Ligand-dependent Notch Signaling Is Involved in Tumor Initiation and Tumor Maintenance in Pancreatic Cancer

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Abstract

Purpose: Aberrant activation of the Notch signaling pathway is commonly observed in human pancreatic cancer, although the mechanism(s) for this activation has not been elucidated.

Experimental Design: A panel of 20 human pancreatic cancer cell lines was profiled for the expression of Notch pathway-related ligands, receptors, and target genes. Disruption of intracellular Notch signaling, either genetically by RNA interference targeting *NOTCH1* or pharmacologically by means of the γ-secretase inhibitor GSI-18, was used for assessing requirement of Notch signaling in pancreatic cancer initiation and maintenance.

Results: Striking overexpression of Notch ligand transcripts was detectable in the vast majority of pancreatic cancer cell lines, most prominently *JAGGED2* (18 of 20 cases, 90%) and *DLL4* (10 of 20 cases, 50%). In two cell lines, genomic amplification of the *DLL3* locus was observed, mirrored by overexpression of *DLL3* transcripts. In contrast, coding region mutations of *NOTCH1* or *NOTCH2* were not observed. Genetic and pharmacologic inhibition of Notch signaling mitigated anchorage-independent growth in pancreatic cancer cells, confirming that sustained Notch activation is a requirement for pancreatic cancer maintenance. Further, transient pretreatment of pancreatic cancer cells with GSI-18 resulted in depletion in the proportion of tumor-initiating aldehyde dehydrogenase – expressing subpopulation and was associated with inhibition of colony formation *in vitro* and xenograft engraftment *in vivo*, underscoring a requirement for the Notch-dependent aldehyde dehydrogenase – expressing cells in pancreatic cancer initiation.

Conclusions: Our studies confirm that Notch activation is almost always ligand dependent in pancreatic cancer, and inhibition of Notch signaling is a promising therapeutic strategy in this malignancy.

Pancreatic cancer is an almost uniformly lethal disease with an overall 5-year survival of ~5%, and this dire prognosis has not markedly improved over the last few decades (1). In the United States, ~34,000 individuals succumb to this malignancy each year. To date, the only potentially curative therapeutic option is complete surgical resection, but unfortunately, the majority of patients are diagnosed at a locally advanced or distant metastatic stage, thus precluding surgical cure (2). Currently available treatment options for advanced pancreatic cancer, such as gemcitabine, have had minimal effect in ameliorating survival. Identification of aberrant signaling pathways that can also form the substrate for targeted therapies has thus become an area of foremost priority.

The reactivation of embryonic signal transduction pathways, such as Notch and Hedgehog, have been reported in a variety of human cancers (3, 4); further, the availability of potent small-molecule inhibitors has meant that these pathways can be targeted in these cancers, as we and others have recently shown

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Translational Relevance

Potent therapeutic strategies are urgently needed for pancreatic cancer, a disease of near uniform lethality. Activation of the Notch signaling pathway is commonly observed in pancreatic cancer, suggesting that pathway blockade with small-molecule inhibitors might be a feasible therapeutic strategy. In this study, we first systematically document the mechanisms of Notch activation in pancreatic cancer and show this to be ligand driven rather than mutationally activated. Second, we show the requirement of sustained Notch signaling for pancreatic cancer maintenance using genetic and pharmacologic approaches toward inhibition of the pathway. Finally, we confirm the presence of a highly Notch-dependent tumor-initiating population in pancreatic cancer that has been implicated as the putative source for disease recurrence and systemic metastases. Our studies underscore the emerging paradigm that cancers are heterogeneous populations composed of tumor-initiating "stem cells" and the "bulk" tumor population, and therapeutic success will be engendered by dual targeting of both compartments.

(5–7). The Notch signaling pathway is an evolutionarily conserved pathway that plays a major role in cell fate decisions in various tissues during the development of multicellular organisms (8). In adult tissues, Notch signaling prevents cells from undergoing terminal differentiation, thus maintaining pools of undifferentiated stem/progenitor cells (9, 10). Activation of the Notch signaling pathway has previously been described in several human malignancies, including pancreatic cancer (4, 11, 12). For example, our group has shown that expression of Notch gene targets is observed not only in invasive pancreatic cancers but also in the noninvasive precursor lesions of this malignancy (13). In a series of elegant studies, Wang and colleagues (14–17) have shown a requirement for active Notch signaling for tumor maintenance in pancreatic cancer, with down-regulation of NOTCH1 contributing to growth inhibition and apoptosis of cancer cells through inhibition of key survival pathways, such as nuclear factor-κB. However, the underlying mechanisms causing aberrant Notch signaling in pancreatic cancer are poorly understood.

