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Notch2 is required for progression of pancreatic intraepithelial neoplasia and development of pancreatic ductal adenocarcinoma

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Pancreatic cancer is one of the most fatal malignancies lacking effective therapies. Notch signaling is a key regulator of cell fate specification and pancreatic cancer development; however, the role of individual Notch receptors and downstream signaling is largely unknown. Here, we show that Notch2 is predominantly expressed in ductal cells and pancreatic intraepithelial neoplasia (PanIN) lesions. Using genetically engineered mice, we demonstrate the effect of conditional Notch receptor ablation in *Kras*^{G12D}-driven pancreatic carcinogenesis. Deficiency of *Notch2* but not *Notch1* stops PanIN progression, prolongs survival, and leads to a phenotypical switch toward anaplastic pancreatic cancer with epithelial–mesenchymal transition. By expression profiling, we identified increased Myc signaling regulated by Notch2 during tumor development, placing Notch2 as a central regulator of PanIN progression and malignant transformation. Our study supports the concept of distinctive roles of individual Notch receptors in cancer development.

genetically engineered mice | K-Ras | Myc | Notch | pancreatic cancer

Pancreatic ductal adenocarcinoma (PDAC) remains a devastating disease despite tremendous therapeutical efforts. PDAC derives from several preneoplastic lesions, including pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasm, and mucinous cystic neoplasm (MCN), of which PanINs are the most common precursors (1). PanINs typically progress through defined histological and molecular stages, with the most advanced PanIN3 lesion being defined as carcinoma in situ (2). Because of early metastatic spread, PanIN3 represents the latest curable precursor lesion. Thus, defining the regulators of PanIN initiation and progression is of utmost importance.

Recapitulation of human pancreatic carcinogenesis was greatly advanced by generating mice with pancreas-specific activation of endogenous oncogenic *Kras*^{G12D} (3). The ongoing characterization of relevant signaling pathways in pancreatic carcinogenesis using genetically engineered mouse models has helped to depict the enormous plasticity in precursors to PDAC. Despite activation of cell fate regulating signaling pathways such as Hedgehog, Wnt, and Notch signaling (3–9), the precise role of these pathways remains largely unclear.

The Notch signaling pathway plays a pivotal role in cell fate and differentiation decisions, and its activation early in the carcinogenic process suggests a role in initiation of transformation. Although the cell of origin in PDAC has not been decisively identified, activation of Notch signaling during PanIN initiation probably presents a pivotal step for transformation. In several murine models of PDAC, expression of the Notch target gene *Hes1* was increased in PanIN lesions (3, 5, 8, 9). In a recent study, chemical inhibition of Notch activation completely blocked tumor progression in vivo (10). Conversely, Murtaugh and co-workers (11) described a PanIN-promoting effect of Notch activation in *Kras*^{G12D}-driven PanIN development. However, the specific role of individual Notch receptors and the downstream events have so far not been determined.

Here, we describe the effect of pancreas-specific ablation of *Notch1* and *Notch2* in *Kras*^{G12D}-driven pancreatic carcinogenesis, taking advantage of the nonessential role of Notch1 and Notch2 during pancreatogenesis (12). We show that Notch1 and Notch2 are expressed in pancreatic acinar and ductal cells, respectively. Conditional ablation of *Notch2* but not *Notch1* leads to an abrogation of PanIN progression, development of MCN-like lesions, and increased survival. Identification of Notch2-regulated Myc signaling during carcinogenesis points to a central role of Notch2 in controlling PanIN progression and tumor differentiation.

Results

Notch1 and Notch2 Are Expressed in Different Compartments in Adult Pancreata and Are Activated in *Kras* Mice During PanIN Development. To determine the expression of members of the Notch signaling family during pancreatic carcinogenesis, *Kras*$^{+/LSL\text{-}G12D}$ mice were crossed to *Ptf1a*$^{+/Cre(ex1)}$ mice (referred to as *Kras*; Fig. S1*C*), as previously described (9). Notch1 and Notch2 were predominantly expressed in whole-tissue mRNA from WT and *Kras*^{G12D}-induced pancreata compared with low expression of Notch3 and Notch4 (Fig. 1*A*). In *Kras* pancreata at 9 wk of age, when only a few PanIN1 lesions are notable, increased expression of Notch2 and the Notch target gene Hes1 but not Notch1 was observed, similar to previous reports (5). During progression, we noted a significant increase in Notch2 and Hes1 expression, whereas Notch1 was further reduced. Notch3 was also increased, albeit at lower total expression levels (Fig. 1*B*). This expression pattern correlated well with an increase in CK19 and a decrease in amylase expression, suggesting that Notch2 is expressed in CK19$^+$ PanINs, whereas Notch1 may be predominantly expressed in acinar cells. To test this hypothesis, we used transgenic *Notch1-GFP* and *Notch2*^{lacZ} knockin reporter mice (13, 14) to localize Notch1 and Notch2 expression in WT and *Kras* mice. In WT pancreata, we found X-Gal as a surrogate for Notch2 expression in ductal but not acinar or islet cells (Fig. 1*C*). Moreover, X-Gal$^+$ cells were notable in the typical centroacinar position thought to be a presumed progenitor cell compartment (15) (Fig. 1*C*). In

