



Hes1 plays an essential role in *Kras*-driven pancreatic tumorigenesis

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Abstract

Most pancreatic ductal adenocarcinoma (PDAC) develops from pancreatic epithelial cells bearing activating mutant *KRAS* genes through precancerous lesions, i.e. acinar-to-ductal metaplasia (ADM) and pancreatic intraepithelial neoplasia (PanIN). During pancreatic tumorigenesis, Hes1 expression starts with the transition from acinar cells to ADM, and continues during PanIN and PDAC formation, but the role of Hes1 in pancreatic tumorigenesis is not fully elucidated. Here we show that Hes1 plays an essential role in the initiation and progression of *KRAS*-driven pancreatic tumorigenesis. In vitro, activation of MAPK signaling due to EGF or mutant *KRAS* activation induced sustained Hes1 expression in pancreatic acinar cells. In vivo, acinar cell-specific activation of mutant *KRAS* by *Elastase1-CreERT2;Kras*^{G12D} induced ADM/PanIN formation with Hes1 expression in mice, and genetic ablation of *Hes1* in these mice dramatically suppressed PanIN formation. Gene expression analysis and lineage tracing revealed that Hes1 regulates acinar-to-ductal reprogramming-related genes and, in a *Hes1*-deficient state, mutant *Kras*-induced ADM could not progress into PanIN, but re-differentiated into acinar cells. In the *Elastase1-CreERT2;Kras*^{G12D};*Trp53*^{R172H} mouse PDAC model, genetic ablation of *Hes1* completely blocked PDAC formation by keeping PanIN lesions in low-grade conditions, in addition to reducing the occurrence of PanIN. Together, these findings indicate that mutant *KRAS*-induced Hes1 plays an essential role in PDAC initiation and progression by regulating acinar-to-ductal reprogramming-related genes.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related death in the United States [1], and a highly lethal disease with a 5-year survival rate of only 5–10% [1, 2]. The extremely poor prognosis is due to the lack of early detection methods, preventive strategies, and effective treatments. Overcoming such a negative situation critically requires elucidation of the underlying molecular mechanisms of PDAC initiation and progression, as well as exploration of novel risk factors and treatment targets.

PDAC is believed to arise from a spectrum of pre-neoplastic mucinous lesions with a ductal morphology, among which pancreatic intraepithelial neoplasia (PanIN) is most commonly observed in humans [3]. PanIN lesions are considered to progress stepwise to PDAC by accumulating multiple genetic alterations [2, 3]. Among these, activating mutations of the *KRAS* gene have been detected at the earliest stage and are thought to be essential for the initiation of PanIN. The significance of the *KRAS* mutation has

been demonstrated in pancreatic cancer mouse models in which expression of mutant *Kras* in pancreatic cells recapitulates the development of PanIN and subsequent PDAC [4]. The cells of origin for PDAC have been explored using these mouse models, and acinar cells are currently recognized as the putative origin of PDAC, at least in mice [5]. Acinar cells bearing mutant *Kras* transform into PanIN through acinar-to-ductal metaplasia (ADM), which is characterized by the replacement of acinar cells with cells co-expressing ductal markers as well as acinar markers. This acinar-to-ductal reprogramming (ADR) is promoted by factors that induce ductal cell identity, such as Sox9 and Klf4, but suppressed by factors that preserve acinar cell properties, such as Ptf1a and Mist1 [5–8]. Important signaling pathways for pancreatic development, including Notch signaling, are involved in ADR [9], but it is still unknown how a *KRAS* mutation affects and controls signaling.

Hes1 is a transcriptional factor and thought to be a main target of the Notch signaling pathway [10]. In the embryonic stage, Hes1 is expressed in stem/progenitor cells and is thought to maintain these cells and play a role in their cell fate decision. Observations of pancreas hypoplasia following global *Hes1* gene deletion in mice suggest that Hes1 has essential roles in pancreatic development [11]. In the adult pancreas, Hes1 is expressed in duct cells but not in acinar cells under normal conditions. In contrast, during tumorigenesis, Hes1 expression starts with the transition from acinar cells to ADM, and continues during PanIN and PDAC formation, suggesting an essential role in the initiation and progression of PDAC [4, 9]. Indeed, the importance of Hes1 in PDAC development was recently highlighted in a study reporting that embryonal *Hes1* gene deletion accelerates ADM and subsequent PDAC formation in a *Kras*^{G12D}-driven PDAC mouse model [12]. Because of the essential role of Hes1 in pancreatic development, we considered that *Hes1* should be deleted in the adulthood rather than in the embryonic stage, especially for analyzing the PDAC mouse model.

In the present study, we first demonstrated that sustained Hes1 expression was induced in pancreatic cells by mutant *KRAS* activation. Furthermore, contrary to the previous report of embryonal *Hes1* gene deletion, conditional knockout of *Hes1* in adult pancreatic acinar cells dramatically suppressed the formation of *Kras*^{G12D}-driven PanIN and PDAC lesions in mice. Here we provide evidence that Hes1 plays essential roles in multiple steps of PDAC initiation and progression by regulating ADR-related genes. Given our previous report showing the antitumor effect of a novel Hes1 inhibitor on pancreatic cancer cells in vitro and in vivo [13], Hes1 could be a new preventive or therapeutic target for PDAC.

Results

Sustained Hes1 expression is induced by mutant *KRAS* activation in pancreatic carcinogenesis

To evaluate the role of Hes1 in pancreatic carcinogenesis, we first used immunohistochemistry to evaluate HES1 protein expression in normal pancreatic tissues and pancreatic lesions in surgically resected specimens. In normal pancreatic tissues, HES1 expression was detected in duct cells and centroacinar cells, but not in acinar cells, as previously reported [9] (Fig. 1a). On the other hand, HES1 was broadly expressed in precancerous lesions (i.e., ADM and PanIN) to PDAC (Fig. 1a). Additionally, analysis of The Cancer Genome Atlas (TCGA) database revealed that higher expression of *Hes1* mRNA in PDAC specimens was significantly correlated with poor prognosis of PDAC patients (Fig. 1b), suggesting an essential role of Hes1 in PDAC formation.

Recent studies revealed that at least in mice, *Kras*^{G12D}-driven PanIN and PDAC develop from acinar cells via ADM [5]. Therefore, the absence of Hes1 expression in normal acinar cells but its presence in ADM/PanIN suggests that Hes1 is induced by the activation of mutant *Kras* and involved in ADM/PanIN formation. Indeed, expression of pERK1/2, which indicates mitogen-activated protein kinase (MAPK) signaling activation, was observed in ADM, PanIN, and PDAC, but not in acinar cells in human pancreatic specimens (Fig. 1a).

To analyze the relationship between MAPK signaling activation and Hes1 expression, we performed in vitro experiments using 266-6 cells, a mouse acinar cell-derived cell line established by SV40 T antigen induction without *Kras* mutation. Upon treatment with epidermal growth factor (EGF) to activate MAPK signaling, phosphorylation of Erk1/2 peaked in 5 min and *Hes1* mRNA was transiently increased for 90 min (Fig. 2a). This induction of Erk1/2 phosphorylation and *Hes1* expression was completely suppressed by the Mek inhibitor U0126 (Fig. 2b), suggesting MAPK-specific Hes1 induction in pancreatic acinar cells. To activate of MAPK signaling by mutant *Kras*, tamoxifen-inducible mutant *KRAS* (*ER-KRAS*^{G12V}) [14] was induced in 266-6 cells using a lentivirus expression system. Upon tamoxifen treatment, strong and persistent phosphorylation of Erk1/2 by mutant *KRAS* was confirmed in this model. Notably, this mutant *KRAS* activation induced a sustained *Hes1* mRNA increase (Fig. 2c). Again, U0126 treatment completely repressed Erk1/2 phosphorylation and the subsequent *Hes1* mRNA increase, suggesting a direct association of mutant *Kras* activation and Hes1 induction (Fig. 2d). To assess the involvement of Notch signaling in this Hes1 induction, we next analyzed the effect of

