We sought to determine when Notch/Hes1 signaling carries out its critical function in postnatal

400   pancreatic progenitor cell maturation after performing its role in exocrine/endocrine cell fate

401   determination at the embryonic stage. We, therefore, analyzed *Pdx1-Cre;Hes1^{f/f}* mice in

402   comparison with *Ptf1a^{cre/+};Hes1^{f/f}* mice. As a result, impaired postnatal exocrine maturation was

403   observed only in the pancreatic tail in *Ptf1a^{cre/+};Hes1^{f/f}* mice. However, this was observed in both

404   the pancreatic head and tail in *Pdx1-Cre;Hes1^{f/f}* mice. We speculate that this phenotypic difference

405   in the pancreatic head between *Ptf1a^{cre/+}* and *Pdx1-Cre* is due to their difference in timing of

406   genetic recombination, as mentioned above [2]. The appearance of this phenotype in *Pdx1-Cre* but

407   not in *Ptf1a$^{cre/+}$* may indicate the importance of Notch signaling activation between Pdx1 and Ptf1a

408   expression in progenitor cells, at least in the pancreatic head. The greater effect by earlier *Hes1*

409   deletion is further confirmed by our previous observation that adult pancreatic tissue is resistant to

410   *Hes1* deletion [14]. Interestingly, unlike *Ptf1a$^{cre/+}$;Hes1$^{f/f}$* mice, pancreatic atrophy was not

411   observed in *Pdx1-Cre;Hes1$^{f/f}$* mice. This is probably because the loss of X-gal positive / *Hes1*

412   knockout cells was replaced by X-gal negative exocrine cells that escaped the recombination of

413   *Hes1* gene.

414   In conclusion, in addition to the previously reported role of exocrine/endocrine cell fate

415   determination, Notch signaling in pancreatic progenitor cells plays an essential role in the postnatal

416   maturation of exocrine tissue, partly through centroacinar cell formation.

417

418

Competing interests

The authors declare that they have no conflict of interest.

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472

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$^{f/f}$; Rosa26^{LacZ})* and

control (Ctrl) *(Ptf1a$^{+/+}$; Hes1$^{f/f}$; Rosa26^{LacZ}, Ptf1a$^{cre/+}$;Hes1$^{+/+}$; Rosa26^{LacZ} or Ptf1a$^{cre/+}$;Hes1$^{f/+}$;*

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

µm.

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 µm (**a**), 50 µm (**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$^{f/f}$;Rosa26^{LacZ})*

and control *(Ptf1a$^{cre/+}$;Hes1$^{f/+}$;Rosa26^{LacZ})* mice at indicated weeks of age. *Hes1 cKO* mice showed

497 atrophic changes in the pancreatic tail compared to control mice. **b** H&E staining and

498 immunohistochemical staining for amylase, cytokeratin, insulin, and glucagon in *Hes1 cKO* mice

499 and control mice at 4 weeks of age. Impaired development of exocrine cells and fat replacement

500 was found in *Hes1 cKO* mice, whereas no change was observed in endocrine cells. Scale bars: 50

501 μm (**b**).

502

503 **Fig. 4**

504 *Hes1* **knockout in Ptf1a-positive progenitor cells induced more apoptotic cells in the**

505 **pancreatic tail.**

506 **a** Immunohistochemical analysis of Ki67 and cleaved caspase3 in the pancreatic head and tail in

507 *Hes1 cKO* (*Ptf1a$^{cre/+}$;Hes1$^{f/f}$*) and control (*Ptf1a$^{cre/+}$;Hes1$^{f/+}$*) mice at age P0. Representative

508 immunohistochemical images (**a**) and their graphic representation (**b, c**). Arrow heads show

509 apoptotic cells in pancreas. Arrows show apoptotic cells in duodenum (positive control).

510 Significantly more apoptotic cells were detected in the pancreatic tail in *Hes1* cKO mice than in

511 control mice (**a, c**), whereas there was no difference in the number of Ki67-positive cells (**a, b**).

512 Scale bars: 50 μm (**a**, Ki67 and Cleaved Caspase3 of pancreatic tail), 100 μm (**a**, Cleaved Caspase3

513 of pancreatic head). *P < 0.05.

514

515 **Fig. 5**

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

517 **a** H&E staining and immunohistochemical images of Hes1 and ALDH1A1 in *Hes1 cKO*

518 (*Ptf1a$^{cre/+}$;Hes1$^{f/f}$*) and control (Ctrl, *Ptf1a$^{cre/+}$;Hes1$^{f/+}$*) mice at P0. Magnifications of the broken

519 line are shown. Centroacinar cells expressed Hes1 and ALDH1A1 in control mice, whereas there

520 was a vacant space in the center of acini of *Hes1 cKO* mice. **b, c** The ratio of acini without

521 centroacinar cells at P0 (**b**) and P3 (**c**) in the pancreatic head and tail of *Hes1 cKO* and control

522 mice are shown. The ratio of acini without centroacinar cells was significantly higher in *Hes1 cKO*

523 than in control mice at P0 (**b**). Centroacinar cell reduction was still observed in the pancreatic tail

524 of *Hes1 cKO* mice at P3 (**c**). **d-f**: Microarray analysis of pancreatic head and tail tissue in *Hes1*

525 *cKO* and control mice at P7. Expression of exocrine-related genes was reduced in the pancreatic

526 tail of *Hes1 cKO* mice (**d**), whereas expression of endocrine-related genes was elevated (**e**)

527 compared to in the control group or head of *Hes1 cKO* mice. Expression of Aldh1, a centroacinar

528 cell marker, was lower in the pancreatic tail of *Hes1 cKO* mice (**f**). **g** Quantitative analysis of

529 Aldh1a1 mRNA expression in the pancreatic head and tail of *Hes1 cKO* and control mice. Scale

530 bars: 50 μm (**a**, H&E), 15 μm (**a**, ALDH1A1). *P < 0.05.

