GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Hes1 Controls Exocrine Cell Plasticity and Restricts Development of Pancreatic Ductal Adenocarcinoma in a Mouse Model

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Perturbation of pancreatic acinar cell state can lead to acinar-to-ductal metaplasia (ADM), a precursor lesion to the development of pancreatic ductal adenocarcinoma (PDAC). In the pancreas, Notch signaling is active both during development and in adult cellular differentiation processes. *Hes1*, a key downstream target of the Notch signaling pathway, is expressed in the centroacinar compartment of the adult pancreas as well as in both preneoplastic and malignant lesions. In this study, we used a murine genetic *in vivo* approach to ablate *Hes1* in pancreatic progenitor cells (*Ptf1a*^{+/-Cre}; *Hes1*^{fl/fl}). Using this model, we studied the role of *Hes1* in both acinar cell plasticity and pancreatic regeneration after caerulein-induced pancreatitis and in *Kras*^{G12D}-driven PDAC development. We show that, although pancreatic development is not perturbed on the deletion of *Hes1*, terminal acinar differentiation in the adult pancreas is compromised. Moreover, the loss of *Hes1* leads to the impaired regeneration of the exocrine compartment, accelerated fatty metaplasia, and persistent ADM after acute caerulein-induced pancreatitis. In *Kras*^{G12D}-driven carcinogenesis, *Hes1* ablation resulted in increased ADM, decreased formation of high-grade pancreatic intraepithelial neoplasias, and accelerated development of PDAC with shortened survival time. In conclusion, *Hes1* plays a key role in acinar cell integrity and plasticity on cellular insults. Furthermore, *Hes1* is an essential component of the pancreatic intraepithelial neoplasias—to-PDAC route in *Kras*^{G12D}-driven mouse pancreatic carcinogenesis. (*Am J Pathol* 2016; 186: 2934–2944; <http://dx.doi.org/10.1016/j.ajpath.2016.07.025>)

Pancreatic ductal adenocarcinoma (PDAC) has a frightening 5-year survival rate of 5% and is currently the fourth leading cause of cancer-related death in the Western world.¹ Activating oncogenic *Kras* mutations are the key genetic alterations in virtually all PDACs.² Genetically engineered mouse models with an active *Kras*^{G12D} mutation recapitulate formation and progression of preneoplastic lesions to PDAC.³ Acute and chronic pancreatitis accelerate the induction of PDAC in the context of *Kras*^{G12D} activation, with acinar cells adopting ductal features in the process of acinar-to-ductal metaplasia (ADM). ADM development precedes or emerges concomitantly with low-grade

pancreatic intraepithelial neoplasia (PanIN) lesions.^{4–7} Despite the ductal phenotype of most PDACs, acinar cells undergoing SOX9-dependent ADM have been proposed to act as cell of origin of PDACs^{8,9} and acinar dedifferentiation

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has been shown to be critical for malignant transformation.^{10,11} We and others have recently provided evidence for ADM and related atypical flat lesions to be PDAC precursors in mice and humans.^{12–14}

Notch signaling is a key regulator of cell fate and maintenance of multipotent progenitors.^{15–17} Notch receptors are present at the membrane, and on ligand binding, a series of protease cleavages release Notch intracellular domain (NICD). Eventually, NICD translocates to the nucleus to activate the expression of target genes, such as hairy and enhancer of split-1 (*Hes1*).¹⁸ In the pancreas, Notch signaling is required during embryonic development,¹⁹ and to control the plasticity of adult acinar cells.^{20,21} During early embryonic development, *Hes1* is expressed in multipotent progenitors driving a ductal phenotype. Later in embryogenesis, *Hes1* is expressed in exocrine-restricted progenitors, promoting ductal differentiation at the expense of acinar cell fates. In the adult pancreas, *Hes1* is restricted to centroacinar cells and ducts, where it is thought to be involved in the maintenance of a progenitor cell pool, and for repressing the potential of these cells to shift toward acinar differentiation.^{20,22,23} In agreement with its role as negative regulator of pancreatic differentiation, *Hes1*-null mice display pancreatic hypoplasia because of premature differentiation of precursor cells.²⁴ On caerulein-mediated insult, acinar cells transiently dedifferentiate²⁵ and express a progenitor genetic program.^{26,27} In this context, *Hes1* up-regulation is indicative of active Notch signaling, which has been shown to be a requirement for exocrine regeneration.²¹ Finally, Notch signaling is also involved in tumor initiating processes, by influencing the differentiation status of pre-malignant cells, including the development of ADM^{20,28,29} and PanIN lesions,^{3,5,30} and in tumor progression.^{20,31}