In the present study, we examine the mechanisms of Notch pathway activation in the setting of pancreatic cancer. We find that endogenous overexpression of Notch ligands, specifically *JAGGED2* and *DLL4*, seems to be the most common mechanism; uncommonly, genomic amplification of the *DLL3* locus on chromosome 19q13 contributes to Notch activation in this malignancy. In contrast to hematologic malignancies such as T-cell leukemia (18), mutational activation of Notch is rare to absent in pancreatic cancer. Our studies also show that sustained Notch signaling is required for the viability of a subpopulation of pancreatic cancer cells with tumor initiation properties (i.e., "cancer stem cells"), further supporting the utility of targeting this pathway as a therapeutic strategy in this malignancy.

Materials and Methods

Cell lines and culture conditions. Twenty pancreatic cancer cell lines (PANC-1, CAPAN-1, Colo-357, CFPAC, MIAPaCa-2, BxPC-3, AsPc-1, L3.6PL, PL-4, PL-5, PL-8, PL-9, PL-12, PL-13, XPA-1, XPA-3, XPA-4, Panc-8.13, Panc-3.27, and Panc-4.30) were grown as previously described (19). Immortalized nonmalignant human pancreatic epithelial cells (hTERT-HPNE) were cultured as described elsewhere (20). The hTERT-HPNE cells were used for normalization of expression levels for Notch pathway components among the 20 cancer cell lines.

RNA interference–mediated transcript knockdown. For knockdown of *NOTCH1* transcripts, PANC-1 and CAPAN-1 cells were transiently transfected with gene-specific or scrambled small interfering RNA (siRNA) using Oligofectamine (Invitrogen) following the standard procedure recommended by the manufacturer. Efficacy of knockdown was confirmed by quantitative reverse transcription-PCR (qRT-PCR). The sequences for the synthetic siRNAs against *NOTCH1* (Dharmacon) have been previously described (21). Similarly, RNA interference (RNAi) against *DLL3* was done in PANC-1 and SU86.86 cell lines using SMARTpool siRNA (Dharmacon) followed by qRT-PCR to confirm efficacy of *DLL3* knockdown.

Stable overexpression of Notch 1 intracytoplasmic domain in PANC-1 cells. Generation of PANC-1 cells stably overexpressing the NOTCH1 intracytoplasmic domain (N1ICD) was accomplished as previously described (21). Empty vector was used for mock transfection.

Notch pathway inhibitor GSI-18. Synthesis of the γ-secretase inhibitor [11-endo]-*N*-(5,6,7,8,9,10-hexahydro-6,9-methanobenzo[a][8]annulen-11-yl)-thiophene-2-sulfonamide (also known as GSI-18) and its ability to block Notch pathway activity in cancer cells have been previously described (21–23).

Notch reporter assays. Assessment of Notch activity following GSI-18 administration was done using a CBF-1–binding site luciferase reporter (8X-Luc), as previously described, in PANC-1 cells (13). *Renilla* luciferase was used as transfection control.

Cell viability assay. Growth inhibition was measured using the CellTiter 96 AQ_{ueous} Cell Proliferation Assay (Promega), which relies on the conversion of a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS)] to a colored formazan product by the activity of living cells. Briefly, 2,000 cells per well were plated in 96-well plates and treated with 2, 5, and 10 μmol/L concentrations of GSI-18, for 96 h, at which point the assay was terminated, and relative growth inhibition compared with vehicle-treated cells was measured using the CellTiter 96 reagent, as described in the manufacturer's protocol. A panel of six human pancreatic cancer cell lines was examined (PANC-1, CAPAN-1, BxPC-3, MIAPaCa-2, Panc-8.13, and Panc-3.27) in the MTS assays. Cell viability assays were also done for PANC-1 and SU86.86 cells following RNAi against *DLL3* using scrambled siRNA as control. All experiments were set up in triplicates to determine means and SDs.

Anchorage-independent growth. Anchorage-independent growth was assessed in PANC-1 and CAPAN-1 cells following either genetic inhibition (*NOTCH1* siRNA) or with pharmacologic inhibition of Notch signaling with GSI-18 (5 μmol/L). Soft agar assays were set up in six-well plates, each well containing a bottom layer of 1% agarose (Invitrogen), a middle layer of 0.6% agarose including 10,000 cells, and a top layer of medium only. For the pharmacologic inhibition experiments, mixtures in each well were supplemented with GSI-18 at the respective concentration or solvent only, and the plates were incubated for 3 wk. An independent series of colony assays was done in PANC-1 and SU86.86 cells, following genetic knockdown of *DLL3* using siRNA. To assess colony formation, the medium was removed, and 1.5 mL of 0.5% Wright's staining solution were added to each well. After incubation at 4°C for 12 h, removal of the staining solution, and washing twice with PBS, colonies were visualized by trans-UV illumination and counted using the analysis software Quantity One (Bio-Rad).