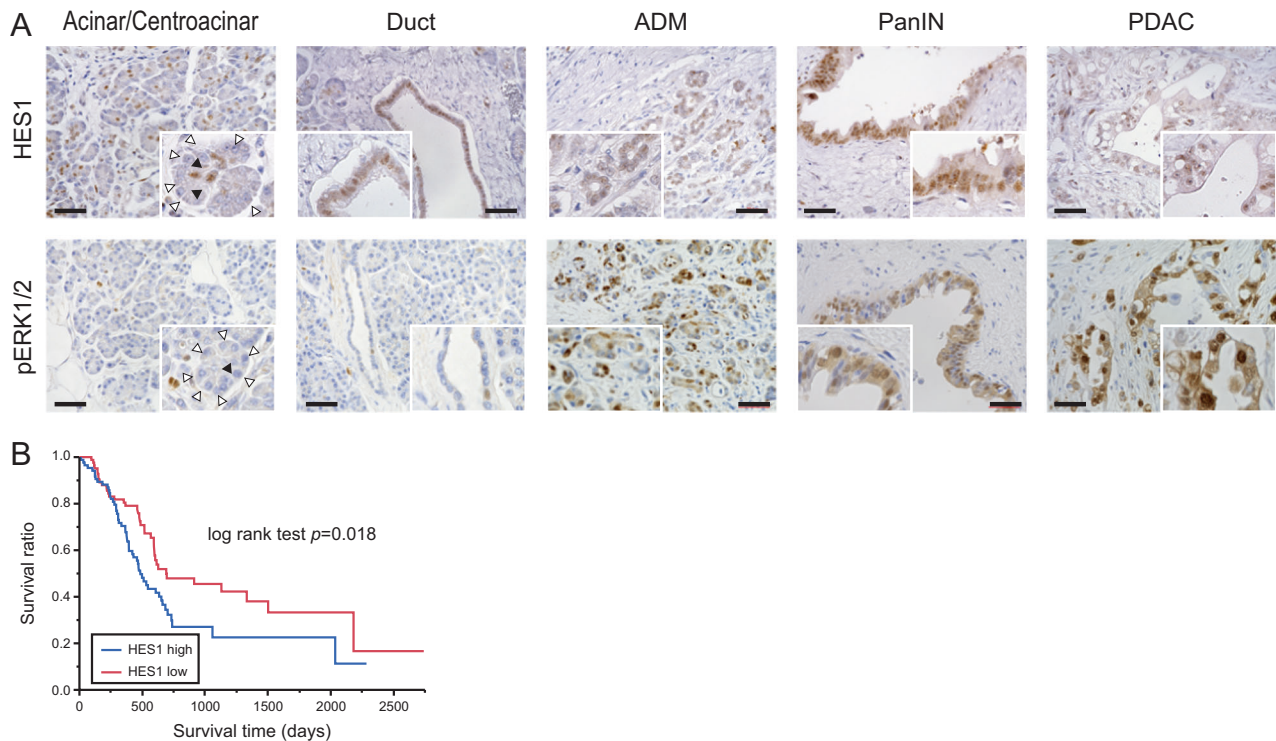


Fig. 1 HES1 expression in human normal pancreatic tissues, pre-cancerous lesions, and pancreatic ductal adenocarcinoma (PDAC). **a** Immunohistochemical analysis of human pancreatic tissues and lesions. White arrowheads indicate acinar cells and black arrowheads

indicate centroacinar cells. **b** Kaplan-Meier survival curve for PDAC patients in Hes1-high or Hes1-low groups analyzed from The Cancer Genome Atlas database. Scale bars: 50 μ m

inhibition of Notch signaling by gamma secretase inhibitor N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-(S)-phenylglycine t-butyl ester (DAPT) in 266-6 cells. As a result, DAPT partially repressed *Hes1* mRNA expression in a dose-dependent manner (Fig. S1A). However, Hes1 upregulation by mutant *Kras* activation was not inhibited by treatment with DAPT, suggesting that Hes1 was induced by MAPK signaling in a Notch signaling-independent manner (Fig. 2e). These findings indicate that sustained Hes1 expression was induced by mutant *Kras* activation via MAPK signaling in pancreatic acinar cells, and may have an important role in pancreatic tumorigenesis.

Hes1 is not essential for maintaining the homeostasis of adult pancreatic acinar cells

Hes1 has an essential role in pancreatic development [11], but its role in adult acinar cells is not fully elucidated. To elucidate the role of Hes1 in adult pancreatic acinar cells by excluding the influence of *Hes1* deletion at the developmental stage, we generated *Elastase1-CreERT2;Hes1^{flox/flox}* (*E-Hes1-KO*) mice in which the *Hes1* gene can be deleted specifically in acinar cells in a tamoxifen-dependent manner (Fig. S2A). *E-Hes1-KO* mice and *Elastase1-CreERT2;Hes1^{wt/wt}* (*E-Hes1-WT*) mice were administered tamoxifen

subcutaneously at 4 weeks of age and evaluated up to 7 months of age (Fig. S2B). Compared with *E-Hes1-WT* mice, the *E-Hes1-KO* mice showed no obvious morphologic differences in pancreas as evaluated by hematoxylin and eosin staining, as well as by immunohistochemistry for amylase, cytokeratin, insulin, and glucagon after 6 months (Fig. S2C). Because *Elastase1-Cre* does not affect centroacinar cells and duct cells, Hes1 expression was observed in these cells in both types of mice (Fig. S2C). Also, body weight, pancreatic weight, pancreas/body weight ratio, fasting blood glucose, and mRNA expression of several pancreatic differentiation marker genes were not significantly different between the two groups (Fig. S2D-S2G).

We then evaluated the role of Hes1 in pancreatic regeneration after acute pancreatitis. For this purpose, *E-Hes1-WT* and *E-Hes1-KO* mice were administered tamoxifen at 4 weeks of age and injected with cerulein for 2 consecutive days at 1 week after the tamoxifen administration. Pancreatic tissues were evaluated 2 and 7 days after cerulein administration (Fig. 3a, b). In *E-Hes1-WT* mice at day 2, abundant ADMs expressing Hes1 developed, and some cleaved caspase-3-positive cells, indicating apoptosis, were observed (Fig. 3c). At day 7, as previously reported [15], *E-Hes1-WT* mice almost completely recovered from acute pancreatitis and showed normal pancreatic