531

532 **Fig. 6**

533 **Transgenic *Notch1* expression compensates for the *Hes1* knockout phenotype in Ptf1a-**

534 **positive progenitor cells.**

535 Analysis of $Ptf1a^{cre/+}$; $Hes1^{f/f}$; $Rosa26^{NICD}$ mice and control ($Ptf1a^{+/+}$; $Hes1^{f/f}$; $Rosa26^{NICD}$) at P0

536 (**a**) and at 16 weeks of age (**b, c**). **a** H&E staining showed almost normal pancreatic development

537 in $Ptf1a^{cre/+}$; $Hes1^{f/f}$; $Rosa26^{NICD}$ mice at P0, although some acini showed a vacant space in the

538 center without centroacinar cells. **b, c** At 16 weeks, H&E staining, immunohistochemistry of

539 amylase and cytokeratin, and immunofluorescent staining for insulin and glucagon showed normal

540 pancreatic tissue development in $Ptf1a^{cre/+}$; $Hes1^{f/f}$; $Rosa26^{NICD}$ mice compared to $Ptf1a^{+/+}$; $Hes1^{f/f}$;

541 $Rosa26^{NICD}$ mice. Scale bars: 50 μm (**a-c**).

542

543 **Fig. 7**

544 ***Hes1* knockout in Pdx1-positve progenitor cells induces impaired exocrine development in**

545 **the pancreatic head and tail.**

546 **a** Analysis of *Pdx1-Cre;Hes1^f/f* mice and control *(Hes1^f/f)* mice at P0. Hematoxylin and eosin

547 (H&E) staining showed normal exocrine/endocrine cells and some acini without centroacinar cells.

548 **b** The ratio of acini without centroacinar cells was higher in *Pdx1-Cre;Hes1^f/f* than control mice at

549 P0. **c** Time-course analysis of *Pdx1-Cre;Hes1^f/f;Rosa26^LacZ* and control *(Pdx1-*

550 *Cre;Hes1^f/+;Rosa26^LacZ)* mice by X-gal staining at indicated weeks of age. Broken lines show the

551 pancreas. X-gal staining showed gradual reduction of exocrine cells in *Pdx1-cre;Hes1^f/f;Rosa26^LacZ*

552 mice. **d** Microscopic image of X-gal staining of pancreatic tissues in *Pdx1-Cre;Hes1^f/f;Rosa26^LacZ*

553 and control *(Pdx1-Cre;Hes1^f/+;Rosa26^LacZ)* mice at P0 and 8 weeks of age. Only endocrine cells

554 and a few exocrine cells were stained blue, and pancreatic tissue was replaced by non-stained

555 exocrine cells in both the pancreatic head and tail at 8 weeks of age. Scale bars: 50 μm (**a, d**), 1000

556 μm (**a**). An arrow head shows an islet (**d**).

557

558

559 **Supplementary Figure S1**

560 **Mouse strains**

561 **a** The protein-coding region of *Ptf1a* was precisely replaced with that of *Cre*, encoding the

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

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

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

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

566 mouse *Pdx1* promoter.

567

568 **Supplementary Figure S2**

Hes1 immunohistochemistry

Hes1 expression in pancreatic tissue was analyzed by immunohistochemistry in *Hes1 cKO* (*Ptf1a$^{cre/+}$;Hes1$^{f/f}$;Rosa26^{LacZ}*) and control (Ctrl, *Ptf1a$^{+/+}$;Hes1$^{f/f}$;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* (*Ptf1a$^{cre/+}$;Hes1$^{f/f}$;Rosa26^{LacZ}*) and control (Ctrl, *Ptf1a$^{+/+}$;Hes1$^{f/f}$;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$^{f/f}$;Rosa26^{LacZ}*) and control mice (*Ptf1a$^{cre/+}$;Hes1$^{f/+}$;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 μm (**b, d**).

Supplementary Figure S5

a Western blot analysis for LC3-I and its lapidated 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.

593 **Supplementary Figure S6**

594 **a** Immunofluorescence images of Hes1 and Aldh1a1 in *Hes1 cKO* (*Ptf1a$^{cre/+}$;Hes1$^{f/f}$*) and control

595 (Ctrl, *Ptf1a$^{+/+}$;Hes1$^{f/f}$*) mice at P3. Scale bars: 50 μm. **b** Microarray analysis of pancreatic head and

596 tail tissues of *Hes1 cKO* and control mice at P7. A heatmap of genes related to pancreatic

597 development is shown.

598

599 **Supplementary Figure S7**

600 **a** FACS isolation of Aldh-positive cells with Aldefluor reagent. FACS gating was established by

601 negative control. The number of Aldh-positive cells were decreased in *Hes1 cKO*

602 (*Ptf1a$^{cre/+}$;Hes1$^{f/f}$*) mice compared with that in control mice at P7 (**b**). The number of organoid

603 formation from pancreatic cells (**c, d**). *$P < 0.05$.