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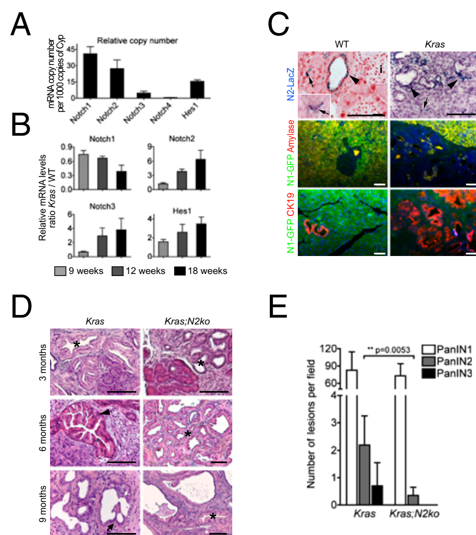

Fig. 1. Expression analysis of Notch receptors in WT and $Kras^{G12D}$-induced pancreata. (*A*) Transcript levels of Notch receptors and Hes1 in relation to cyclophilin gene expression in WT pancreata (n = 3). (*B*) Quantification of Notch receptor and Hes1 gene expression at indicated time points in *Kras* pancreatic tissue. Values represent WT-to-*Kras* tissue ratios of relative expression levels (n = 4). (*C*) Expression of Notch1 and Notch2 in distinct compartments of 18-wk-old WT and *Kras* pancreas using Notch1 and Notch2 reporter mice. Arrows indicate centroacinar cells, and arrowheads point to X-Gal⁺ ducts and PanINs. i, islets. (*D*) H&E staining of 3-, 6-, and 9-mo-old *Kras* and *Kras;N2ko* pancreata. Asterisks indicate PanIN1, arrowhead points to PanIN2, and arrow indicates PanIN3 lesions. Note the absence of PanIN2/3 in *Kras;N2ko* mice. (Scale bars: 50 μm.) (*E*) Quantification of PanINs in 9-mo-old *Kras* (n = 4) and *Kras;N2ko* (n = 5) mice shows a significant reduction in PanIN2 and absence of PanIN3 lesions in *Kras;N2ko* mice.

Kras:Notch2^{lacZ} mice, X-Gal expression was detectable in PanIN lesions and the surrounding stroma (Fig. 1*C*). GFP expression as a surrogate for Notch1 was found in normal acinar cells, as previously described (16), but was hardly ever detectable in PanIN lesions (Fig. 1*C*). In summary, these expression data are consistent with Notch2 as the predominant Notch receptor in ductal, centroacinar, and PanIN cells as suggested previously (5).

PanIN Development and Progression in *Notch*-Ablated Pancreata. To analyze the effect of *Notch1* and *Notch2* deficiency in pancreatic carcinogenesis, we crossed previously described floxed *Notch1*$^{fl/fl}$ and *Notch2*$^{fl/fl}$ mice (17) with *Ptf1a*$^{+/Cre(ex1)}$ mice (18) for generation of *Ptf1a*$^{+/Cre(ex1)}$;*Notch1*$^{fl/fl}$ and *Ptf1a*$^{+/Cre(ex1)}$;*Notch2*$^{fl/fl}$ mice, respectively (called *N1ko* and *N2ko* mice hereafter). These mice were born at the expected Mendelian ratio, and successful recombination of the floxed loci was confirmed by PCR (Fig. S1 *A* and *B*). *N1ko* mice have been previously described to show no major pancreatic abnormalities (16). Similarly, *N2ko* adult pancreata displayed no obvious morphological or functional abnormalities (Fig. S2). However, in mice older than 12 mo of age, we often noted a slight to moderate degree of focal exocrine atrophy with adipose tissue accumulation.

To study the role of Notch1 and Notch2 during pancreatic carcinogenesis, we crossed *N1ko* and *N2ko* mice with *Kras* mice for generation of *Kras;N1ko* and *Kras;N2ko* mice, respectively. Notably, *Kras;N2ko* mice showed no PanIN progression over time, whereas *Kras* and *Kras;N1ko* mice developed higher grade PanIN lesions, suggesting that Notch2 is involved in PanIN progression (Fig. 1 *D* and *E*). PanIN lesions from all genotypes expressed typical markers such as CK19 and MUC5AC and, somewhat surprisingly, HES1 (Tables S1–S3).