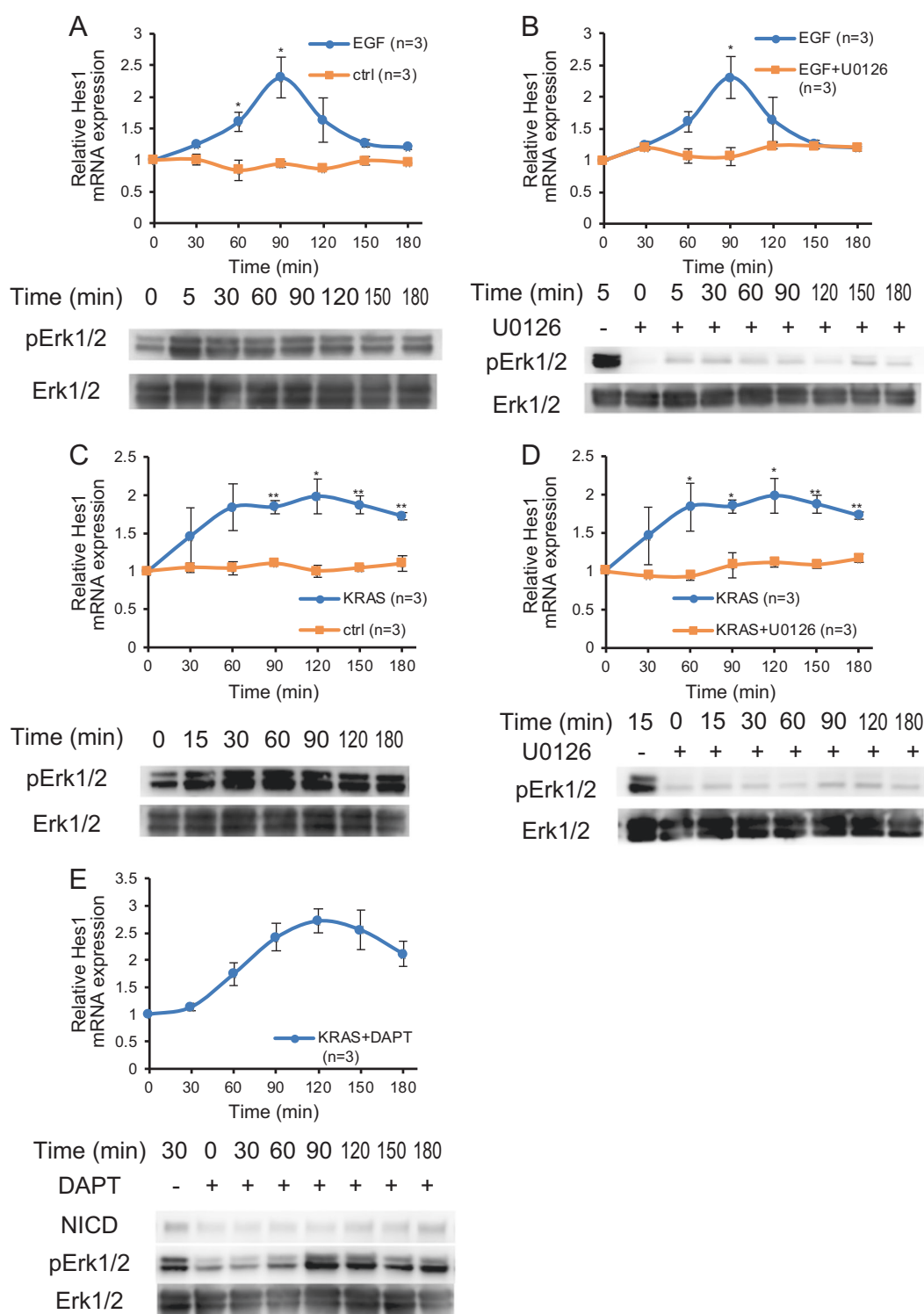


Fig. 2 Sustained Hes1 expression is induced by mutant *KRAS* activation. **a–e** Quantitative PCR analysis of *Hes1* mRNA expression, and western blot analysis of Erk1/2, pErk1/2, and cleaved Notch1

expression in 266-6 acinar cells after epidermal growth factor (EGF) treatment or mutant *KRAS* induction with or without U0126 and DAPT. * $P < 0.05$ and ** $P < 0.01$

morphology (Fig. 3d). Notably, *E-Hes1-KO* mice also developed an equivalent level of abundant ADMs at day 2 as compared with *E-Hes1-WT* mice, but were negative for Hes1, confirming successful deletion of the *Hes1* (Fig. 3c).

Furthermore, *E-Hes1-KO* mice showed no differences in the induction of apoptosis at day 2 (Fig. 3c, e), recovery from acute pancreatitis at day 7 (Fig. 3d), or in the pancreas/body weight ratio at day 7 (Fig. 3f). Taken together, our data

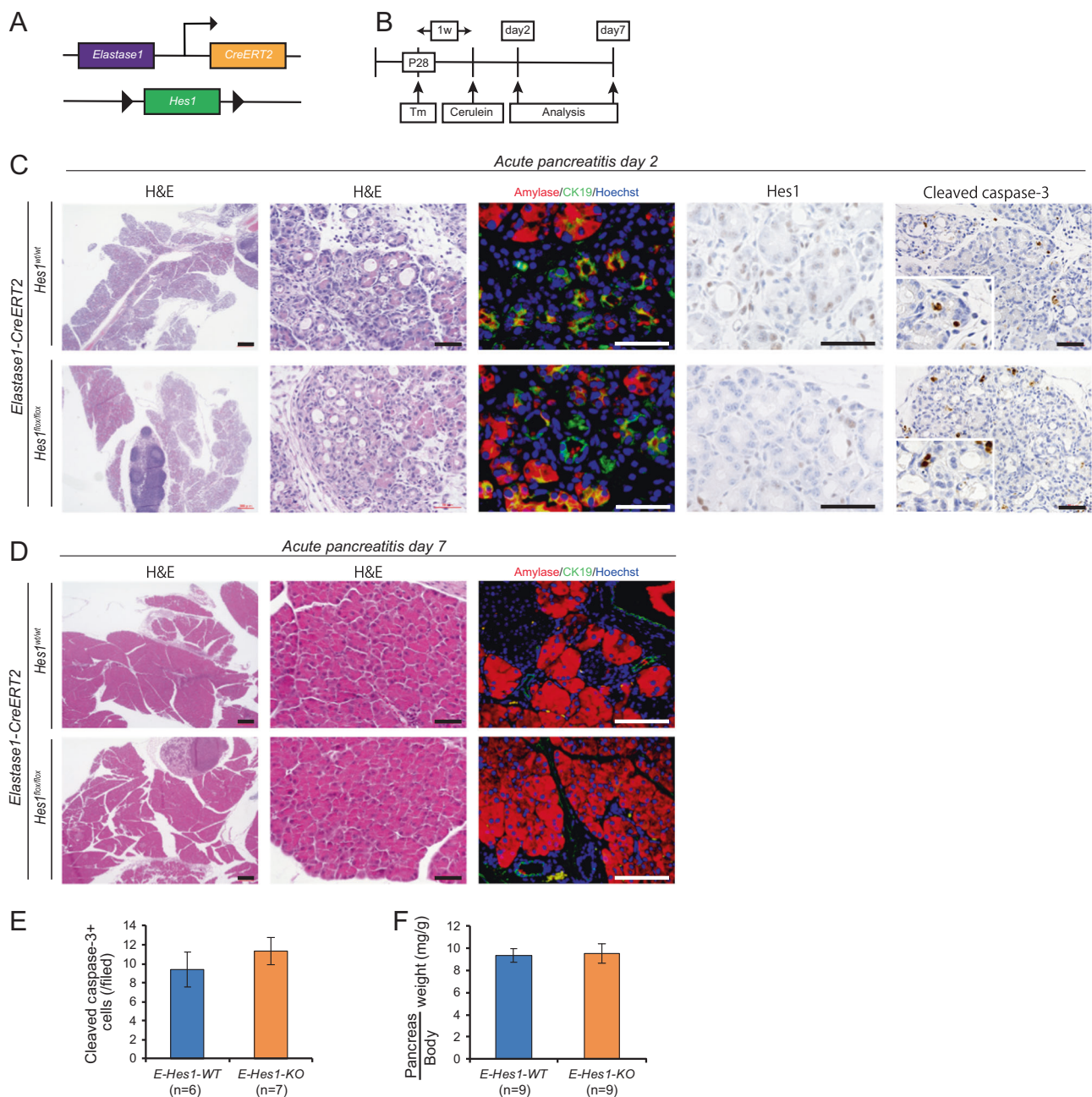


Fig. 3 Hes1 is not essential for maintaining the homeostasis of adult acinar cells. **a** Tamoxifen-induced Cre-mediated pancreatic acinar cell-specific *Hes1* ablation. **b** Schematic of the experimental design. **c, d** Pancreatic tissues were evaluated by hematoxylin and eosin staining, immunofluorescent staining for amylase and CK19, and

immunohistochemistry for Hes1 and cleaved caspase-3. **e** Number of cleaved caspase-3-positive cells of *Elastase1-CreERT2*; *Hes1*^{wt/wt} (*E-Hes1-WT*) mice and *Elastase1-CreERT2*; *Hes1*^{flox/flox} (*E-Hes1-KO*) mice at day 2. **f** Pancreas weight to body weight ratio in *E-Hes1-WT* and *E-Hes1-KO* mice at day 7. Scale bars: 50 μ m

suggest that *Hes1* deletion in adult acinar cells does not affect the maintenance of mature acinar cell homeostasis under both normal and injured conditions.