604

605 **Supplementary Figure S8**

606 **a** H&E staining and immunohistochemical staining for Hes5 and Hey1 of control mice,

607 *Ptf1a$^{cre/+}$;Hes1$^{f/f}$* mice (*cKO*), *Ptf1a$^{cre/+}$;Rosa26^{NICD}* mice, and *Ptf1a$^{cre/+}$;Hes1$^{f/f}$;Rosa26^{NICD}* mice

608 at P0. Marked increase of Hes5 expression was observed in *Ptf1a$^{cre/+}$;Hes1$^{f/f}$;Rosa26^{NICD}* mice.
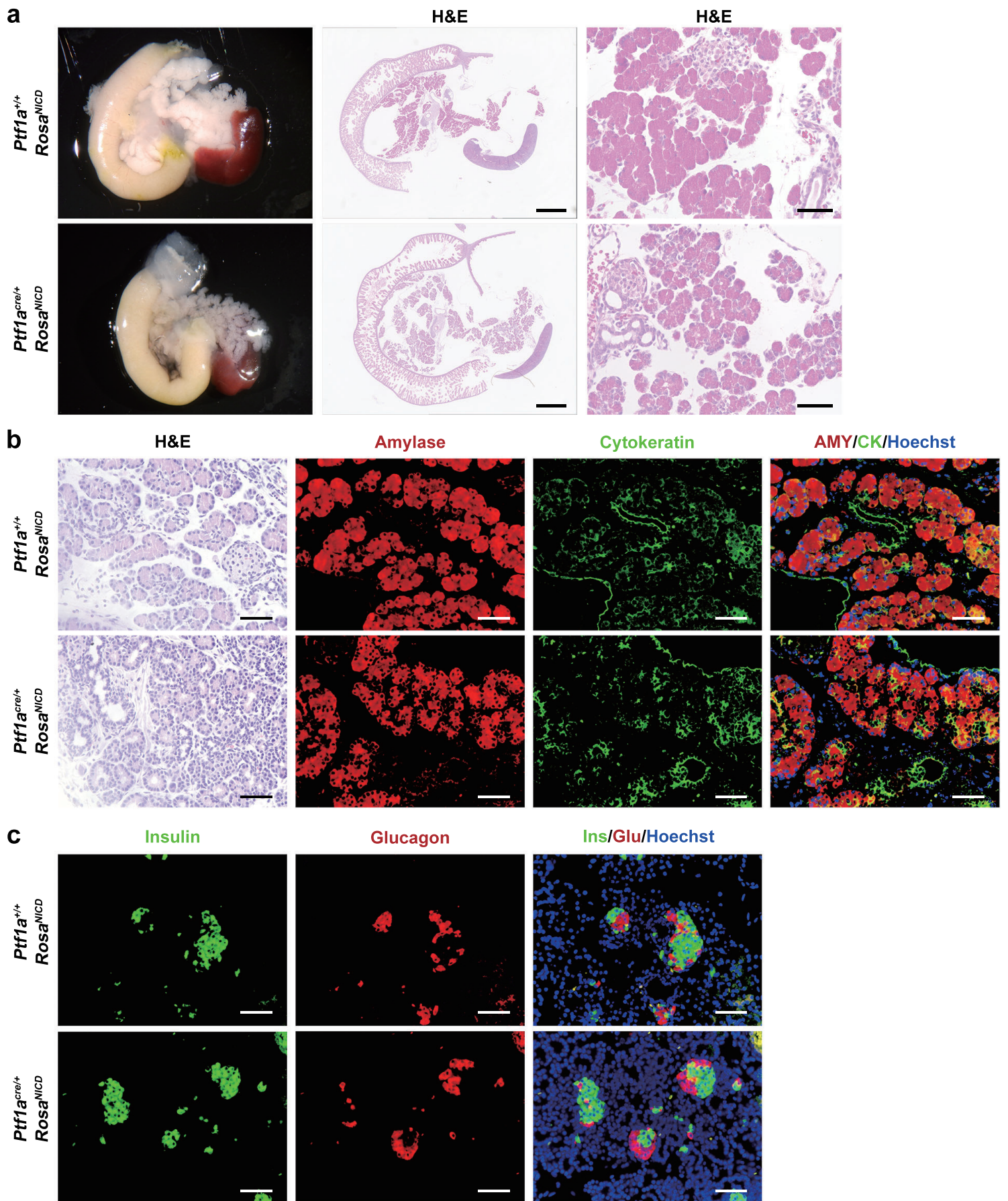
609 Scale bars: 50 μm.