Development of MCN-Like Lesions in *Kras;N2ko* Mice. Frequently, albeit not in all mice, *Kras;N2ko* mice developed moderate to very large multilocular cysts. These cysts most often developed in the splenic part of the pancreas and showed a mucinous columnar epithelium resembling human MCN (Fig. S3 *A* and *B*). Rarely, goblet cells, high-grade dysplasia, and invasion into the adjacent stroma were noted. To characterize these lesions further, various markers, including those found in human MCNs, were analyzed. The cystic epithelial cells expressed PDX1, MUC5AC, and HES1, thus showing similar characteristics as PanIN lesions (Table S3). Consistent with the observation of an MCN-like preneoplastic lesion, we found an ovarian-like stroma surrounding the cystic lesions with estrogen receptor (ER)-positive and progesterone receptor-positive nuclei characteristic for human MCNs (19) (Fig. S3*B* and Table S7). To see whether the MCN-like lesions were derived from *Notch2*-deficient cells, cell lineage analysis was performed by crossing the *Rosa26R*$^{+/LSL\text{-}lacZ}$ reporter strain to *Kras;N2ko* mice. Indeed, we found all PanIN and MCN lesions to be X-Gal⁺ (Fig. 2*C*).

Distinct Roles for Notch1 and Notch2 During Tumor Development. For analysis of PDAC development, a cohort of mice was followed for signs of disease progression or death. *Kras* and *Kras;N1ko* mice developed PDAC with similar characteristics regarding age of tumor development, tumor differentiation, rate, and sites of metastasis (Tables S4–S6). *Kras;N1ko* mice showed a slight, albeit not significant, reduction in median survival compared with *Kras* mice, supporting a nononcogenic role of Notch1 in *Kras*^{G12D}-driven pancreatic carcinogenesis (Fig. 2*A*). However, in *Kras;N2ko* mice, a largely altered carcinogenic process was notable. These mice survived significantly longer than *Kras* and *Kras;N1ko* mice and only very rarely developed PDAC with ductal differentiation. Instead, *Kras;N2ko* mice either died without development

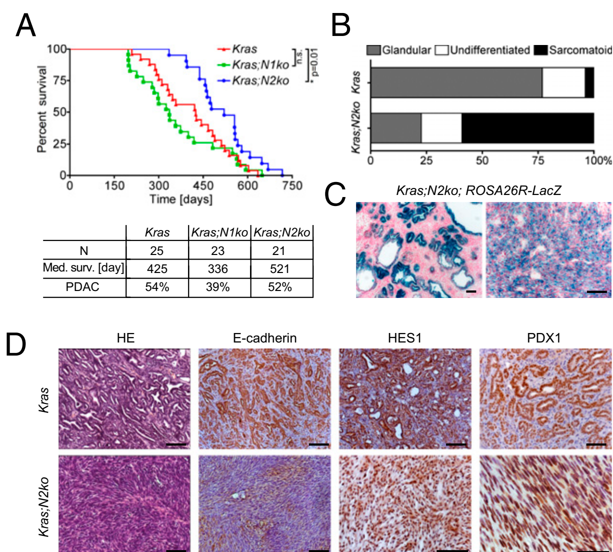

Fig. 2. Deficiency of *Notch2* prolongs survival and delays development of anaplastic PDAC. (*A*) Kaplan–Meier survival data and PDAC development of *Kras*, *Kras;N1ko*, and *Kras;N2ko* mice. *Kras;N2ko* mice have significantly prolonged survival compared with *Kras* and *Kras;N1ko* mice ($P < 0.02$). n.s., not significant. (*B*) Tumor differentiation analysis reveals more anaplastic PDAC in *Kras;N2ko* mice compared with *Kras* mice. (*C*) Positive X-Gal staining shows Cre-induced recombination in cells of MCN-like cysts and anaplastic PDAC in *Kras;N2ko;Rosa26R*$^{+/LSL\text{-}lacZ}$ mice. (*D*) Histological and immunohistochemical analysis of *Kras* and *Kras;N2ko* tumors. Expression of E-cadherin in *Kras* PDAC and low to absent expression in *Kras;N2ko* tumors. The Notch targets HES1 and PDX1 are expressed in tumors derived from both genotypes. (Scale bars: 50 μm.)

of PDAC or developed highly aggressive anaplastic PDAC at a very advanced age (Fig. 2 *A* and *B* and Tables S4–S6). Histologically, most of these tumors were very large, showing a sarcomatoid cell pattern with a high proliferative index. Although we observed tumor areas that displayed features of poorly differentiated PDAC, we practically never observed G1/2 grades. Anaplastic PDAC showed an absence or low expression of E-cadherin and expressed PDX1, indicating its pancreatic origin (Fig. 2*D*). Lineage tracing showed PanIN and anaplastic PDAC development from *Notch2*-ablated pancreatic cells (Fig. 2*C*). Surprisingly, as was seen in MCN-like lesions, many cells expressed HES1, suggesting Notch2-independent regulation (Fig. 2*D*). *Kras;N1ko* and *Kras;N2ko* PDAC showed an absence of the respective Notch receptor, whereas expression was notable in *Kras* cancer cells (Figs. S1*D* and S4). To determine whether deficiency of *Notch2* led to up-regulation of other Notch receptors, we tested *Kras* and *Kras;N2ko* PDAC cells for expression of Notch1–4. Here, we did not detect a consistent compensatory expression pattern of other Notch receptors in *Kras;N2ko* mice (Fig. S4).