Despite this mounting evidence, the functional role of the Notch downstream target, *Hes1*, has not yet been addressed in PDAC development. Herein, we generated mice with conditional inactivation of *Hes1* in pancreatic progenitor cells. We show that *Hes1* is required to maintain acinar cell identity, because deletion leads to fatty metaplasia and ADM, both in caerulein-induced pancreatitis and in *Kras*^{G12D}-based genetically engineered mouse models. In addition, *Hes1* ablation in the *Kras*^{G12D}-driven PDAC model leads to accelerated ADM and PDAC development in concomitance with less advanced PanIN lesions, leading to a significantly shortened overall survival. In summary, we demonstrate a key role of *Hes1* in regulating the plasticity of acinar cells during inflammation and tumor development in a functional *in vivo* approach.

Materials and Methods

Mouse Strains

All animal experiments were conducted in accordance with German Federal Animal Protection Laws and approved by the Institutional Animal Care and Use Committee at the Technical

University of Munich (Munich, Germany). *Hes1*^{fl/fl}²⁴ mice were bred to *Ptf1a*^{+/-Cre}³² and *Kras*^{+/-LSL-G12D3} mice to generate *Ptf1a*^{+/-Cre}; *Hes1*^{fl/fl} (*Hes1*^{Δ/Δ}) and *Ptf1a*^{+/-Cre}; *Kras*^{+/-LSL-G12D}; *Hes1*^{fl/fl} (*Kras*^{G12D}; *Hes1*^{Δ/Δ}). *Hes1*^{fl/fl} and *Ptf1a*^{+/-Cre}; *Kras*^{+/-LSL-G12D} mice served as controls. Mouse experiments were performed according to the German Federal Animal Protection Laws and were approved by the Institutional Animal Care and Use Committees of the government of Bavaria and the Technical University of Munich.

Pancreatic Weight Analysis

Mice were sacrificed, and the whole pancreas was carefully excised without any adjacent tissue and immediately weighed in a precision scale under sterile technique.

Immunohistochemical Staining

Immunohistochemistry was performed as previously described.³³ Histological images were taken on a Zeiss Axiovert Imager (Zeiss, Oberkochen, Germany). The following primary antibodies were used: rat anti-HES1 (1:500; Biozol, Eching, Germany), rat anti-Notch2IC (1:1000; DSHB, Iowa City, IA), rabbit anti-amylase (1:1000; Sigma-Aldrich, Taufkirchen, Munich, Germany), rabbit anti-SOX9 (1:1000; Millipore, Darmstadt, Germany), rabbit anti-E-cadherin (1:500; Cell Signaling, Leiden, the Netherlands), guinea pig anti-insulin (1:500; Dako, Hamburg, Germany), guinea pig anti-glucagon (1:500; Dako), rat anti-CK19 (1:200; DSHB), mouse anti-MUC5AC (1:500; Neomarkers, Fremont, CA), rabbit anti-Ki-67 (1:500; Abcam, Cambridge, UK), goat anti-clusterin (1:500; Santa Cruz, Heidelberg, Germany), and rabbit anti-CLAUDIN18 (1:500; Invitrogen, Karlsruhe, Germany). Immunofluorescence was performed as previously described³³ with the following primary antibodies: goat anti-amylase (1:100; Santa Cruz) and rabbit anti-SOX9 (1:200; Millipore).

Glucose Tolerance Test

Age-matched littermates were fasted overnight before the initial measurement of serum glucose levels. Glucose levels were determined using blood collected from the tail vein (OneTouch Ultra Glucometer; LifeScan, Neuss, Germany). After initial measurement of glucose levels, 1.5 g glucose/kg body weight of 220% glucose solution was injected i.p. Glucose levels were subsequently measured 30, 60, 90, and 120 minutes after initial glucose injection.

Caerulein Treatment

Induction of pancreatitis in 6-week-old mice was performed using the previously described regeneration protocol that consists of 8 hourly i.p. injections of caerulein (Sigma-Aldrich) (50 μg/kg) on 2 consecutive days.²⁷ Mice were sacrificed 1, 3, and 5 days after the final caerulein injection.