Hes1 is required for *Kras*^{G12D}-driven PanIN formation

We then explored the role of *Hes1* in the initiation of pancreatic tumorigenesis using a *Kras*^{G12D}-driven mouse PanIN

model. We generated *Elastase1-CreERT2*; *Kras*^{G12D}; *Hes1*^{wt/wt} mice (*KE-Hes1-WT*) and *Elastase1-CreERT2*; *Kras*^{G12D}; *Hes1*^{flox/flox} mice (*KE-Hes1-KO*) (Fig. 4a). *KE-Hes1-WT* and *KE-Hes1-KO* mice were administered tamoxifen at 4 weeks of age and evaluated for up to 6 months after the administration (Fig. 4b). In *KE-Hes1-WT* mice, some PanIN lesions positive for alcian blue staining and Hes1 immunostaining developed after 2 months (Fig. 4c). In contrast, in *KE-Hes1-*

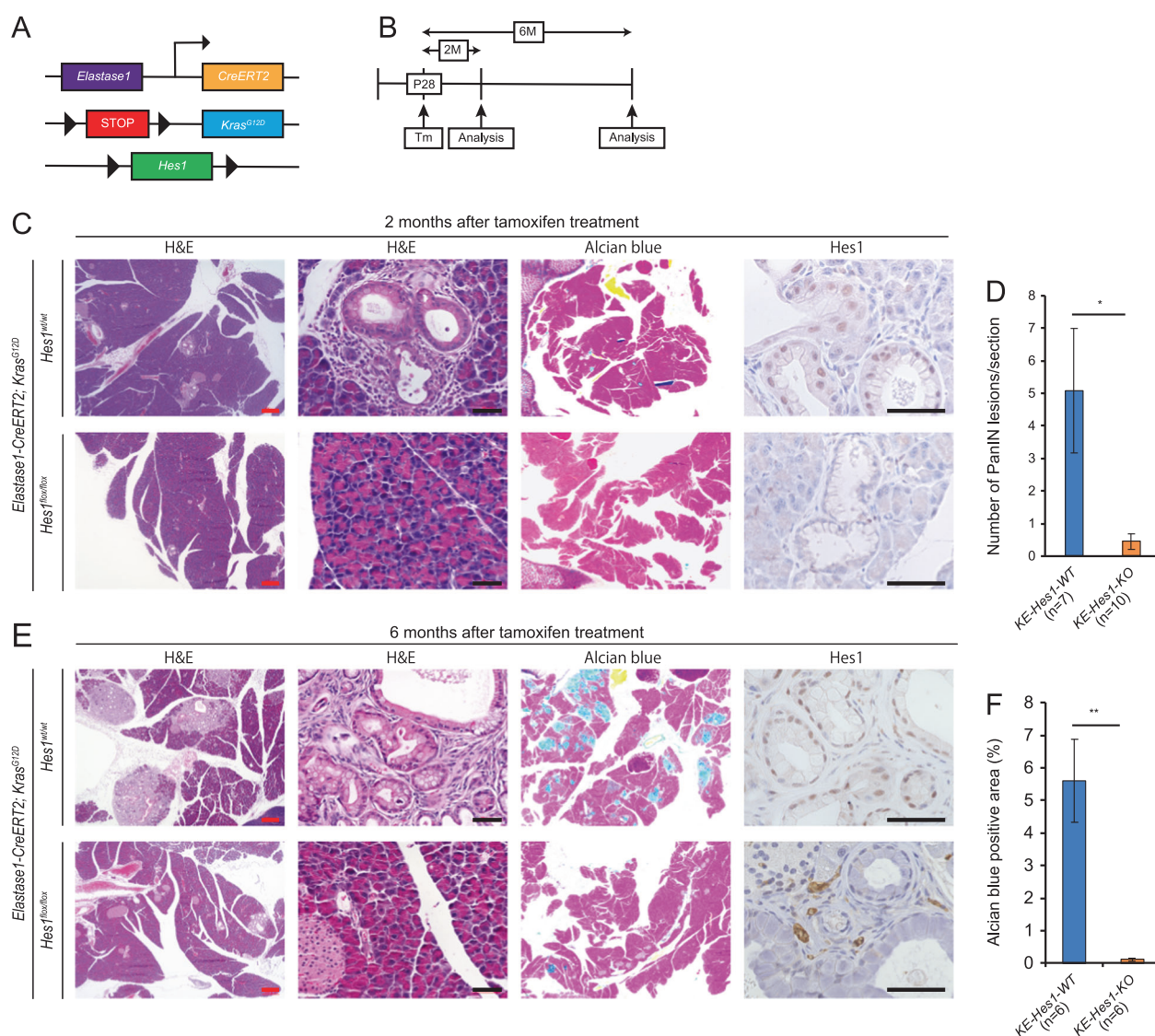


Fig. 4 Hes1 is required for *Kras*^{G12D}-driven pancreatic intraepithelial neoplasia (PanIN) formation. **a** Tamoxifen-induced Cre-mediated pancreatic acinar cell-specific *Kras*^{G12D} induction and *Hes1* ablation. **b** Schematic of the experimental design. **c–e** Histologic lesions observed in pancreas of *Elastase1-CreERT2*;*Kras*^{G12D};*Hes1*^{wt/wt} (*KE-Hes1-WT*) mice and *Elastase1-CreERT2*;*Kras*^{G12D};*Hes1*^{flx/flx} (*KE-Hes1-KO*)

mice. Representative images showing the results of hematoxylin and eosin staining, alcian blue staining, and immunohistochemistry for Hes1. **d** Number of PanIN lesions. **f** Percentage of alcian blue-positive area. **P* < 0.05 and ***P* < 0.01. Red scale bars: 300 μm, black scale bars: 50 μm

KO mice, only a few PanIN lesions without Hes1 immunostaining due to gene deletion were observed (Fig. 4c). The number of PanIN lesions in *KE-Hes1-KO* mice was significantly lower than that in *KE-Hes1-WT* mice (Fig. 4d). This difference was more evident after 6 months. In *KE-Hes1-WT* mice, a number of PanIN lesions developed across the pancreas, whereas only focal lesions were found in *KE-Hes1-KO* mice (Fig. 4e). The area of PanIN lesions positive for alcian blue was significantly smaller in *KE-Hes1-KO* mice compared with *KE-Hes1-WT* mice (Fig. 4f). Thus, *Hes1* deletion dramatically suppressed *Kras*^{G12D}-driven PanIN formation in mice, suggesting an essential role of Hes1 in the development of PanIN, i.e., the initiation of pancreatic carcinogenesis.

Because our *KE-Hes1-WT* mice developed multiple lung tumors, as previously reported [16], we could not observe these mice for more than 6 months, which prevented us from evaluating PDAC formation (Fig. S3A–S3B).

Hes1 is not required for *Kras*^{G12D}-driven ADM formation, but is necessary for further progression to PanIN

Pancreatitis can significantly accelerate the formation of ADM and subsequent PanIN in a *Kras*^{G12D}-driven mouse model [17]. To evaluate the precise role of Hes1 in the stepwise development of ADM and PanIN, we applied