Molecular Analysis of Key Signaling Pathways in *Notch2*-Deficient PDAC. Analysis of genetic alterations typically found in PDAC showed no differences in *p16Ink4a*, *p19Arf*, *p53*, and *Smad4* status between low-passage cancer cells isolated from *Kras* and *Kras;N2ko* PDAC (Tables S8 and S9). Consistent with low E-cadherin expression, we found increased levels of Twist, Snail, Slug, vimentin, and TGF-β1 in *Kras;N2ko* cancer cells, suggesting a high rate of epithelial-to-mesenchymal transition (EMT) (Fig. 3*A*). Because EMT has been associated with TGF-β signaling, we next tested integration of the pathway. Using a wound-healing assay, we found significantly increased cell migration of *Notch2*-deficient cancer cells (Fig. 3*B*). Gene set enrichment analysis (GSEA) was performed using pancreatic tissue at 7 d of age and cancer cells isolated from *Kras* and *Kras;N2ko* PDAC, as previously described (9), and revealed significant enrichment of several TGF-β signatures in *Kras;N2ko* preneoplastic tissue and cancer cells (Fig. 3*C* and Tables S10 and S11). Next, expression of E- and N-cadherin was studied in the presence of a TGF-β receptor inhibitor. Here, we found a reversed EMT process with increased expression of E-cadherin and down-regulation of N-cadherin (Fig. 3*E*), whereas addition of TGF-β led to down-regulation of E-cadherin and translocation of SMAD4 to the nucleus (Fig. 3*D*). These results suggest that TGF-β signaling is increased in *Kras;N2ko* PDAC yet responsive to either inhibition or activation in the absence of Notch2.

Deficiency of *Notch2* Modulates Myc Signaling. To elucidate the oncogenic role of Notch2 further, we screened *Kras* and *Kras;N2ko* preneoplastic pancreatic tissue and cancer cells using GSEA. Here, we noted highly significant enrichment of several Myc signatures, suggesting that Notch2 modulates Myc signaling (Fig. 4*A* and Tables S12 and S13). Compatible with deregulation of Myc signaling during early carcinogenesis, we found increased Myc expression in PanIN lesions as well as increasing mRNA levels in $Kras^{G12D}$-induced pancreatic tissue during preneoplastic progression (Fig. 4 *B* and *C* and Tables S1–S3). We next examined *Kras* and *Kras;N2ko* cancer cells and found reduced mRNA and, most importantly, reduced protein levels in *Kras;N2ko* cells (Fig. 4*D*). Immunohistochemistry of Myc in PDAC of *Kras* mice and anaplastic PDAC of *Kras;N2ko* mice revealed a heterogeneous yet decreased expression pattern in *Kras;N2ko* mice (Fig. 4*E* and Tables S1–S3), suggesting that Myc protein expression is indeed down-regulated in *Notch2*-ablated preneoplastic and malignant pancreatic cells.

Recently, several Notch/Rbpj binding sites in the murine *Myc* promoter have been described (20). To analyze transcriptional regulation of *Myc* further, we considered three Notch/Rbpj signaling binding sites of interest in the *Myc* promoter (Fig. 5*A*). To test the relevance of each binding site, we transfected *Kras;N2ko* cancer cells with activated Notch2 (N2IC) and luciferase reporter vectors with one, two, or all three Notch/Rbpj sites mutated. As shown in Fig. 5*B*, all three sites seemed to be functional for transcriptional regulation. Intriguingly, we found *Myc* promoter induction through Notch2 in every cell line tested. We next performed ChIP to substantiate the reporter assay results in *Kras* cancer cells. ChIP demonstrated Notch2 and RbpJ binding to the *Myc* promoter. In fact, the increased *Myc* promoter occupation by Notch2 and RbpJ was comparable to that of Notch2 binding to the *Hes1* promoter (Fig. 5*C*). Intriguingly, a similar result was obtained in the human PDAC cell lines MiaPaCa2 and Panc1, in which two Notch/Rbpj binding sites are conserved between humans and mice (Fig. S5). We next tested whether N2IC would increase Myc expression in *Kras;N2ko* and Panc1 cells. As shown in Fig. 5*D*, Myc mRNA and protein expression was increased in N2IC-transfected cells, suggesting transcriptional regulation.