Morphometric Quantification

Ki-67 and cleaved-caspase 3 positive cells were scored from images of whole pancreatic tissue sections ($N = 3$ for each genotype). For proliferation analysis, mice received i.p. injections with 100 mg/kg bromodeoxyuridine (Sigma-Aldrich) 2 hours before sacrifice. Counting was performed with AxioVision Viewer software version 4.8 (Zeiss). The percentage of positive cells was calculated by dividing the number of cells positively stained with the respective antibody by the total number of cells in the regions of pancreatic tissue, excluding fatty, edema, or inflammatory tissue. For quantification of MUC5AC-positive PanIN lesions, two slides representing two different pancreatic tissue sections were analyzed using AxioVision Viewer software version 4.8 ($N = 3$ to 6 mice per group).

Histological alterations in pancreata, such as ADM, fibrosis, lipomatosis, ductectasia, and cystic changes, were semiquantitatively determined by an experienced pathologist (B.S.). A numerical system was assigned from 4 to 0 to generate graphs or tables, as follows: strong-indicates 4 (ADM >50% of pancreatic area), moderate indicates 3 (ADM 10% to 50% of pancreatic area), mild indicates 2 (ADM up to 10% of pancreatic area), minimal/slight indicates 1 (single ADMs), and no lesion indicates 0 (no ADM). For specific lesion quantification, ADM and PanINs were manually counted using $\times 200$ magnification (0.98 mm^2) by an experienced pathologist (B.S.). Depending on the size of the pancreas, four to eight fields were quantified, and the ratio of PanIN/ADM was calculated. Differentiation of the murine PDACs was graded as follows: grade 1, tumors with well-formed ducts and low or moderate nuclear polymorphism; grade 2, tumors with moderate-to-high nuclear polymorphism and diminished duct formation; and grade 3, tumors almost, exclusively, composed of solid cell nests with high nuclear polymorphism and, occasionally, of spindle cells.

RNA Isolation and Quantitative RT-PCR

RNA from pancreatic tissue was homogenized with the RNAlater stabilization reagent (Qiagen, Hilden, Germany). Subsequently, RNA was isolated using the RNA-easy kit (Qiagen). SuperScript II Reverse Transcriptase (Invitrogen) was used for cDNA synthesis, according to manufacturer’s protocol. RT-PCR was performed as previously described³³ using the SYBR Green master mix (Roche, Unterhaching, Germany) on a Lightcycler480 system (Roche). Primers used are described in Table 1. Melting curve analysis was performed to ensure product quality and specificity. Expression levels of each transcript were normalized to murine *Cyclophilin* using the $\Delta\Delta C_t$ method. All RT-PCR experiments were performed with $N = 3$ to 6 individual biological samples per group.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism program version 5 (GraphPad Software, Inc., La Jolla, CA). Intergroup comparison was performed using the Mann-Whitney test for nonnormal distributed unpaired data. Differences with a $P < 0.05$ were considered significant. For P values, the following scale was applied: $P < 0.05$, $P < 0.01$, and $P < 0.001$. Data are presented as means $\pm$ SEM. Kaplan-Meier curves were calculated using the survival time for each mouse from the littermate groups. The log-rank test was used to address significant differences between the groups.

Results

Hes1 Is Dispensable during Pancreatic Organogenesis But Required for Acinar Cell Compartment Maintenance and Differentiation

We have previously shown that HES1 is expressed in the adult pancreas and during *Kras*^{G12D}-initiated pancreatic