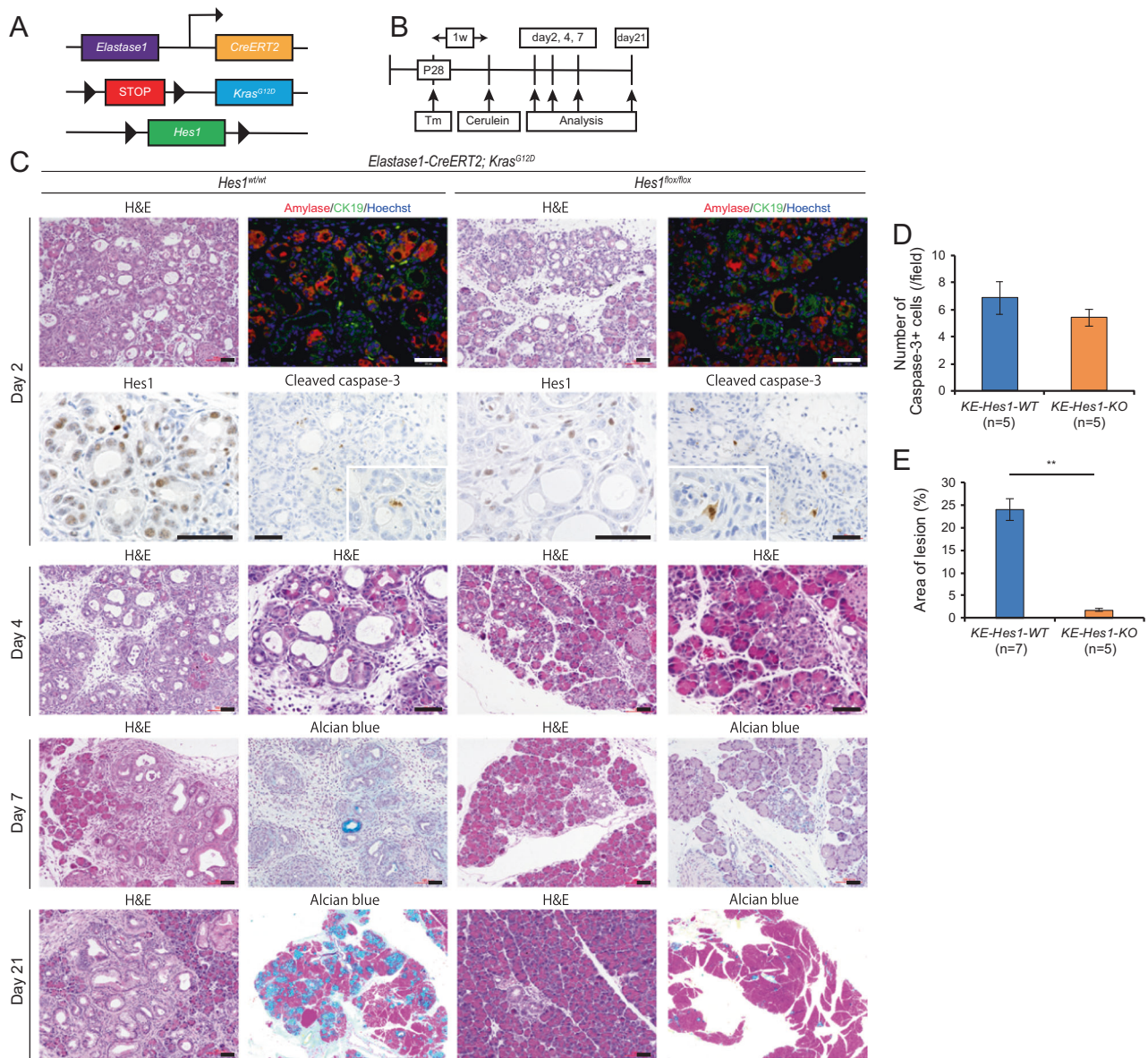


Fig. 5 Hes1 is essential for progression from acinar-to-ductal metaplasia to pancreatic intraepithelial neoplasia. **a** Tamoxifen-induced Cre-mediated pancreatic acinar cell-specific *Kras*^{G12D} induction and *Hes1* ablation. **b** Schematic of the experimental design. **c** Histologic analysis of *Elastase1*-CreERT2;*Kras*^{G12D}; *Hes1*^{wt/wt} (KE-Hes1-WT)

mice and *Elastase1*-CreERT2;*Kras*^{G12D}; *Hes1*^{lox/lox} (KE-Hes1-KO) mice. Representative images showing the results of hematoxylin and eosin staining, alcian blue staining, and immunohistochemistry for Hes1 and cleaved caspase-3. **d** Number of cleaved caspase-3-positive cells. **e** Area of lesions in each group. ***P* < 0.01. Scale bars: 50 μ m

cerulein-induced acute pancreatitis to our *Kras*^{G12D}-driven mouse model. *KE-Hes1-WT* and *KE-Hes1-KO* mice were administered tamoxifen at 4 weeks of age and injected with cerulein for 2 consecutive days at 1 week after the tamoxifen administration. Pancreatic tissues were evaluated at day 2, 4, 7, and 21 (Fig. 5a, b). In *KE-Hes1-WT* mice, widespread ADM lesions with Hes1 immunostaining developed by 2 days, and were still observed on day 4. A few PanIN lesions positive for alcian blue were observed on day 7, and, at 21 days, pancreatic acinar cells were replaced by widespread PanIN lesions (Fig. 5c). Thus, as previously

reported, cerulein-induced pancreatitis strongly accelerated the formation of PanIN via ADM in *KE-Hes1-WT* mice. In stark contrast to these observations, in *KE-Hes1-KO* mice, widespread ADM lesions developed to the same extent as in *KE-Hes1-WT* mice in 2 days, but these Hes1-negative ADM lesions gradually regressed by day 4. Notably, *KE-Hes1-KO* mice almost completely recovered from acute pancreatitis within 7 days (Fig. 5c), as is observed in *E-Hes1-WT* or *E-Hes1-KO* mice (Fig. 3d). In addition, the PanIN lesions rarely formed by 21 days (Fig. 5c), and the area of the lesions in *KE-Hes1-KO* mice was significantly smaller than

that in *KE-Hes1-WT* mice (Fig. 5e). Taken together, *Hes1* deletion did not affect ADM formation, but almost completely suppressed *Kras*^{G12D}- and pancreatitis-induced ADM prolongation and the subsequent formation of PanIN. These results suggest an essential role of *Hes1* in the progression from ADM to PanIN.

***Hes1*-deficient ADM lesions escape *Kras*^{G12D}-driven progression to PanIN by re-differentiating into acinar cells**

How did *Hes1*-deficient ADM lesions escape PanIN progression even with *Kras*^{G12D} induction? We considered two possibilities: (1) *Hes1*-deficient ADM lesions are eliminated by cell death, or (2) the lesions re-differentiate into acinar cells. To elucidate this, we generated mice in which the lineage of cells can be traced by crossing *Rosa26-LSL-tdTomato* mice with *E-Hes1-WT*, *E-Hes1-KO*, *KE-Hes1-WT*, or *KE-Hes1-KO* mice (Fig. 6a). Once cerulein-induced acute pancreatitis developed, these labeled acinar cells transitioned to ADM lesions. If the ADM lesions are eliminated by cell death, the pancreatic tissue will be compensated for by non-labeled cells (i.e. non-recombined acinar cells or duct cells), and the ratio of labeled cells will decrease after recovery from pancreatitis. On the other hand, if the ADM lesions re-differentiate into acinar cells, the pancreatic tissue will be repaired by the labeled (i.e., recombined) cells, and the ratio of labeled cells will not change, as previously reported [18]. Under these conditions, tamoxifen was administered to 4-week-old mice, and gene recombination efficiency in pancreatic acinar cells was evaluated by analyzing the ratio of tdTomato-labeled cells to amylase-positive acinar cells 1 week later (day 0). Another cohort of mice was injected with cerulein over 2 consecutive days, and the ratio of tdTomato-labeled cells to amylase-positive acinar cells was evaluated 2 and 14 days later (day 2 and 14) (Fig. 6b). In *E-Hes1-WT* mice, 75.8% of acinar cells were labeled with tdTomato on day 0, and most of these cells transitioned to ADM lesions on day 2 and re-differentiated into acinar cells expressing amylase on day 14 (Fig. 6c), resulting in an unaltered tdTomato-positive cell ratio (74.3%) as previously described [18]. On the other hand, in *KE-Hes1-WT* mice, most tdTomato-labeled acinar cells progressed into PanIN that was positive for tdTomato and CK19 but negative for amylase on day 14 (Fig. 6e). Interestingly, not only in *E-Hes1-KO* mice but also in *KE-Hes1-KO* mice, the ratio of tdTomato-labeled cells was not different between day 0 and 14 (77.5 and 74.5% in *E-Hes1-KO* mice, respectively; Fig. S4; 79.6 and 74.3% in *KE-Hes1-KO* mice, respectively; Fig. 6d) as is observed in *E-Hes1-WT* mice. In addition to these results, no significant differences in the number of cleaved caspase-3 positive cells were observed between *KE-Hes1-WT* mice

and *KE-Hes1-KO* mice at acute pancreatitis day 2 (Fig. 5c, d). These results indicate that even with *Kras*^{G12D} induction, *Hes1*-deficient ADM lesions did not progress to PanIN lesions, but instead re-differentiated into acinar cells, suggesting an essential role of *Hes1* in *Kras*^{G12D}-driven ADM prolongation and progression to PanIN.