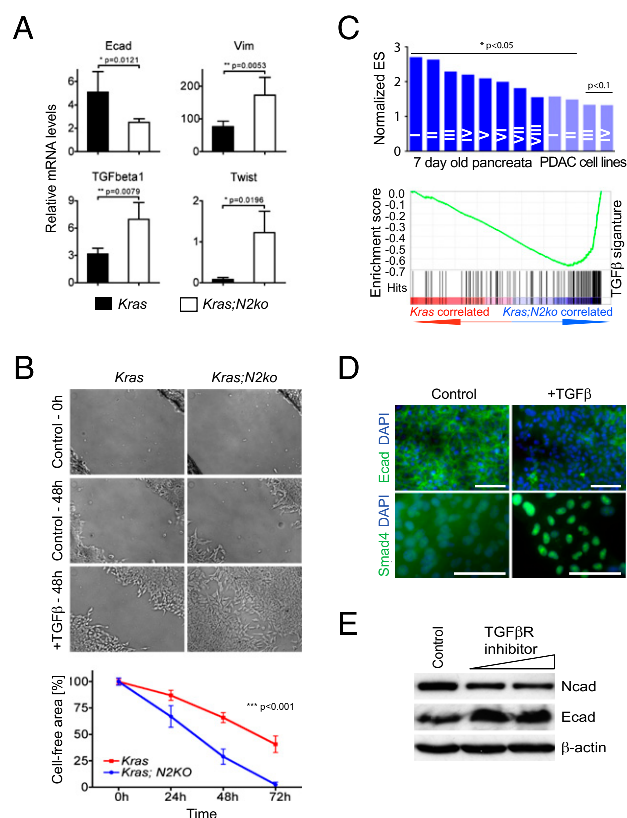

Fig. 3. EMT is a prominent feature in *Kras;N2ko* PDAC. (*A*) Quantitative RT-PCR analysis of EMT-associated genes expressed by cancer cells from *Kras* and *Kras;N2ko* PDAC (*n* = 4 for each genotype). (*B*) Assessment of cell migration in wound closure assays performed in *Kras* and *Kras;N2ko* cells treated with TGF-β. Wound closure is delayed in *Kras* cells compared with *Kras;N2ko* cells. Quantification of wound closure is plotted as the percentage of the cell-free area over time. (*C*) Comparison of TGF-β gene sets by GSEA reveals significantly up-regulated TGF-β signatures in *Kras;N2ko* pancreata isolated from 7-d-old mice (dark blue, *n* = 2 and 4) and cancer cells (light blue, *n* = 6 each). A positive normalized enrichment score indicates elevated TFG-β–associated gene expression. Roman numbers refer to the detailed analysis in Tables S10 and S11. (*D*) *Kras;N2ko* cells reveal morphological and molecular responses characteristic of EMT in response to TGF-β, including loss of E-cadherin expression and nuclear translocation of SMAD4. (Scale bars: 50 μm.) (*E*) Treatment with the TGF-β receptor inhibitor SB431542 is sufficient to reverse the EMT-associated cadherin switch, suggesting that EMT in *Kras;N2ko* cells is dependent on a TGF-β autocrine loop.

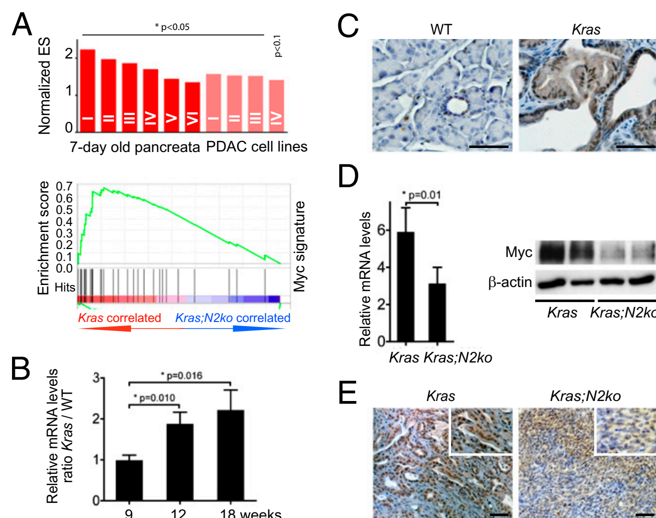

Fig. 4. Myc is up-regulated during pancreatic carcinogenesis and down-regulated in *Kras;N2ko* mice. (*A*) GSEA shows significantly enriched Myc signatures in *Kras* vs. *Kras;N2ko* pancreata isolated from 7-d-old mice (dark red, $n = 2$ and 4) and primary cancer cells (light red, $n = 6$ each). Roman numbers refer to detailed analysis in Tables S12 and S13. (*B*) Myc transcript levels increase during carcinogenesis in *Kras* pancreata at indicated time points. Values represent WT-to-*Kras* ratio of relative expression levels ($n = 3$ for each time point). (*C*) Expression of Myc is low in the normal pancreas and increases in PanIN lesions of *Kras* mice. (*D*) *Kras;N2ko* cancer cells ($n = 4$) show decreased Myc mRNA and protein expression compared with *Kras* cells ($n = 5$). (*E*) Immunohistochemical staining in *Kras;N2ko*-derived anaplastic PDAC shows lower expression of Myc compared with *Kras* PDAC. (Scale bar: 50 μm.)