Table 1 PCR Primers Used for RT-PCR

Forward primer	Sequence (5'-3')	Reverse primer	Sequence (5'-3')
<i>Amy2a_Fw</i>	5'-TGGTCAATGGTCAGCCTTTTTC-3'	<i>Amy2a_Rw</i>	5'-CACAGTATGTGCCAGCAGGAAG-3'
<i>Cyclophilin_Fw</i>	5'-ATGGTCAACCCACCGTGT-3'	<i>Cyclophilin_Rw</i>	5'-TTCTGCTGTCTTTGGAACCTTTGTC-3'
<i>Cela2a_Fw</i>	5'-GCCTGCTGGTTGTGGACTAT-3'	<i>Cela2a_Rw</i>	5'-GTAGTTGCAGCCAGAGAGG-3'
<i>Delta-like1_Fw</i>	5'-CCTGGCTGTGTCAATGGAGT-3'	<i>Delta-like1_Rw</i>	5'-TGGCAGTCCTTTCCAGAGAA-3'
<i>Hes1_Fw</i>	5'-AAAGCCTATCATGGAGAAGAGGCG-3'	<i>Hes1_Rw</i>	5'-GGAATGCCGGGAGCTATCTTTCTT-3'
<i>Hes3_Fw</i>	5'-CCCTGCTTAGCACTGCTGAGA-3'	<i>Hes3_Rw</i>	5'-CAGGGCTCAGAAGGCACTAAA-3'
<i>Hes5_Fw</i>	5'-AGATGCTCAGTCCCAAGGAG-3'	<i>Hes5_Rw</i>	5'-TAGCCCTCGCTGTAGTCCTG-3'
<i>Hes7_Fw</i>	5'-ATCAACCGCAGCCTAGAAGA-3'	<i>Hes7_Rw</i>	5'-CACGGCGAACTCCAGTATCT-3'
<i>Hey1_Fw</i>	5'-CACTGCAGGAGGGAAGGTTATT-3'	<i>Hey1_Rw</i>	5'-GCCAGGCATTCCCGAAAC-3'
<i>HeyL_Fw</i>	5'-TGCCAGGAGCATAGTCCCAA-3'	<i>HeyL_Rw</i>	5'-TGGTAGAACACTGCTCCCGC-3'
<i>Mist1_Fw</i>	5'-GCGCGTACGGCCTCG-3'	<i>Mist1_Rw</i>	5'-GGGCCGGTTTTTGGTCTTCAT-3'
<i>Notch1_Fw</i>	5'-ACATCCGTGGCTCCATTGTCTA-3'	<i>Notch1_Rw</i>	5'-TCTTGTAAGGAATATTGAGGCTGC-3'
<i>Notch2_Fw</i>	5'-GCCTCCCATCGTGACTTTCC-3'	<i>Notch2_Rw</i>	5'-GGGCAACTGGACTGCGTC-3'
<i>Pdx1_Fw</i>	5'-TGCCACCATGAACAGTGAGG-3'	<i>Pdx1_Rw</i>	5'-GGAATGCGCACGGGTC-3'
<i>Rbpj_Fw</i>	5'-TGGATGCAGACGACCTGTAT-3'	<i>Rbpj_Rw</i>	5'-TGGAGTGGCCTGAAATTGG-3'
<i>RbpL_Fw</i>	5'-ATGCCTTGCCACAGAGAAGGT-3'	<i>RbpL_Rw</i>	5'-TGCCAATGATGGTCCAGCA-3'

carcinogenesis in ADM, PanIN, and malignant lesions.²⁸ To investigate the role of *Hes1* in pancreatic organogenesis, we generated *Ptf1a*^{+/Cre};*Hes1*^{fl/fl} mice (hereafter referred to as *Hes1*^{Δ/Δ}^{24,32}) (Supplemental Figure S1A). *Hes1*^{Δ/Δ} mice were born at expected mendelian ratio and showed no apparent macroscopic phenotype. At 4 weeks of age, we did not observe histomorphological abnormalities in the pancreas (Figure 1A). Effective deletion of *Hes1* was confirmed by RT-PCR and Western blot (Supplemental Figure S1, B and C). As expected, *Hes1*^{Δ/Δ} mice did not have HES1 protein expression (Supplemental Figure S1D), but still maintained expression of active NOTCH2 intracellular domain in centroacinar cells (Supplemental Figure S1D). Transcriptional profiling revealed no differences in selected Notch family members between *Hes1*^{Δ/Δ} and control mice (Supplemental Figure S2). Staining for insulin and glucagon showed comparable distribution of both hormones, islets of Langerhans were of comparable size, and no endocrine pancreatic insufficiencies could be observed (Supplemental Figure S1, E and F). Therefore, we observed no obvious defects in pancreatic organogenesis on *Hes1* deletion, which is in agreement with our previous results for *Notch1* and *Notch2* conditional knockout mice.²⁸

Interestingly, we found a modest yet significant decrease in pancreas/body weight ratio in *Hes1*^{Δ/Δ} mice compared to controls, although no differences in body weight could be observed (Figure 1B). However, apoptosis was equally low in both groups, but proliferation was significantly reduced in *Hes1*^{Δ/Δ} mice at 4 weeks of age (Supplemental Figure S3, A and B). Furthermore, aged *Hes1*^{Δ/Δ} mice presented massive pancreatic atrophy and fatty metaplasia (Figure 1C). This could already be observed at the age of 12 weeks, with vacuoles accumulating in the cytoplasm of acinar cells (Figure 1D and Supplemental Figure S3C). Islets were dispersed within the adipose tissue (Figure 1D and Supplemental Figure S3C) but remained functional (Supplemental Figure S3D). This fatty phenotype has previously been reported for other mouse models in the pancreas.^{9,34,35} Overall, the lower pancreas/body weight ratio, reduced proliferation, and fatty metaplasia observed in *Hes1*^{Δ/Δ} mice suggest a critical role for *Hes1* in maintaining acinar cell differentiation and homeostasis.