Hes1 regulates ADR-related genes

Previous reports demonstrated that the *Kras*^{G12D}-driven ADR is promoted by transcriptional factors that induce ductal cell identity, such as *Sox9* and *Klf4* [5, 6], but suppressed by transcriptional factors that preserve acinar cell properties, such as *Ptf1a* and *Mist1* [7, 8]. On the basis of the pivotal role of Notch/*Hes1* signaling in the cell fate decision of pancreatic cells by regulating genes, including *Sox9* and *Ptf1a* [10, 19], we assessed the expression of these genes in our *Kras*^{G12D}-driven mouse model with cerulein-induced acute pancreatitis (Fig. 6a, b). The expression of *Sox9* mRNA in pancreatic tissues was significantly lower in *KE-Hes1-KO* mice compared with *KE-Hes1-WT* mice on day 2 (Fig. 6g) when ADM formation was the most strongly induced. The weaker expression of *Sox9* protein in ADM lesions in *KE-Hes1-KO* mice compared with *KE-Hes1-WT* mice was also confirmed by immunohistochemistry (Fig. 6f). In contrast, expression of *Ptf1a* mRNA was higher in *KE-Hes1-KO* mice than in *KE-Hes1-WT* mice on day 2, although the difference was not significant (Fig. 6h). These alterations in *Sox9* and *Ptf1a* mRNA expression were not observed between *E-Hes1-WT* and *E-Hes1-KO* mice with cerulein-induced acute pancreatitis, suggesting that *Kras*-induced *Hes1* plays an important role in regulating ADR-related genes in PanIN formation (Fig. S5A and S5B). To further investigate the relationship between *Hes1* and *Sox9*, we performed in vitro experiments using 266-6 cells. Although we failed to create *Hes1* homozygous knockout cells, probably because *Hes1* is essential for surviving, we successfully established *Hes1* heterozygous knockout cells using the CRISPR/Cas9 technique and also *Hes1*-overexpressing cells using lentivirus expression systems. After confirming the stepwise expression of *Hes1* mRNA (Fig. S6A-S6C), we assessed *Sox9* mRNA in these cells. *Sox9* expression was increased or decreased in a *Hes1* expression-dependent manner (Fig. S6B, S6D). The close relationship between *Hes1* and *Sox9* in the PDAC specimens was also demonstrated by analysis of the TCGA database. The mRNA expression of *Hes1* and *Sox9* (Fig. S6E) was positively correlated, and *Sox9* was one of the genes most strongly correlated with *Hes1* and vice versa (Fig. S6F-S6G). These results suggest that *Hes1* induced by *Kras*^{G12D} could regulate the progression from ADM to PanIN by orchestrating ADR-related genes.

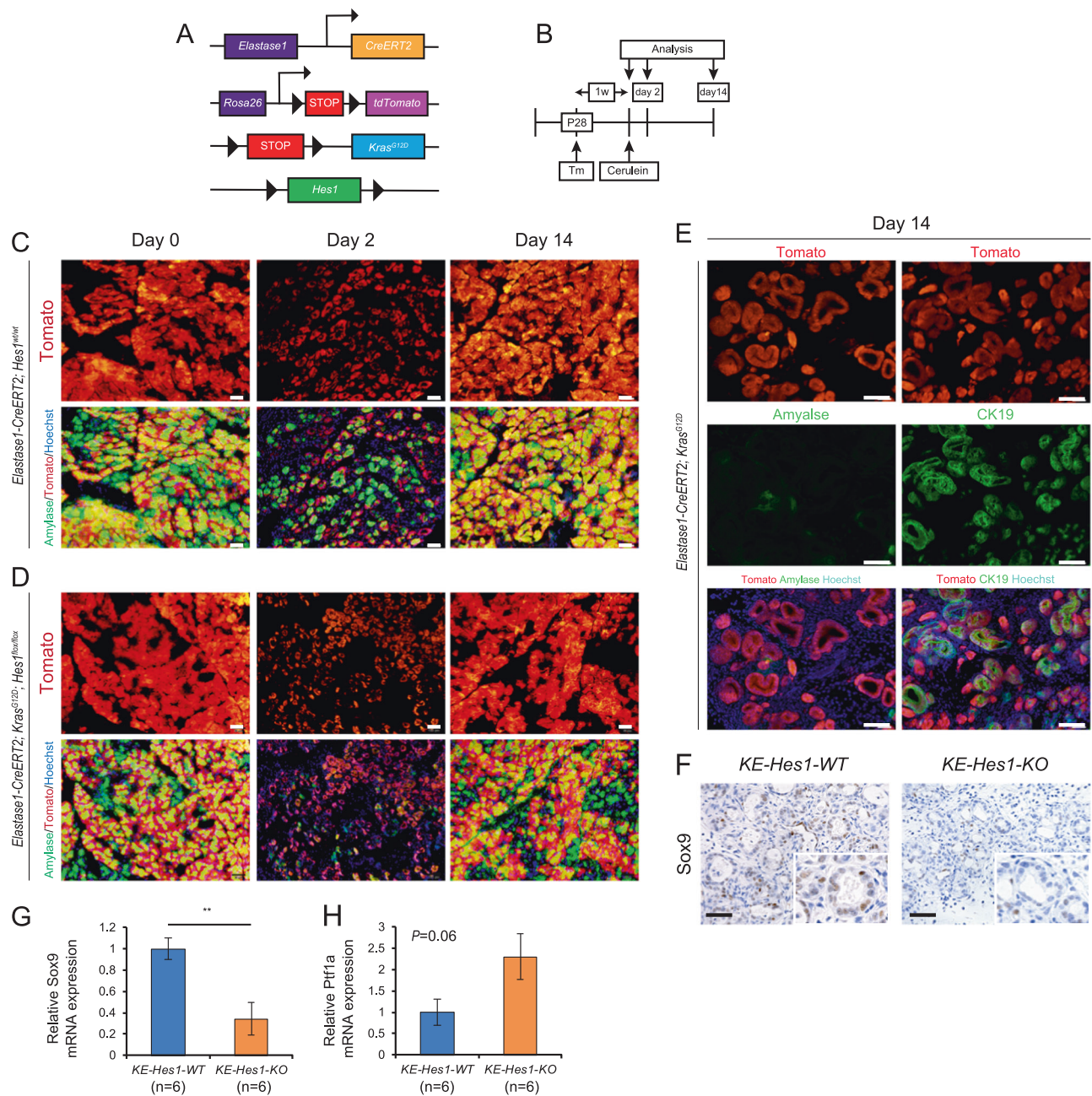


Fig. 6 *Hes1*-deficient ADM lesions escape *Kras*^{G12D}-driven progression to pancreatic intraepithelial neoplasia by re-differentiating into acinar cells. **a** Tamoxifen-induced Cre-mediated pancreatic acinar cell-specific *Kras*^{G12D} and *tdTomato* induction, and *Hes1* ablation. **b** Schematic of the experimental design. **c–e** Representative immunofluorescent images of each mice. **f** Immunohistochemistry for Sox9 in

pancreatic tissues of *Elastase1-CreERT2*;*Kras*^{G12D};*Hes1*^{wt/wt} (*KE-Hes1-WT*) mice and *Elastase1-CreERT2*;*Kras*^{G12D};*Hes1*^{flx/flx} (*KE-Hes1-KO*) mice at day 2. **g, h** *Sox9* and *Ptf1a* mRNA expression in pancreatic tissues of *KE-Hes1-WT* mice and *KE-Hes1-KO* mice at day 2. ***P* < 0.01. Scale bars: 50 μ m

Hes1 is required for progression from PanIN to PDAC

To investigate the role of *Hes1* in the progression from PanIN to PDAC, we established *Elastase1-CreERT2*;*Kras*^{G12D};*Trp53*^{R172H};*Hes1*^{wt/wt} (*KPE-Hes1-WT*) and *Elastase1-CreERT2*;*Kras*^{G12D};*Trp53*^{R172H};*Hes1*^{flx/flx} (*KPE-*

Hes1-KO) mice. Unfortunately, *KPE* mice died from subcutaneous sarcoma at ~3 months after tamoxifen injection prior to PDAC formation (Fig. S7A–S7B), and therefore cerulein was administered to accelerate PDAC formation [20]. *KPE-Hes1-WT* and *KPE-Hes1-KO* mice were administered tamoxifen at 4 weeks of age and injected with cerulein for 2 consecutive days beginning 1 week after