610

611 **Supplementary Table 1.**

612 Primary antibodies used in this study.

613

614

a

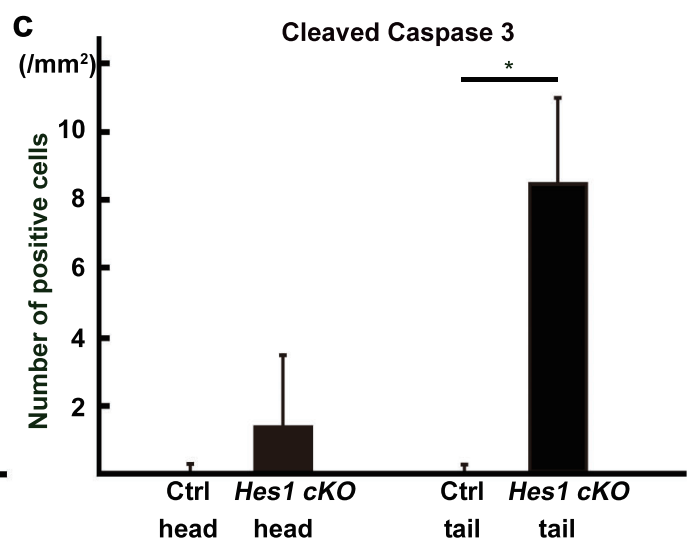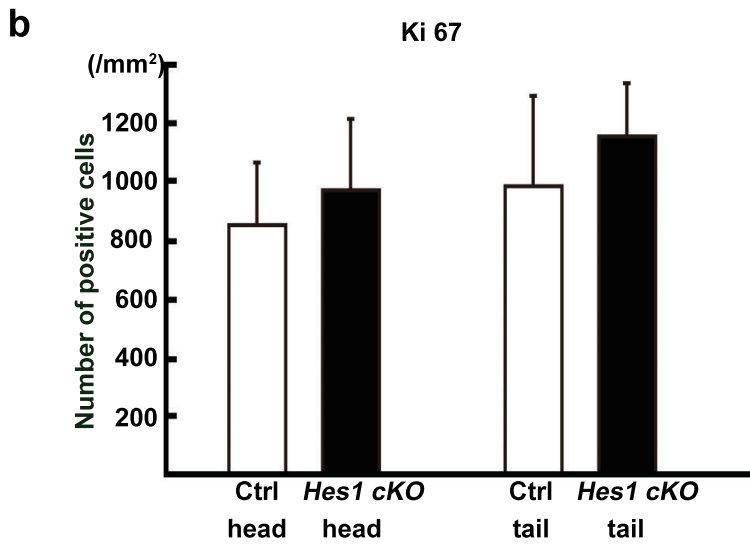|		X-gal staining	H&E
$Ptf1a^{+/+}$ $Hes1^{f/f}$ $Rosa26^{LacZ}$			
$Ptf1a^{cre/+}$ $Hes1^{+/+}$ $Rosa26^{LacZ}$			
$Ptf1a^{cre/+}$ $Hes1^{f/+}$ $Rosa26^{LacZ}$			
$Ptf1a^{cre/+}$ $Hes1^{f/f}$ $Rosa26^{LacZ}$ $(Hes1\ cKO)$			

b

	Amylase	Cytokeratin	Insulin	Glucagon
Ctrl				
Hes1 cKO				

Fig. 1

Fig. 2

a

| | P0 | 1 week | 2 weeks | 4 weeks | 24 weeks |

Ptf1a^cre/+ Hes1^f/+ Rosa26^LacZ (Ctrl)

Ptf1a^cre/+ Hes1^f/f Rosa26^LacZ (Hes1 cKO)

b

| | H&E | Amylase | Cytokeratin | Insulin | Glucagon |

Ctrl pancreatic head

Ctrl pancreatic tail

Hes1 cKO pancreatic head

Hes1 cKO pancreatic tail

Fig. 3

a

Ki 67 | Cleaved Caspase 3

Pancreatic tail | Pancreatic head

Ctrl

Hes1 cKO

b

Ki 67

(/mm²)

Number of positive cells

Ctrl head | *Hes1 cKO* head | Ctrl tail | *Hes1 cKO* tail

c

Cleaved Caspase 3

(/mm²)

Number of positive cells

*

Ctrl head | *Hes1 cKO* head | Ctrl tail | *Hes1 cKO* tail

Fig. 4

a

| | H&E | H&E | Hes1 | ALDH1A1 / Hoechst |

Ctrl

Hes1 cKO

b

(%)   P0

The ratio of acini without centroacinar cells

40

30

20

10

Ctrl head   *Hes1 cKO* head   Ctrl tail   *Hes1 cKO* tail

c

(%)   P3

The ratio of acini without centroacinar cells

50

40

30

20

10

Ctrl head   *Hes1 cKO* head   Ctrl tail   *Hes1 cKO* tail

d

Ctrl
Pnlip
Amy2a4
Sox9

e

Gcg
Chga
Chgb
Isl1
Neurod1

f

Aldh1a1
Aldh1a7

Hes1 cKO tail   *Hes1 cKO* head   Ctrl head   Ctrl tail

g

Aldh1a1

Relative mRNA expresion

4

3

2

1

Ctrl head   *Hes1 cKO* head   Ctrl tail   *Hes1 cKO* tail

Fig. 5

a

H&E

H&E

Ptf1a^{+/+}
Hes1^{f/f}
Rosa^{NICD}

Ptf1a^{cre/+}
Hes1^{f/f}
Rosa^{NICD}

b

H&E

Amylase

Cytokeratin

Ptf1a^{+/+}
Hes1^{f/f}
Rosa^{NICD}

Ptf1a^{cre/+}
Hes1^{f/f}
Rosa^{NICD}

c

Insulin / Glucagon / Hoechst

Ptf1a^{+/+}
Hes1^{f/f}
Rosa^{NICD}

Ptf1a^{cre/+}
Hes1^{f/f}
Rosa^{NICD}

Fig. 6

a

P0

| | H&E | H&E | Insulin / Glucagon / Hoechst |

Hes1^f/f (wild type)

Pdx1-cre Hes1^f/f

b

Ratio of acini without centroacinar cells

(%)

Ctrl head | *Pdx1-Cre Hes1^f/f* head | Ctrl tail | *Pdx1-Cre Hes1^f/f* tail

c

| | P0 | 2 weeks | 54 weeks |

Pdx1-cre Hes1^f/+ Rosa26^LacZ

Pdx1-cre Hes1^f/f Rosa26^LacZ

d

| | P0 | | 8 weeks | |
| | Pancreatic head | Pancreatic tail | Pancreatic head | Pancreatic tail |