To further study the role of *Hes1* in acinar cell integrity, we investigated the expression levels of the acinar-specific genes *Amylase*, *Elastase*, and *Mist1*,³⁶ which were significantly decreased in *Hes1*^{Δ/Δ} mice (Figure 1E). In contrast, markers for epithelial and ductal compartments were equally distributed between both groups (Supplemental Figure S3E). Interestingly, the expression of the progenitor marker SOX9 was markedly increased in acinar cells in *Hes1*^{Δ/Δ} mice, compared to control mice (Figure 1F and Supplemental Figure S3, F and G). Similar results could be observed in our previously described *Notch2*^{Δ/Δ} mice²⁸ (Figure 1G and Supplemental Figure S3H). Furthermore, *Hes1*^{Δ/Δ} mice showed expression of Clusterin, a marker of immature acinar cells²¹ (Figure 1H), suggesting that ablation of *Hes1* leads to compromised terminal acinar cell differentiation.

Hes1 Is Essential for Acinar Regeneration after Acute Pancreatitis

Notch signaling up-regulates its target gene *Hes1* after caerulein-induced pancreatitis, and it is required for exocrine regeneration.²¹ To determine the role of *Hes1* in acinar cell plasticity in a model of regeneration, we used caerulein-induced pancreatitis in 6-week-old *Hes1*^{Δ/Δ} mice. Quantification of total-to-exocrine area at day 3 confirmed that loss of *Hes1* drastically reduced the viable exocrine tissue by half (Figure 2, A and B). At day 1, caerulein-induced pancreatitis led to abundant inflammation, edema, and dedifferentiation of acinar cells in both control and *Hes1*^{Δ/Δ} mice (Figure 2C). Although inflammation and acinar dedifferentiation were comparable in both groups at day 1, partial acinar redifferentiation at day 3 and nearly complete pancreatic regeneration at day 5 were apparent, only, in pancreata of control mice (Figure 2C). In contrast, *Hes1*^{Δ/Δ} mice presented compromised regeneration, persistent ductal metaplasia, and pancreatic fibrosis, combined with severe acinar atrophy (Figure 2C). Interestingly, *Hes1*^{Δ/Δ} mice also displayed heavy replacement of exocrine by fatty tissue (Figure 2C). Despite the fact that the amount of fatty tissue was reduced in the total pancreas area by day 5 (compared to day 3) (Figure 2D), regeneration of the exocrine pancreas in *Hes1*^{Δ/Δ} mice was impaired.

To further characterize the role of *Hes1* in acinar regeneration, we performed expression analysis of pancreatic differentiation markers at day 3. *Hes1*^{Δ/Δ} mice displayed compromised acinar redifferentiation and persistent ductal metaplasia (shown by amylase and CK19 staining), with strong expression of the immature acinar cell marker Clusterin²¹ and the progenitor marker SOX9 in ADM lesions (Figure 2E). All lesions were negative for Alcian blue staining (Figure 2E). Furthermore, we found that immature acinar cells with strong expression of SOX9 were prone to ADM formation, consistent with previous findings.³⁷ Next, we assessed proliferation and apoptosis in this model. We observed a significant decrease in Ki-67 positive cells and a significant increase in cleaved-caspase3 positive cells in *Hes1*^{Δ/Δ} mice compared to control (Figure 2, F and G). These results show that *Hes1* is required for pancreatic regeneration after caerulein-induced cellular insult. Moreover, loss of *Hes1* is associated with decreased cell proliferation and increased apoptosis.

Deletion of *Hes1* in *Kras*^{G12D}-Based Genetically Engineered Mouse Models Results in Highly Proliferative ADM But Fewer High-Grade PanIN Lesions

We next investigated the role of *Hes1* in the context of oncogenic *Kras* activation. We interbred *Hes1*^{Δ/Δ} mice with the KC mouse model, *Ptf1a*^{+/Cre};*Kras*^{+/LSL-G12D} (named *Kras*^{G12D} hereafter³) to generate *Kras*^{G12D};*Hes1*^{Δ/Δ} mice. *Kras*^{G12D};*Hes1*^{Δ/Δ} mice showed an increased replacement of large areas of pancreatic parenchyma by metaplastic ductal