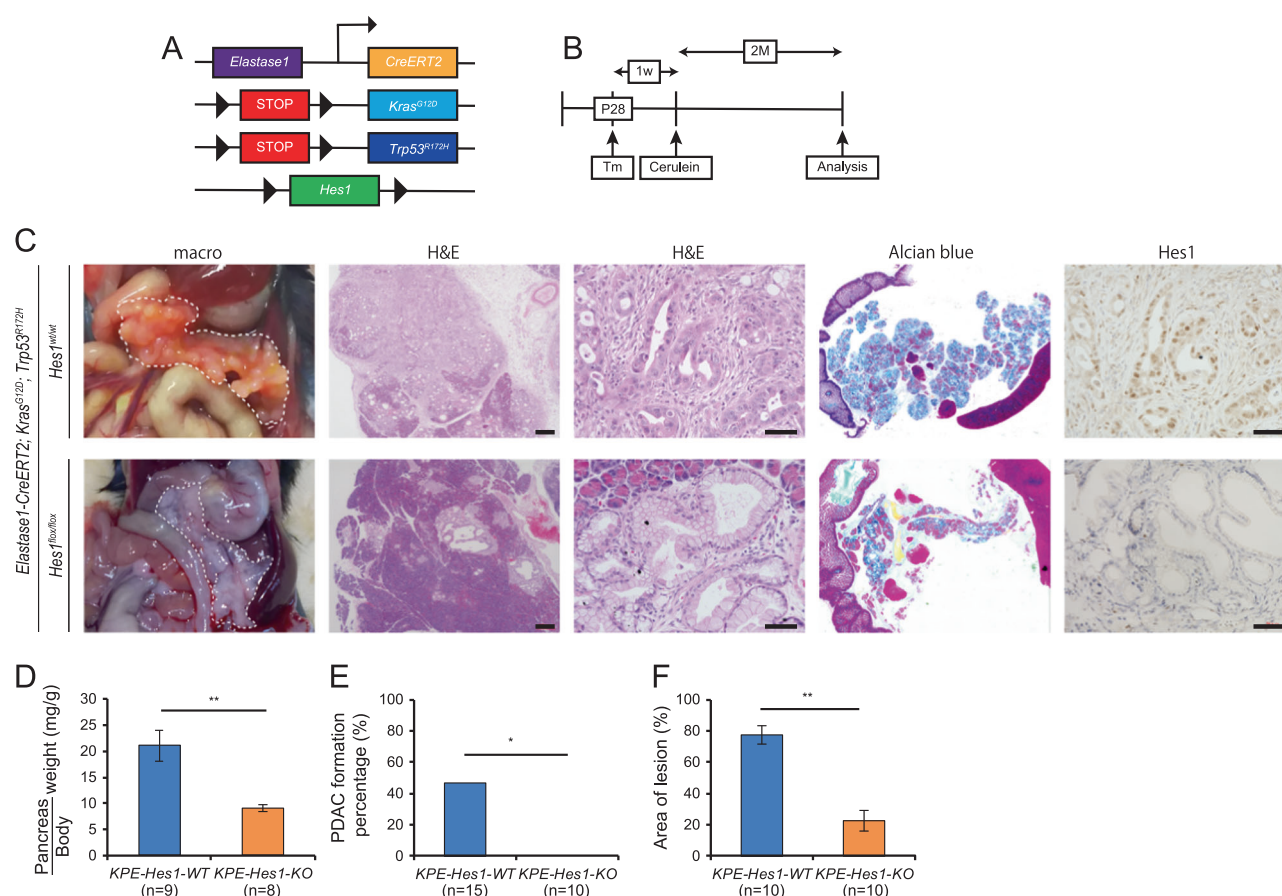


Fig. 7 Hes1 is required for progression from pancreatic intraepithelial neoplasia to pancreatic ductal adenocarcinoma (PDAC). **a** Tamoxifen-induced Cre-mediated pancreatic acinar cell-specific *Kras*^{G12D} and *Trp53*^{R172H} induction, and *Hes1* ablation. **b** Schematic of the experimental design. **c** Macroscopic view of the pancreas and histologic analysis of *Elastase1-CreERT2*; *Kras*^{G12D}; *Trp53*^{R172H}; *Hes1*^{wt/wt} (KPE-

Hes1-WT) mice and *Elastase1-CreERT2*; *Kras*^{G12D}; *Trp53*^{R172H}; *Hes1*^{flx/flx} (KPE-*Hes1*-KO) mice. Representative images showing the results of hematoxylin and eosin staining, alcian blue staining, and immunohistochemistry for Hes1. **d–f** Pancreas to body weight ratio, PDAC formation percentage, and area of lesions in KPE-*Hes1*-WT and KPE-*Hes1*-KO mice. **P* < 0.05 and ***P* < 0.01. Scale bars: 50 μm

tamoxifen administration. Pancreatic tissues were evaluated after 2 months (Fig. 7a, b). The KPE-*Hes1*-WT mouse pancreas was quite enlarged by massively formed PanIN lesions with prominent fibrosis and inflammatory cell infiltration (Fig. 7c). PDAC developed in 46.7% (7/15) of the mice (Fig. 7e). Hes1 expression in these PanIN and PDAC lesions was confirmed by immunohistochemistry (Fig. 7c). In contrast, in KPE-*Hes1*-KO mice, the size of the pancreas was almost normal, and the pancreas/body weight ratio was significantly smaller than that in KPE-*Hes1*-WT mice (Fig. 7c, d). PanIN lesions without Hes1 immunostaining developed (Fig. 7c), but the number of alcian blue-positive PanIN lesions was significantly lower than that in KPE-*Hes1*-WT (Fig. 7f). Notably, no PDAC formation was observed in KPE-*Hes1*-KO mice (0/10) (Fig. 7e). Although to a much lower extent than in the KPE-*Hes1*-WT mice, a substantial number of PanIN lesions were formed in the KPE-*Hes1*-KO mice. In KPE-*Hes1*-WT mice, a broad spectrum of lesions ranging from PanIN1A, 1B, 2, 3 to

invasive carcinoma was observed (Fig. S8A–S8B). In contrast, in KPE-*Hes1*-KO mice, only low-grade PanIN lesions (i.e. PanIN1A, 1B, and 2; most frequently PanIN1A) developed, but high-grade PanIN lesions (i.e. PanIN2 and PanIN3) were rarely observed (Fig. S8A–S8B). Thus, Hes1 was suggested to have an essential role in the development of PanIN but also in its progression and subsequent PDAC formation.

Discussion

Hes1 is not expressed in normal pancreatic acinar cells, but once these cells enter the oncogenic process, Hes1 expression begins during the transition of acinar cells to ADM and continues during PanIN and PDAC formation. These observations as well as the importance of Hes1 in pancreatic development suggest a critical role for Hes1 in pancreatic carcinogenesis, but the detailed functions of Hes1 in adult

pancreatic pathogenesis were not fully elucidated. Here we demonstrated that activation of MAPK signaling due to mutant *KRAS* activation induces sustained Hes1 expression in pancreatic acinar cells, and that this sustained Hes1 activation plays an essential role in the formation of PanIN from ADM by regulating ADR-related genes. We also demonstrated that Hes1 is necessary to progress into PDAC.

Hes1, which is transcriptionally activated by Notch signaling, is reported to have an essential role in early pancreatic development. *Hes1*-ablated mice exhibit severe pancreatic hypoplasia due to the depletion of pancreatic epithelial precursors [10, 11]. A recent report also showed that conditional *Hes1* ablation in pancreatic progenitors disrupts the terminal differentiation of acinar cells [12]. Thus, Hes1 has an essential role in the maintenance and differentiation of pancreatic progenitor cells in the development of the pancreas. On the other hand, consistent with previous reports [4, 9, 21], the present study clearly showed that Hes1 is not expressed in acinar cells in adult pancreas, but is confined to ductal cells. In contrast to detailed previous studies at the developmental stage, the role of Hes1 in adult acinar cells, the putative cell of origin of PDAC [5], had not been analyzed. Therefore, to exclude the effect of *Hes1* ablation on embryogenesis, we utilized *Elastase1-CreERT2* mice for inducible and acinar cell-specific ablation of *Hes1*. As a result, reflecting the absence of Hes1 expression in acinar cells, acinar cell-specific *Hes1* ablation in adult mice induced no obvious changes in the pancreatic morphology, size, or expression of differentiation markers compared with WT mice. Furthermore, even with induction of cerulein-induced acute pancreatitis, acinar cell-specific *Hes1*-ablated mice did not exhibit significant differences in susceptibility to pancreatic injury, ADM formation, or acinar cell regenerative ability compared to WT mice. Thus, we concluded that Hes1 is not essential for maintaining the homeostasis of adult mature acinar cells in either normal or injured conditions. These results were different from the recent report by Hidalgo-Sastre et al., in that *Hes1* ablation impaired acinar cell terminal differentiation and regeneration from caerulein-induced acute pancreatitis. We believe that these differences are due to the difference of Cre-inducing mouse used for *Hes1* ablation, i.e., *Elastase1-CreERT2* mice in our work and *Ptf1a-Cre* in Hidalgo-Sastre's. As previously reported in *Foxa2-Cre;Hes1^{fllox}* mice, conditional deletion of *Hes1* in the endoderm results in a switch from ductal progenitor-to-acinar progenitor in the embryonic pancreas [22]. Although *Ptf1a-Cre*, which induces recombination in pancreatic multipotent progenitor cells in the embryonic stage, may be less efficient than *Foxa2-Cre*, it would be still be expected to induce similar fate switch in at least some pancreatic progenitor cells. We speculate that such a fate change might influence the

properties of resulting adult acinar cells, either directly in transdifferentiated acinar cells or indirectly via secondary, non-cell autonomous effects.