Pdx1-cre Hes1^f/+ Rosa26^LacZ

Pdx1-cre Hes1^f/f Rosa26^LacZ

Fig. 7

a *Ptf1a^{cre/+}*

b *Hes1^{f/f}*

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.
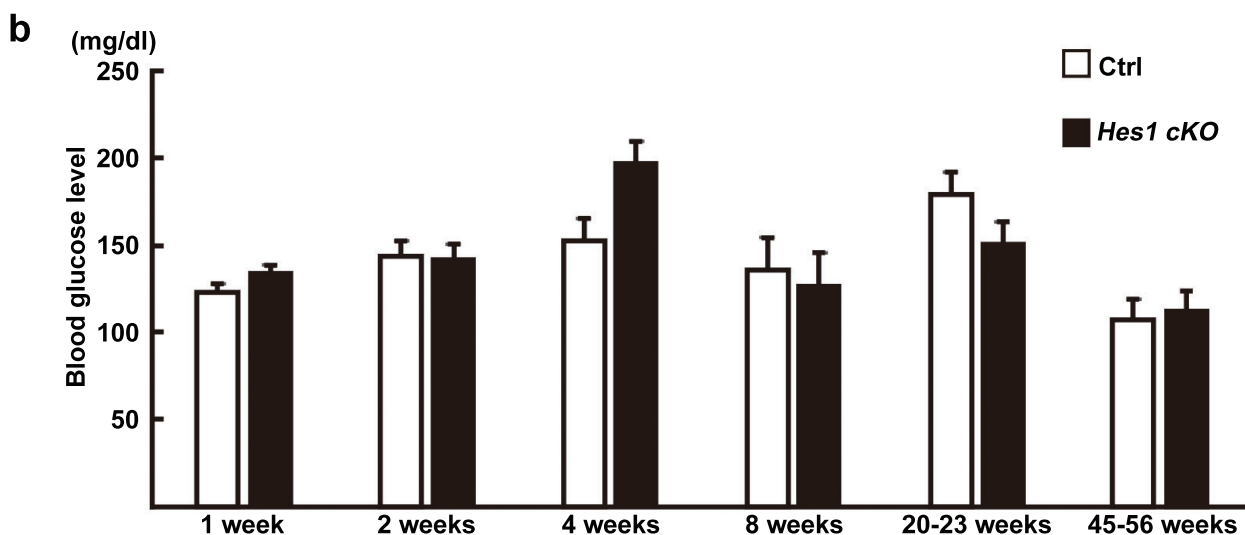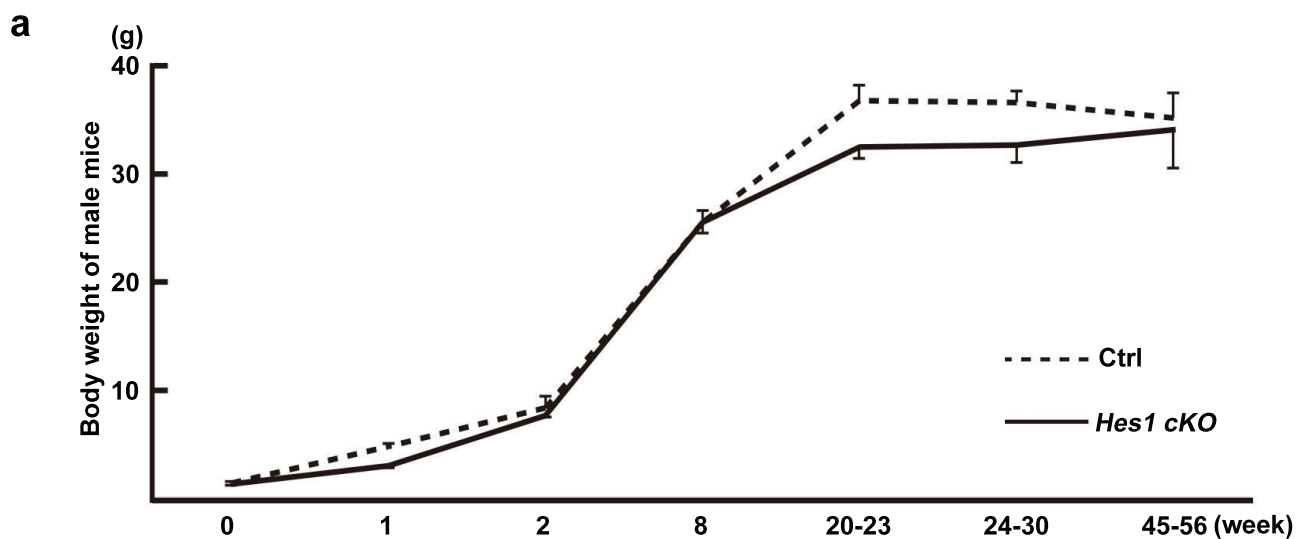
a Hes1

Ctrl

b Hes1

Hes1 cKO

Supplementary Figure S2
Hes1 immunohistochemistry
Hes1 expression in pancreatic tissue was analyzed by immunohistochemistry in *Hes1 cKO*
(Ptf1a$^{cre/+}$;Hes1$^{f/f}$;Rosa26^{LacZ}) and control (Ctrl, *Ptf1a$^{+/+}$;Hes1$^{f/f}$;Rosa26^{LacZ}*) mice at P0.
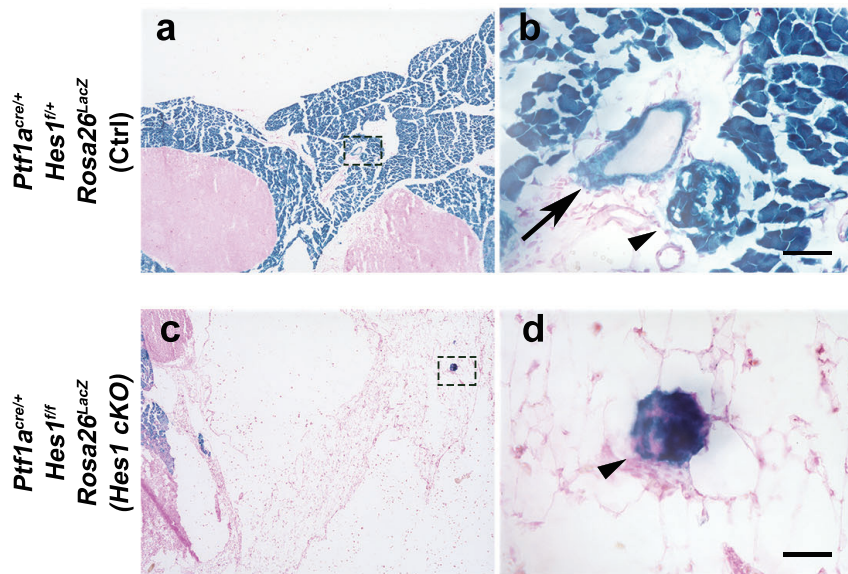Hes1 expression was deleted in *Hes1 cKO* mice (**b**), while it was positive in the ducts and
centroacinar cells of control mice (**a**).