Figure 1 Acinar cell maturation and differentiation is impaired in *Hes1*^{Δ/Δ} mice. **A:** Macroscopic images of pancreata and hematoxylin and eosin (H&E) staining of 4-week-old mice of the indicated genotypes. *Hes1*^{Δ/Δ} mice present normally developed acini (**asterisk**), ducts (**arrowhead**), and islets (**black arrow**). **B:** Body weight and pancreas/body weight ratio of control (ctrl) and *Hes1*^{Δ/Δ} mice. **C:** Macroscopic image of pancreas and H&E staining of 52-week-old *Hes1*^{Δ/Δ} mice. Small areas of pancreatic tissue are still visible but most has been replaced by fatty tissue. **D:** H&E staining of 12-week-old *Hes1*^{Δ/Δ} mice; **black arrows** highlight vacuoles and zymogen granules in the cytoplasm of acinar cells. **E:** RNA expression of acinar markers in 4-week-old mice of the indicated genotypes. **F and G:** Immunofluorescence for the indicated genes in 4-week-old mice. **White arrows** show centroacinar cells. **H:** Immunohistochemistry in 4-week-old mice of the indicated genotypes. **Asterisk** highlights Clusterin deposits. Data are presented as means ± SEM (**B** and **E**). *N* = 6 (**B**, control mice); *N* = 5 (**B**, *Hes1*^{Δ/Δ} mice, and **E**). **P* < 0.05, ***P* < 0.01. Scale bars: 50 μm (**A**, **D**, **G**, and **H**); 5000 μm (**C**); 10 μm (**F**).

Figure 2 Deletion of *Hes1* impairs regeneration of exocrine tissue after acute pancreatitis. **A:** Hematoxylin and eosin (H&E) staining in mice of the indicated genotypes. Although control mice show regenerated acinar tissue, *Hes1*^{Δ/Δ} mice present large areas of exocrine tissue replaced by adipose tissue, as well as persistent inflammation and duct-like structures. **B:** Ratio of total pancreatic area/exocrine area at day 3. **C:** Representative H&E staining of the histological progression of the pancreas after acute pancreatitis induction at the indicated time points. **D:** Ratio of total pancreatic area/exocrine area is similar in control (ctrl) and *Hes1*^{Δ/Δ} mice at day 5. **E:** Immunohistochemical staining for the indicated markers in day 3 tissues. **F:** Representative immunohistochemical staining for the indicated markers. **G:** Morphometric quantification of the staining in F. Values are shown as means ± SEM (B, D, and G). *N* = 3 per group (G). ***P* < 0.01, ****P* < 0.001. Scale bars: 2000 μm (A); 50 μm (C, E, and F).

Figure 3 Loss of *Hes1* leads to strong and highly proliferative ADM in *Kras*^{G12D}-driven carcinogenesis. **A:** Representative hematoxylin and eosin (H&E) staining of mice at 12 weeks of age. The **third panel** highlights the different lesions: PanIN in control mice and ADM in *Kras*^{G12D};*Hes1*^{Δ/Δ} mice. **B:** ADM scoring (0, no lesion; 1, minimal ADM; 2, mild; 3, moderate; 4, strong) and quantification of lesion type in mice of the indicated genotypes. **C:** Immunohistochemistry for the indicated markers. **D:** Immunofluorescence for the indicated proteins in 12-week-old mice. **E:** Immunofluorescence for the indicated proteins in 12-week-old mice of the indicated genotype. **F** and **G:** Real-time PCR for the indicated genes in primary cell lines isolated from mice of the indicated genotypes. Values are shown as means ± SEM (**B**, **F**, and **G**). *N* = 5 (**B**). **P* < 0.05, ***P* < 0.01. Scale bars: 50 μm (**A**, **C**, and **E**); 10 μm (**D**). HPF, high-power field.

structures, abundant cystic transformation, and fatty acinar cells, in comparison to aged matched controls (Figure 3A and Supplemental Figure S4A). Pathological analysis showed increased ADM and low-grade PanINs (Figure 3B). Although CK19-positive ADM (Figure 3C) and cystic lesions were

accompanied by fatty metaplasia (Supplemental Figure S4A), only few and less advanced PanIN lesions, positive for Claudin18, MUC5AC, and Alcian blue, were detected in *Kras*^{G12D};*Hes1*^{Δ/Δ} mice compared to *Kras*^{G12D} controls (Figure 3C). These ADM and PanIN lesions were negative for