In contrast to normal acinar cells, Hes1 expression is broadly observed in ADM, PanIN, and PDAC [4, 9, 21]. In particular, in the *Kras*-driven mouse model, induction of ectopic Hes1 expression was confirmed in the transition of acinar cells to ADM. From these findings, we hypothesized that activation of *KRAS* signaling induces Hes1 in acinar cells. Although several pathways are reported to activate Hes1 expression, such as Notch, Sonic hedgehog, Wnt, etc. [23, 24], our observation of a similar distribution of pErk1/2 and Hes1 in ADM, PanIN, and PDAC supported our hypothesis. Furthermore, using an acinar cell line without *Kras* mutation, we showed that EGF treatment induced Erk1/2 phosphorylation and subsequent *Hes1* mRNA expression, and also that induction of mutant *KRAS* in these cells induced persistent Erk1/2 phosphorylation and sustained Hes1 expression. Notably, these inductions of Hes1 were completely repressed by Mek inhibitor treatment, suggesting a MAPK signaling-dependent Hes1 induction in these cells. In the relationship between Ras/MAPK signaling and Hes1, both Notch signaling-dependent and -independent pathways are reported [9, 25, 26]. Our results from the in vitro experiment that a Notch inhibitor failed to suppress Hes1 induction by mutant *KRAS* activation suggests the existence of a Notch-independent pathway in pancreatic acinar cells. Together, these finding suggests that mutant *KRAS* activation could have a role in the induction of sustained Hes1 expression, especially in the initiation of pancreatic tumorigenesis.

To investigate the role of Hes1 in pancreatic cancer initiation, we utilized a *Kras*-driven mouse model. Recently, using *Ptf1a-Cre;Kras^{G12D};Hes1^{fllox/fllox}* mice, Hidalgo-Sastre et al. reported that pancreas-specific *Hes1* ablation increases ADM formation and accelerates PDAC formation in mice [12]. As described above, however, Hes1 has an essential role in pancreatic development. We had substantial concern about evaluating pancreatic cancer initiation from exocrine cells with altered properties due to *Hes1* ablation by *Ptf1a-Cre* in the embryonic stages. Therefore, to exclude such influences by *Hes1* ablation, we used *Elastase1-CreERT2* mice that were confirmed to have normal homeostasis in adult pancreatic tissue. Surprisingly, contrary to the report by Hidalgo-Sastre et al., *Hes1* ablation almost completely repressed the formation of PanIN lesions in both the *Kras*-driven and cerulein-accelerated *Kras*-driven mouse model. In WT mice, ADM lesions are formed by cerulein-induced pancreatitis and promptly re-differentiate into acinar cells, whereas ADM lesions persist and progress into PanIN lesions in *Kras* mutant mice [15]. We observed that ADM lesions were induced by pancreatitis in *Hes1*-

ablated mice at the same levels as WT mice. More importantly, these *Hes1*-ablated ADM lesions were able to re-differentiate into acinar cells, even with the induction of a *Kras* mutation, based on our precise analyses with daily monitoring or lineage tracing techniques. These findings suggested that *Hes1* is required for sustaining and achieving *Kras*-driven ADR. ADR is promoted by *Sox9* and *Klf4* [5, 6], which induce ductal cell differentiation, and is suppressed by *Ptf1a* and *Mist1* [7, 8], which induce acinar cell differentiation. From this point of view, it is reasonable that *Hes1*, whose expression is restricted to ductal cells, is required for ADR. Indeed, in *Kras*-driven *Hes1*-ablated mice, we demonstrated that *Sox9* expression is significantly reduced, but *Ptf1a* expression is increased after the induction of cerulein-induced pancreatitis. Given the previous reports that *Hes1* and *Sox9* interact with each other [19, 27], it is highly likely that *Hes1* orchestrates these ADR-related genes and promotes *Kras*-driven PanIN formation. This concept is further supported by the results of *Hes1*-dependent *Sox9* expression derived from our in vitro/ in vivo experiment and analysis of the TCGA data. In summary, *Hes1* has an essential role in the formation of PanIN from ADM in the initiation of pancreatic cancer. Sustained *Hes1* expression induced by mutant *Kras* activation may regulate ADR-related genes and promote PanIN formation.

To analyze the role of *Hes1* in pancreatic cancer progression, we utilized a cerulein-accelerated PDAC mouse model in which mutant *Trp53* as well as mutant *Kras* were induced by *Elastase1-CreERT2* so-called KPC mice [28]. As a result, PDAC formation in KPC mice was completely suppressed by *Hes1* ablation. This results suggested that *Hes1* plays an essential roles not only initiation but also progression of pancreatic cancer.

In conclusion, we showed that *Hes1* plays essential roles in multiple steps in PDAC initiation and progression by regulating ADR-related genes.

Materials and methods

Human samples

For immunohistochemistry of human samples, surgically resected specimens were obtained from PDAC patients who had been admitted to Kyoto University Hospital (Kyoto, Japan). For analysis of the relationship between mRNA expression and prognosis in PDAC patients, mRNA expression of all the genes and prognostic data were extracted from the TCGA Research Network (<https://cancergenome.nih.gov/>). Correlations between two genes were explored with cBioPortal (<http://www.cbioportal.org/>) [29, 30].

Animals

All procedures were performed while mice were anesthetized by chloral hydrate (Wako, Japan), isoflurane (Pfizer, New York, NY, USA), or diethyl ether (Sigma-Aldrich, St. Louis, MO, USA), and all efforts were made to minimize the number of animals used and their suffering. The *Hes1* flox mice [31], *Pdx1-Cre* mice [32], *Elastase1-CreERT2* mice [18], *LSL-Kras^{G12D}* mice [33], *LSL-Trp53^{R172H}* mice [34], and *Rosa26-CAG-LSL-tdTomato-WPRE* mice [35] were described previously.

At least five mice in each experimental group were considered to be needed, and randomization and blinding were not used for the analysis of mice model.

Acute pancreatitis was induced by 8 hourly intraperitoneal injections of 100 µg/kg cerulein (Sigma-Aldrich) dissolved in phosphate-buffered saline for 2 consecutive days. For cerulein-induced pancreatitis, the mice were allowed access to food and water ad libitum.

Tamoxifen (Sigma-Aldrich) was dissolved in corn oil at a concentration of 20 mg/ml (Wako). Tamoxifen (200 mg/kg body weight) was subcutaneously administered to 4-week-old mice.

Fasting blood glucose levels from tail vein blood were measured with a glucose meter after overnight fasting.

Histology

Tissue sections were fixed in 10% neutral phosphate-buffered formalin, embedded in paraffin, sectioned at a thickness of 5 µm, and stained with hematoxylin-eosin (Wako).

Cell culture

266-6 cells (American Type Culture Collection, Manassas, VA, USA) were maintained at 37 °C under a humidified atmosphere of 5% CO₂ in Dulbecco's modified Eagle medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Gibco) and 100 µg/ml each of penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA).

Statistics

Statistical analysis was performed using JMP (SAS Institute, Cary, NC, USA) or Excel (Microsoft Corp., Redmond, WA, USA) software. A two-tailed Student's *t* test was used to analyze differences between two groups for continuous data. The chi-squared test was used for categorical data. Pearson's *r* correlation was used to analyze the correlation between two groups. The log-rank test was used in survival studies. A *P* value of <0.05 was considered statistically significant. For *P* values, the following scale was applied:

* $P < 0.05$ and ** $P < 0.01$. Data are presented as means $\pm$ SEM.

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Compliance with ethical standards

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

Ethics approval All animal care and experiments were conducted following the guidelines for the Japan's Act on Welfare and Management of Animals. All animal experiments described herein were approved by the Kyoto University Graduate School (Kyoto, Japan).

Informed consent Written informed consent for the use of resected tissues was obtained from all patients in accordance with the Declaration of Helsinki.

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