a

b

Supplementary Figure S3
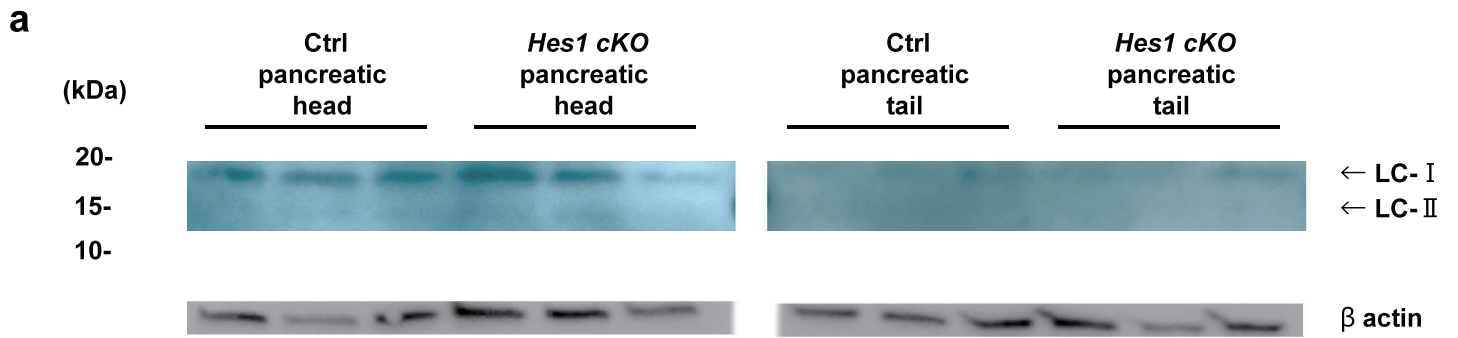**Changes in body weight and blood glucose level**
Changes in body weight (**a**) and blood glucose level (**b**) after birth in *Hes1 cKO (Ptf1a^{cre/+};Hes1^{f/f};Rosa26^{LacZ})*
and control (Ctrl, *Ptf1a^{+/+};Hes1^{f/f};Rosa26^{LacZ}*) mice. The median and the standard error of mean are shown.

Supplementary Figure S4
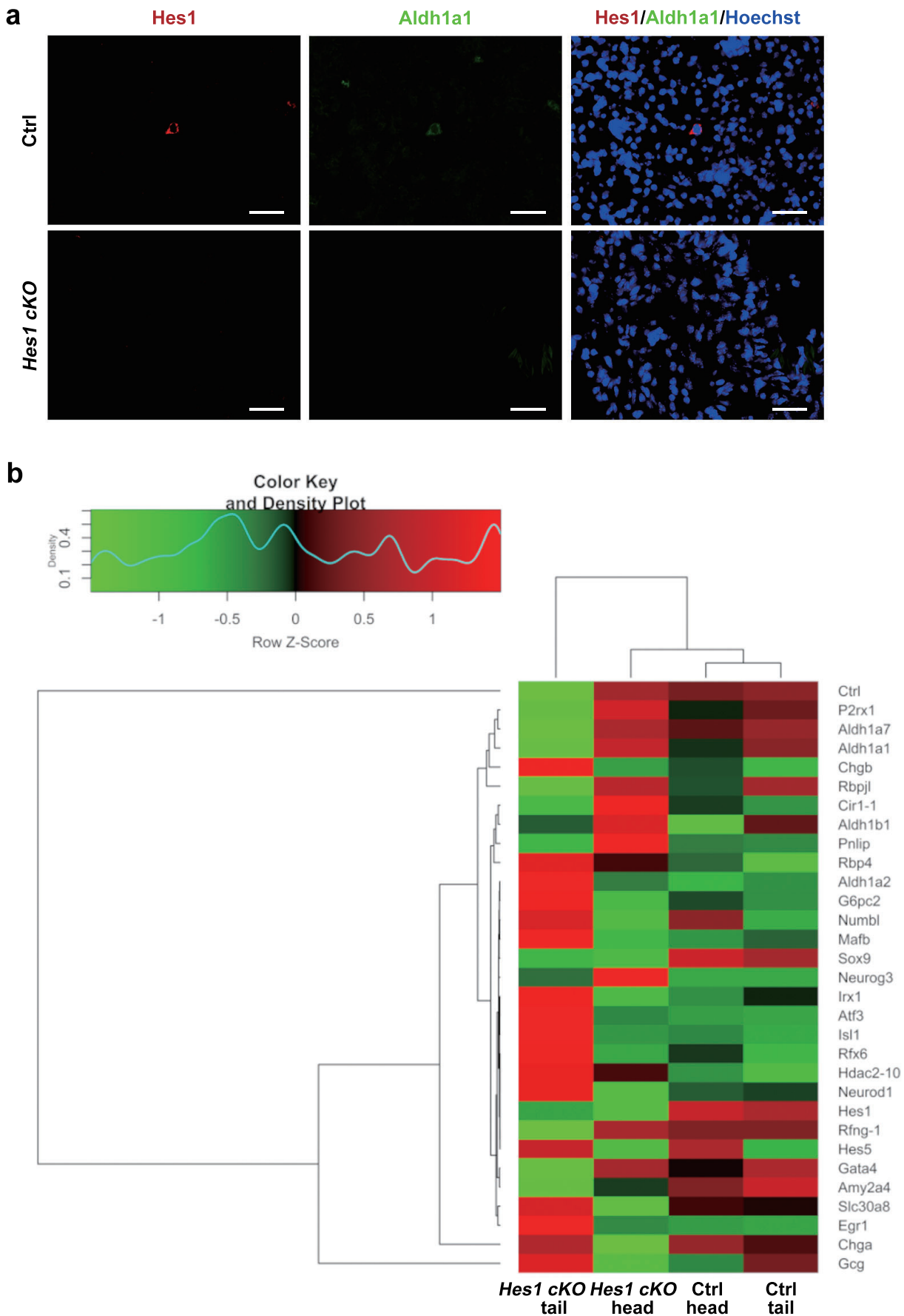***Hes1* knockout in Ptf1a-positive progenitor cells.**
X-gal staining of *Hes1* conditional knockout mice (*Ptf1a$^{cre/+}$;Hes1$^{f/f}$;Rosa26^{LacZ}*) and control mice
(*Ptf1a$^{cre/+}$;Hes1$^{f/+}$;Rosa26^{LacZ}*) at 4 weeks of age. **b** and **d** are a magnification of the broken line in **a** and **c**.
Arrow head: islet, arrow: duct. Scale bars: 50 μm (**b, d**).