HES1 (Supplemental Figure S4B). Moreover, Hes1 mRNA was absent in primary cell lines isolated from PDAC of *Kras*^{G12D};*Hes1*^{Δ/Δ} mice compared to *Kras*^{G12D} controls (Figure 3F). In areas without neoplastic lesions, the SOX9 expression pattern was comparable to that of *Hes1*^{Δ/Δ} and *Kras*^{G12D};*Notch2*^{Δ/Δ} mice (Figure 3, D and E, and Supplemental Figure S4C). Furthermore, we found higher expression of SOX9 mRNA in primary cell lines isolated from tumors from *Kras*^{G12D};*Hes1*^{Δ/Δ} mice compared to *Kras*^{G12D} controls (Figure 3G). These results support a route to PDAC in the absence of *Hes1*.

The higher incidence of ADM and low-grade PanIN (Figure 3B), in *Kras*^{G12D};*Hes1*^{Δ/Δ} mice, held true at advanced ages (4 to 9 months and terminal) (Figure 4A, Supplemental Figure S4, A and B, and Tables 2 and 3). We observed that MUC5AC-negative ADM lesions were highly proliferative in *Kras*^{G12D};*Hes1*^{Δ/Δ} mice compared to controls (Supplemental Figure S4, D and E). In a cohort of *Kras*^{G12D};*Hes1*^{Δ/Δ} animals kept for survival analysis, PDACs developed in 65% of the mice sacrificed when showing signs of morbidity. This contributed to significantly reduce the median survival of *Kras*^{G12D};*Hes1*^{Δ/Δ} mice compared to *Kras*^{G12D} controls (Figure 4B). Notably, pancreata of *Kras*^{G12D};*Hes1*^{Δ/Δ} mice showed no presence of high-grade PanIN lesions (Figure 4A and Table 3). Next, we

analyzed HES1 protein levels in human samples using a human tissue microarray ($N = 56$)^{38–40} (Figure 4C). Higher HES1 levels correlated with lower tumor grades (grades 1 and 2, well to moderately differentiated tumors), whereas low HES1 expression was found in patients with high tumor grade (grade 3, poorly differentiated but more aggressive) (Figure 4C). However, HES1 expression did not correlate with survival (data not shown).

Discussion

Recent studies suggest that perturbation of acinar cell integrity is a key factor for ADM development and, in the setting of oncogenic *Kras*^{G12D} signaling, a potential route to PDAC development.^{8,10,11} Notch signaling has been linked to ADM formation and is essential for pancreatic exocrine regeneration^{15–17,21,22,41} and PDAC development.^{20,31,42} Although it has been shown that NOTCH1 and NOTCH2 have different roles in PDAC development,^{21,28} the role of the main NOTCH downstream target, *Hes1*, has not been elucidated in this setting so far.

Herein, we investigated the role of *Hes1* in acinar cell regeneration and PDAC development using a genetic approach. We show that deletion of *Hes1* results in delayed exocrine tissue regeneration, accelerated fatty pancreatic

Figure 4 Concomitant loss of *Hes1* and activation of *Kras*^{G12D} leads to accelerated pancreatic ductal adenocarcinoma (PDAC). **A:** Hematoxylin and eosin (H&E) staining at 6 and 9 months of age and terminal stage. **B:** Kaplan-Meier survival curve. *Kras*^{G12D} mice have a significantly prolonged survival compared to *Kras*^{G12D};*Hes1*^{Δ/Δ} mice. **C:** Immunohistochemistry in human PDAC. Graph showing relative intensity of HES1 expression in a human tissue microarray according to tumor grading [grades (G) 1, 2, and 3]. $N = 56$ (C). $^{**}P < 0.01$. Scale bar = 50 μ m (A and C).

Table 2 Pathological Analysis of Pancreata Lesions from *Kras*^{G12D};*Hes1*^{Δ/Δ} Mice at the Indicated Time Points

Age (days)	ADM	Fibrosis	Lipomatosis	Ductectasia	Cystic transformation	PanIN			Tumor	Comments
						1	2	3		
133	4	4	3	2	0	3	2	0		PanINs low proliferating
133	3	4	0	0	4	0	0	0		Epithelia of cysts low proliferating
133	3	3	3	2	0	3	0	0		
140	3	4	3	4	3	2	0	0	Incipient grade 3 carcinoma	Flat atypia, focal moderately proliferating
161	4	4	4	0	4	0	0	0		
224	4	4	3	3	0	0	2	0		Mucocele-like lesions; flat atypia, moderately proliferating, epithelia of cysts low proliferating