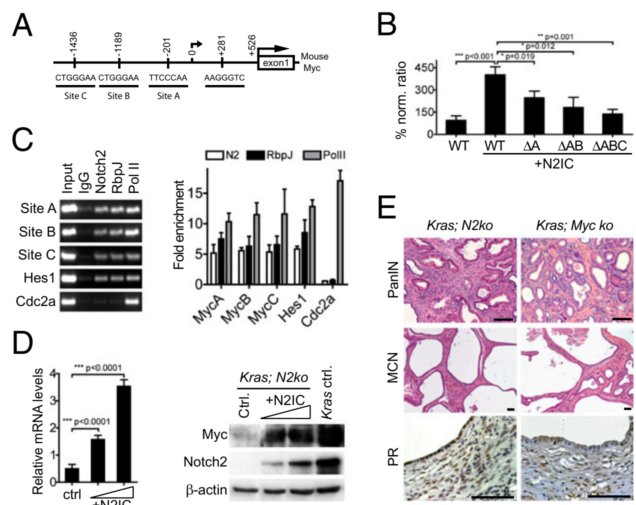

Fig. 5. Myc is a downstream target of Notch, and its ablation resembles features of the *Notch2*-deficient phenotype. (*A*) Analysis of Notch/Rbpj binding sites in the mouse *Myc* promoter using the consensus RTGGGAA motif reveals three sites: A, B, and C. (*B*) Activity of a *Myc* promoter fragment containing binding regions A, B, and C was analyzed using luciferase reporter assays. *Kras;N2ko* cells were cotransfected with *Myc* luciferase plasmids and N2IC. Mutations in the respective binding sites decrease activation of Myc. Activities were corrected for transfection efficiency by normalizing with Renilla luciferase activity and are expressed as a percentage of induction. (*C*) ChIP analyses using the indicated antibodies were analyzed by PCR for sites of interests. Products of the exponential phase of PCR are shown. *Hes1* promoter primer served as positive control, and *Cdc2a* promoter primers as negative control. Quantitative PCR indicates that Notch2 binds to regions A, B, and C of the *Myc* promoter comparable to a binding site in the *Hes1* promoter. (*D*) Transfection of N2IC stimulates Myc expression in *Kras;N2ko* cells in a dose-dependent manner. Notch2 and Myc expression levels of *Kras* control are shown for comparison. (*E*) *Myc* and *Notch2* ablation in *Kras* mice results in similar phenotypes. *Kras;N2ko* and *Kras;Myc-ko* mice develop PanIN1 but not advanced PanIN2/3 lesions and MCN-like lesions with progesterone receptor-positive (PR^+) surrounding stroma. Brightness and contrast levels were adjusted across the whole image for each panel. (Scale bar: 50 μm.)

To analyze Myc signaling in pancreatic carcinogenesis in vivo, we interbred previously described $Myc^{fl/fl}$ mice (21) with *Pdx1-Cre;* $Kras^{+/LSL-G12D}$ mice to obtain *Myc*-ablated *Kras* mice. Although breeding was hindered by exocrine atrophy occurring in most animals, we could analyze two mice 11 and 12 mo of age that showed a phenotype of only PanIN1 but not higher grade lesions, strongly supporting our hypothesis of Myc signaling being essential for PanIN progression. Additionally, we observed the development of MCN-like lesions with ovarian-like stroma, similar to *Kras;N2ko* mice (Fig. 5*E*).

Discussion

Notch Signaling Activation in Pancreatic Carcinogenesis. In this study, we have evaluated the role of the Notch receptors 1 and 2 in pancreatic carcinogenesis in vivo using the well-established conditional $Kras^{G12D}$ model generated by Tuveson and co-workers (3). Although inhibition of PanIN progression in *Kras;N2ko* mice goes along with the results of inhibition of Notch signaling through γ-secretase inhibitor treatment (10), some differences between the models are notable. Plentz et al. (10) found a high relative increase of Notch3 mRNA in duct cells derived from PanIN-bearing pancreata and cells isolated from PDAC. Although we also found an increase in expression of Notch3 in PanIN-bearing compared with WT pancreata, expression was low compared with Notch1 and Notch2 levels. Reasons may include use of different mouse models as well as analysis of different tissue samples. In cancer cells isolated from PDAC of *Kras* mice, however, we also found much lower mRNA and protein levels of Notch3 compared with Notch2. In fact, Notch2 was by far the most prominently expressed Notch receptor during PanIN development and in PDAC, a finding supported by earlier studies (5). Importantly, we found no consistent up-regulation of any other Notch receptor in *Notch2*-deficient PDAC cells, suggesting that these cells could not easily reconstitute loss of Notch2 by any other Notch receptor. Interestingly, we did not observe loss of HES1 expression in either *Notch1*- or *Notch2*-ablated pancreata, suggesting that Hes1 may be regulated by other signaling pathways, as suggested previously (12, 16).