a

	Ctrl pancreatic head	Hes1 cKO pancreatic head	Ctrl pancreatic tail	Hes1 cKO pancreatic tail

(kDa)

20-

15-

10-

← LC-Ⅰ
← LC-Ⅱ

β actin

Supplementary Figure S5
a Western blot analysis for LC3-I and its lapidated 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.
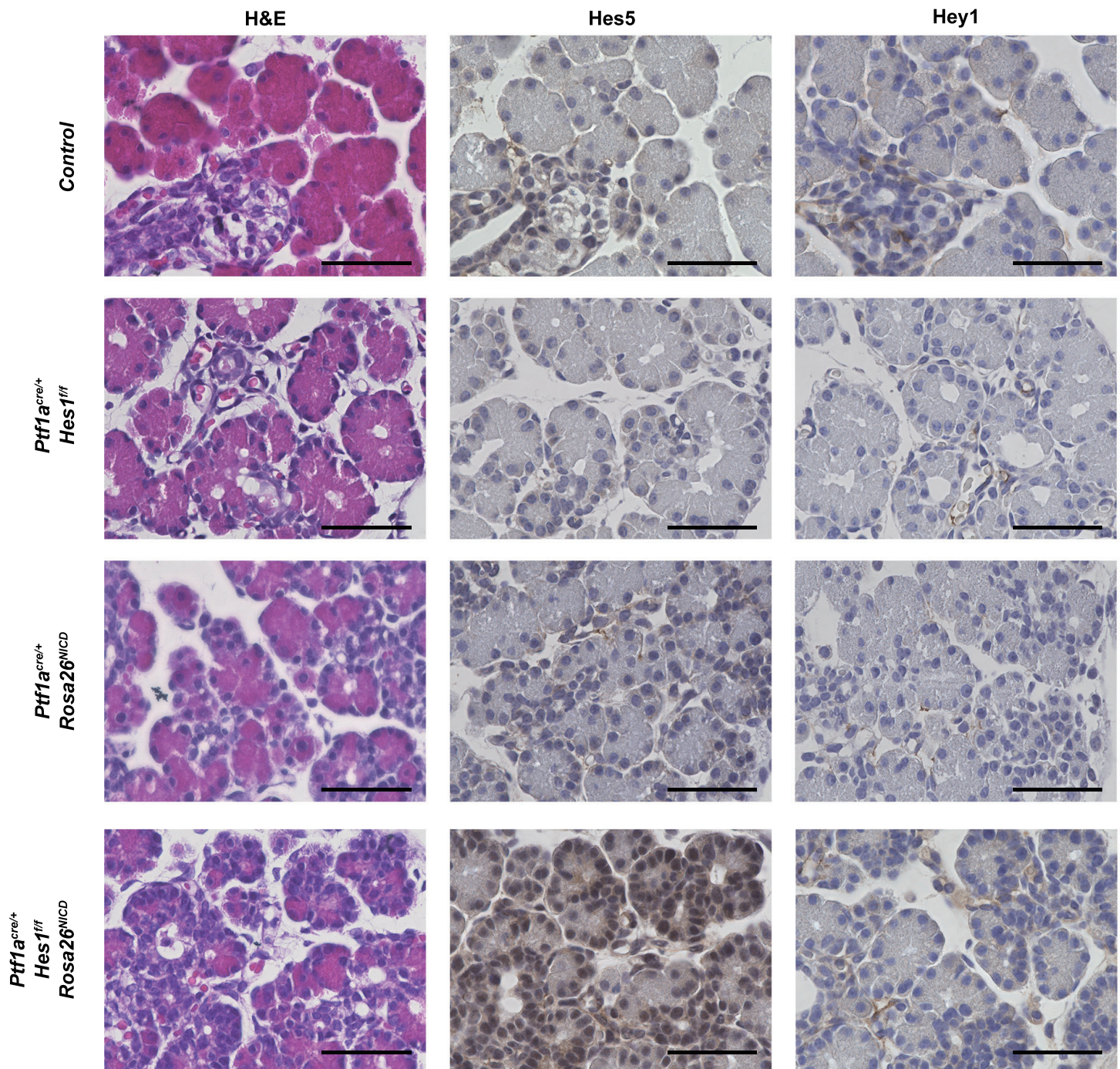
Supplementary Figure S6
a Immunofluorescence images of Hes1 and Aldh1a1 in *Hes1 cKO (Ptf1a^{cre/+};Hes1^{f/f})* and control
(Ctrl, *Ptf1a^{+/+};Hes1^{f/f}*) mice at P3. Scale bars: 50 μm. **b** Microarray analysis of pancreatic head and tail tissues of
Hes1 cKO and control mice at P7. A heatmap of genes related to pancreatic development is shown.
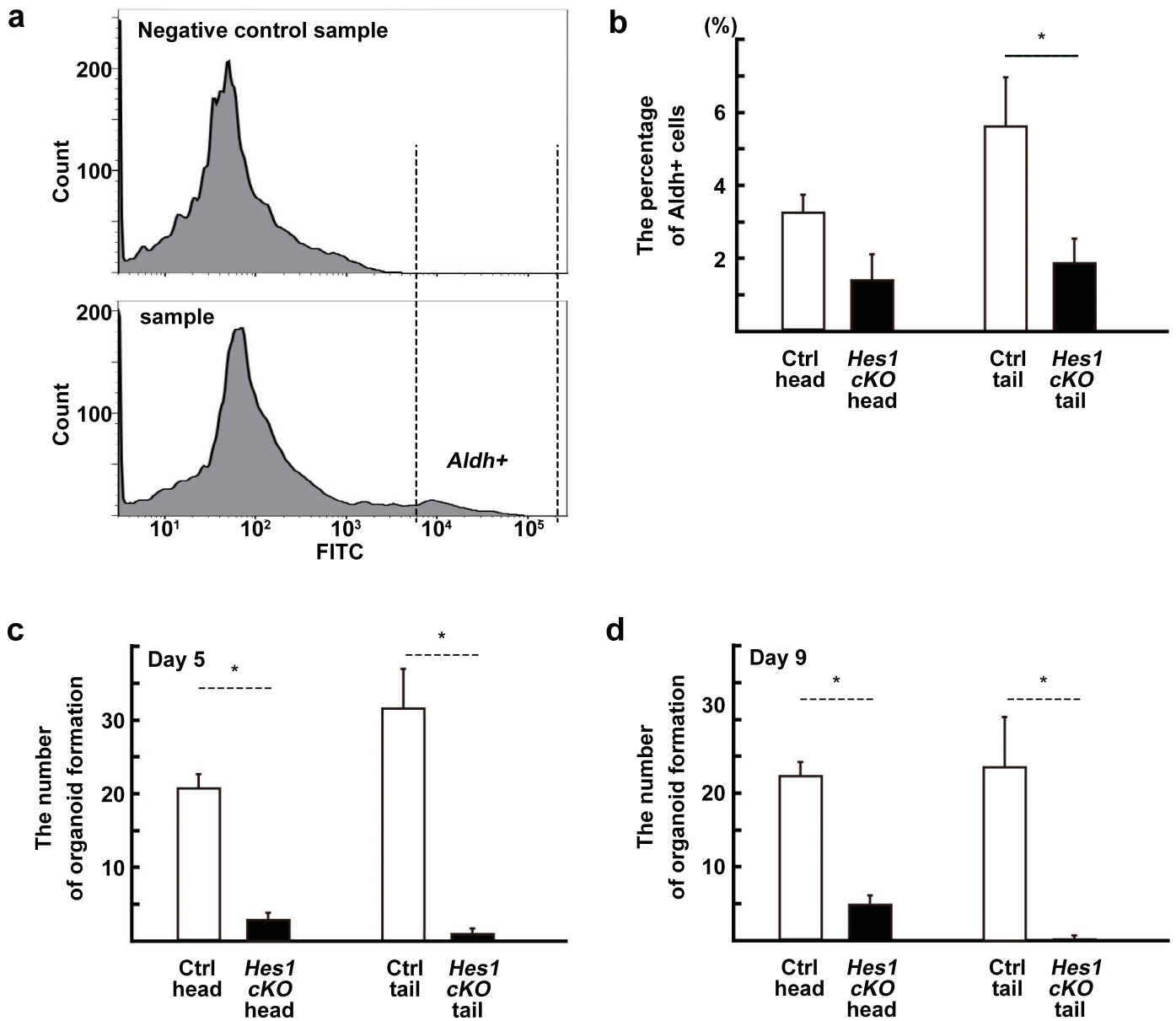
Supplementary Figure S7
a FACS isolation of Aldh-positive cells with Aldefluor reagent. FACS gating was established by negative control. The number of Aldh-positive cells were decreased in *Hes1 cKO (Ptf1a^{cre/+};Hes1^{f/f})* mice compared with that in control mice at P7 (**b**). The number of organoid 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, Ptf1a$^{cre/+}$;Hes1$^{f/f}$ mice (*cKO*), *Ptf1a$^{cre/+}$;Rosa26^{NICD}* mice, and *Ptf1a$^{cre/+}$;Hes1$^{f/f}$;Rosa26^{NICD}* mice at P0. Marked increase of Hes5 expression was observed in *Ptf1a$^{cre/+}$;Hes1$^{f/f}$;Rosa26^{NICD}* mice. Scale bars: 50 μm.

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
Hey1	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.