Histological alterations in pancreata, such as ADM, fibrosis, lipomatosis, ductectasia, and cystic transformation, were semiquantitatively determined, as described in [Materials and Methods](#).
ADM, acinar-to-ductal metaplasia; PanIN, pancreatic intraepithelial neoplasia.

atrophy, and ADM development on caerulein-induced acute pancreatitis. Furthermore, absence of *Hes1* maintains the acinar cell compartment in a dedifferentiated state, with lower proliferative activity. This phenotype supports previous studies that link impaired acinar cell maturation with altered NOTCH signaling.^{9,11,43,44} Thus, we hypothesize that the regenerative ability of NOTCH1 requires its major target gene, *Hes1*. In the adult pancreas, NOTCH1 is expressed in acinar cells, whereas NOTCH2 expression is restricted to the ductal compartment and centroacinar cells. Therefore, it is conceivable that regeneration after pancreatitis occurs from the acinar compartment, whereas acinar maintenance and integrity may involve centroacinar cells.

Multiple studies found Notch signaling to promote ADM and PanIN formation,^{20,21,29,31,44} suggesting a role during PDAC initiation and progression. In addition, NOTCH mediates transforming growth factor- α -induced changes in epithelial differentiation during pancreatic tumorigenesis²⁰; and on deletion of the NOTCH transcription factor *Rbpj*, centroacinar cells rapidly transform into acinar cells.²² Herein, we describe that loss of *Hes1* in a *Kras*^{G12D}-based endogenous PDAC mouse model enhances ADM formation and tumor development while preventing high-grade PanIN

formation, resembling features of the *Notch2* knockout phenotype in pancreatic cancer development.²⁸ The comparable increased expression pattern of SOX9 (a key regulator of acinar-to-ductal reprogramming³⁷) in *Hes1* and *Notch2* knockout mice also supports this observation. Furthermore, the ADM lesions in *Kras*^{G12D};*Hes1*^{Δ/Δ} mice were highly proliferative, as seen in other studies.¹² These results suggest a direct ADM-to-PDAC route, in agreement with the existence of different progenitors leading to PDAC development, including a direct ADM/atypical flat lesion-to-PDAC route in mice and humans.^{14,28} Loss of *Hes1* in centroacinar cells allows differentiation into acinar cells^{20,22,23}; and because impaired redifferentiation within the acinar compartment has been proposed to promote PDAC development,^{5,13} *Hes1* may, in fact, protect acinar cells from undergoing KRAS-driven malignant transformation. Finally, the fact that HES1 expression is decreased in human poorly differentiated but highly aggressive grade 3 tumors may correlate with the higher proliferation and accelerated development of tumors observed in our *Kras*^{G12D};*Hes1*^{Δ/Δ} mice.

In many cancer types, including PDAC, Notch signaling has been considered an attractive therapeutic target. However,

Table 3 Pathological Analysis of Lesion Types in *Kras*^{G12D};*Hes1*^{Δ/Δ} Mice at the End-Point Stage

Age (days)	PanIN	PDAC	Grade	Parenchyma
112	Minimal 1a	No		Marked ductectasia
160	Moderately 1a	No		Almost complete cystic
177	No residual pancreas	Yes	2–3	Ductectasia
186	Marked 1b, few 2	No		Complete fibrosis/ADM
245	Moderately 1a–b, focally 3, flat atypia?	Yes	3	Moderate ductectasia
269	No residual pancreas	Yes	2–3	No residual pancreas
387	Marked 1b and 2	No		Complete fibrosis/ADM
399	No residual pancreas	Yes	1–2	No residual pancreas
445	Moderately 1a–b, few 2	Yes	ND	Marked fibrosis/ADM
469	Marked 1a–b	Yes	2–3	Marked fibrosis/ADM
479	1a	Yes	1–2	Marked ductectasia

PanIN lesion determination and grading of the murine PDACs were performed as described in [Materials and Methods](#).
ADM, acinar-to-ductal metaplasia; ND, not determined; PanIN, pancreatic intraepithelial neoplasia; PDAC, pancreatic ductal adenocarcinoma.

our current and previous studies²⁸ highlight the importance of homeostatic maintenance of Notch signaling levels in the pancreatic exocrine compartment. Furthermore, the observed cell-type and context-dependent effects of Notch and its targets, including *Hes1*, advise caution when evaluating Notch inhibition in PDAC treatment.

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Supplemental Data

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