Although the downstream signaling of different Notch receptors and ligand specificity are complex, the differential pancreatic expression of Notch1 and Notch2 is noteworthy. The predominant expression of Notch1 in acinar cells goes along with our previous result of impaired regeneration in conditional *Notch1*-deficient mice during acute pancreatitis (16). Interestingly, Murtaugh and co-workers (11) found Notch1-activated mature acinar cells to be susceptible to PanIN initiation and progression. The hypothesis of acinar cells as potential cells of origin for PDAC has recently gained much interest because of the plasticity of this cell type, its potential for initiation of preneoplastic lesions (22–25), and the involvement of Notch signaling (5, 11). Although Notch1 is expressed in the acinar compartment, expression was absent in PanIN lesions when analyzed using transgenic *Notch1-GFP* reporter mice. Along this line, we did not observe fewer PanINs when Notch1 was ablated in our model. Instead survival and tumor incidence was reduced, although this finding was not significant. Of note, Notch1 ablation in *Pdx1-Cre;*$Kras^{G12D}$ mice was recently shown to result in increased PanIN progression, supporting the concept that Notch1 has no oncogenic role in pancreatic carcinogenesis (26).

Expression of Notch2 in ductal cells has been described previously and increases in metaplastic ductal cells (27, 28). Recently, centroacinar cells were described to show features of progenitor cells, including respective marker expression, sphere formation ability, and differentiation into different pancreatic lineages (15).

MEDICAL SCIENCES

These and our results suggest that a potential progenitor compartment in small ducts such as centroacinar cells expresses Notch2, a hypothesis supported by our expression studies using *Notch2*$^{+/lacZ}$ reporter mice. Because we observed PanIN1 initiation but no higher grade PanINs in *Kras;N2ko* mice, activation of Notch2 may be required for progression of PanIN lesions. However, other explanations remain possible. Because PanIN1 lesions are often encountered in pancreata of elderly people, it is possible that PanIN1 lesions may not actually precede PanIN2 and PanIN3 lesions but are mainly default lesions that may form from different pancreatic cells, including the acinar compartment. Consistent with this hypothesis is the induction of PanIN lesions but usually no development of invasive PDAC from acinar cells in *Ela-Cre-ER*;*Kras*^{G12D} mice. Although our study did not directly address this intriguing question, it remains possible that PanIN1 lesions may originate from acinar cells, whereas initiation or progression of PanIN2/3 lesions may require a Notch-regulated potential progenitor compartment or an additional stimulus such as ongoing inflammation (25, 29).

Development of MCN-Like Lesions and Anaplastic PDAC in *Kras;N2ko* Mice. The blockade of PanIN progression and PDAC development in *Notch2*-deficient *Kras*^{G12D} mice goes along with the longer survival of these mice. Eventually, these mice develop large cysts resembling MCNs and succumb from either pancreatic insufficiency or from the development of anaplastic PDAC. Development of MCN-like lesions may thus be a bypass route for pancreatic cells undergoing oncogenic stress. However, two scenarios are possible with either (*i*) a common cell of origin for PanIN and MCN development, in which the route to higher grade PanINs is blocked by Notch2 deficiency, or (*ii*) different cells of origin for each lesion type that respond differentially to *Kras*^{G12D} in the presence or absence of Notch2.

Interestingly, an association of anaplastic PDAC and MCN has been repeatedly described in patients (30). However, we do not have enough evidence to conclude that MCNs are the direct precursors for PDAC in *Kras;N2ko* mice. Further analysis is required to understand the cellular and molecular cues in *Notch2*-deficient malignant transformation. However, the clinical and experimental observations of the combined occurrence of MCN and anaplastic PDAC highlight the potential predictive capability of genotype-phenotype correlations in complex cancer mouse models.

TGF-β Signaling and EMT in *Notch2*-Deficient PDAC. Molecular characterization of the anaplastic PDAC in *Kras;N2ko* mice showed evidence of EMT. Several reports have described an activating role of increased Notch signaling in EMT by regulation of E-cadherin repressors such as Snail or interaction with TGF-β signaling (31–34). TGF-β is known to play an ambivalent role in cancer biology. In the pancreas, conditional inactivation of TGF-β receptor 2 led to accelerated development and progression of well-differentiated PDAC (35). The development of late-occurring anaplastic PDAC with increased EMT is compatible with the dual role of TGF-β signaling in epithelial tumorigenesis. The effect of TGF-β receptor inhibition on E- and N-cadherin expression and exogenous TGF-β–induced nuclear translocation of SMAD4 suggest an intact TGF-β signaling axis. Indirect regulation of TGF-β may occur through deregulated Myc signaling, which is known to suppress the activation of TGF-β–induced genes such as p21CIP1, which has been shown to interact with Notch in various organs (36, 37). However, we could not detect consistent differences in p21CIP1 expression or related signatures between *Kras* and *Kras;N2ko* tumors.

Myc Signaling Is Regulated by Notch2 in PDAC. Decreased Myc signaling in *Kras;N2ko* mice supports the hypothesis of Notch2-dependent Myc signaling as a key regulator of the carcinogenic process in the pancreas. Deregulation of Myc in PDAC has been described in many studies, and amplification occurs in about 30% of human PDAC as well as in murine PDAC (38–40). In recent studies, Myc signaling has been identified to play a key role in cell cycle regulation of PDAC cells (41, 42). Although these studies demonstrate the importance of deregulated Myc signaling in PDAC, our results suggest an early role during PanIN progression supported by early Myc amplification in precursor lesions (38). In a recent quantitative proteomic screen of preneoplastic PanIN lesions, Myc expression was identified in PanIN3 lesions (43).

We and others have previously characterized the important role of Myc in progenitor and acinar cell proliferation during development and adult homeostasis (21, 44, 45). Consistently, we found increased Myc expression throughout PanIN development in *Kras* mice. It is tempting to speculate that Myc and Ras signaling cooperatively promote tumor progression in a setting of active Notch. Notch signaling has been reported to cooperate with Ras, and several studies have reported direct transcriptional regulation of Myc by Notch1 (20, 46–48). Our finding that active Notch2 induces Myc expression in PDAC cells supports these reports. Although preliminary, the phenotypical similarities of *Notch2* and *Myc*-ablated *Kras*^{G12D}-induced pancreata with development of cystic lesions and a PanIN progression stop strongly support this hypothesis. Of consideration is the use of different Cre mice, *Ptf1a*$^{+/Cre(ex1)}$ and *Pdx1-Cre* mice, in *Kras;N2ko* and *Kras;Myc-ko* mice, respectively, because of extensive exocrine hypoplasia and early postnatal death of *Ptf1a*$^{+/Cre(ex1)}$;*Myc*$^{fl/fl}$ mice (21). Although we cannot rule out different target compartments in both Cre lines, this seems unlikely, given the similar phenotype in *Kras*^{G12D}-activated mice (3).

The results from luciferase reporter and ChIP assays suggest that all three reported Notch/Rbpj binding sites in the *Myc* promoter are relevant for transcriptional regulation of Myc. On the basis of our findings, we report that Myc is regulated by Notch2. Why *Notch1* ablation did not lead to similar alterations in early tumor progression in our model is not clear. A possible explanation would be a context- and cell-specific role of Myc and its regulation through Notch. A possible scenario may thus be that a progenitor cell (e.g., within the centroacinar compartment) is the target cell for cooperative Myc-Ras–induced tumor development propagated by Notch2 activation. The success of Notch inhibition as a chemopreventive approach to inhibit PanIN progression has been shown (10). This outcome is supported by our results. Of note, the same group has reported *Myc* amplification in *Kras*^{G12D}-driven PDAC mouse models, adding evidence for a key role of this signaling pathway during the carcinogenic process (40). It will be of great interest to study the integration of the transcriptional programs regulated by Myc and Notch signaling in further detail, which may eventually help to explain the permissive signals regulating pancreatic plasticity and malignant transformation.

In summary, our results provide evidence for an essential role of Notch2 and Myc in the initiation of a neoplastic transformation program in pancreatic cells, whereas Notch1 has no oncogenic role, supporting the concept of distinctive roles of individual Notch receptors in cancer development. In addition, the data demonstrate the integrative interaction of regulators of cell fate and cell cycle signaling, thereby enhancing our biological understanding for unique approaches in this still untreatable disease.

Materials and Methods

Mouse Strains. *Kras*$^{+/LSL\text{-}G12D}$, *Notch1*$^{fl/fl}$, *Notch2*$^{fl/fl}$, *Myc*$^{fl/fl}$, *Ptf1a*$^{+/Cre(ex1)}$, *Pdx1-Cre*, and Rosa26$^{+/LSL\text{-}lacZ}$ mice have been described before (3, 9, 17, 21). All experiments were performed according to the guidelines of the local animal use and care committees.

Detailed descriptions of additional procedures, including protein and mRNA analysis, immunohistochemistry, microarray/GSEA, luciferase-based reporter assays, and ChIP, are provided in *SI Text*.

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