Evaluation of aldehyde dehydrogenase activity. Aldehyde dehydrogenase (ALDH) expression was determined at baseline and after pharmacologic Notch inhibition in two pancreatic cancer cell lines, E3LZ10.7 and CAPAN-1, where we have previously shown that inhibition of Hedgehog signaling selectively depletes the ALDH "bright"

subpopulation (5, 6). After incubation with either vehicle or GSI-18 (5 μmol/L) for 24 h, E3LZ10.7 and CAPAN-1 cells were stained for ALDH expression using the Aldefluor reagent (StemCell Technologies) according to the manufacturer's instructions and analyzed on a FACSCalibur flow cytometer (Becton Dickinson). ALDH-positive cells were quantified by calculating the percentage of total cells that displayed greater fluorescence compared with a control staining reaction containing the ALDH inhibitor diethylaminobenzaldehyde, as we have previously described (5, 6, 24).

Pretreatment with GSI-18. We have previously shown that transient *ex vivo* pretreatment with Hedgehog antagonists inhibits both anchorage-independent growth and *in vivo* tumorigenicity of pancreatic cancer and glioblastoma cell lines (6, 24). To determine the effects of Notch antagonism on tumor initiation, pancreatic cancer cell lines E3LZ10.7 and CAPAN-1 cells were pretreated with either vehicle or GSI-18 for 24 h (2 and 5 μmol/L) and allowed to recover in full serum for 24 h. Thereafter, equal numbers of viable cells from each condition, quantified using trypan blue dye exclusion assay, were plated in soft agar for colony assays, as described above. Pretreated and serum-recovered E3LZ10.7 and PANC-1 cells were also injected in athymic (nude) mice for tumor engraftment studies, as described below.

Colony assays with LY294002, an Akt/phosphatidylinositol 3-kinase pathway inhibitor. To confirm the specificity of Notch inhibition against the tumor-initiating component and exclude the potential for artifact, we did a series of experiments using CAPAN-1 cells treated with LY294002, a small-molecule inhibitor of the oncogenic Akt/phosphatidylinositol 3-kinase pathway. Two parallel sets of anchorage-independent assays were done. First, a "pretreatment" experiment mirroring the GSI-18 study, with two doses of LY294002 (5 and 10 μmol/L). In this experiment, CAPAN-1 cells were exposed to the drug for 24 h followed by full serum recovery and plating in soft agar. The second set of experiments, with the same dosages, used a "continuous" (conventional) approach, where the cells were incubated in soft agar with continuous exposure to LY294002 for 2 wk. Colony counts were done as described above.

Generation of murine s.c. xenografts. All animal experiments conformed to the guidelines of the Animal Care and Use Committee of Johns Hopkins University and animals were maintained in accordance to guidelines of the American Association of Laboratory Animal Care. A total of 5×10^6 E3LZ10.7 or PANC-1 cells in a volume of 200 μL of 1/1 (v/v) PBS/Matrigel, pretreated with either vehicle or GSI-18 at 5 μmol/L and allowed to recover in full serum for 24 h, were injected s.c. into male CD1 *nu/nu* athymic mice (Charles River). Tumor volumes (V) were determined after measuring the larger (a) and smaller (b) diameters as $V = \frac{ab^2}{2}$, as previously described (5, 6).

Statistical analysis. Kruskal-Wallis analysis was done using Statistical Package for the Social Sciences version 15.0.1 for Microsoft Windows, and two-tailed t test, one-way ANOVA, and linear regression analysis (Pearson's test) were done using GraphPad Prism for Windows version 5. $P < 0.05$ was regarded as statistically significant. Results in bar diagrams are plotted as means and SDs if not otherwise indicated.

Results

Endogenous overexpression of Notch ligands in pancreatic cancer. Quantitative real-time qRT-PCR analysis of 20 human pancreatic cancer cell lines compared with hTERT-HPNE cells confirmed variable expression of NOTCH1 through NOTCH4 transcripts, with most cell lines not showing any evidence of receptor mRNA overexpression. Thus, compared with hTERT-HPNE cells, only 8 of 20 (40%) pancreatic cancer lines had equal or greater expression of *NOTCH1*, 5 of 20 (25%) had equal or greater expression of *NOTCH4*, and 4 of 20 (20%) had equal or greater expression of *NOTCH2* transcripts, respectively (Fig. 1A). Curiously, *NOTCH3* mRNA expression was lower than hTERT-HPNE cells in all 20 cancer cell lines, with several lines not expressing any detectable transcripts. In contrast to receptor mRNA levels, marked up-regulation of two of four Notch pathway ligand transcripts (specifically *JAGGED2* and *DLL4*) were observed in the majority of pancreatic cancer cell lines. This was particularly striking for *JAGGED2* where 18 of 20 (90%) of cell lines had higher transcript levels than observed in hTERT-HPNE (with the majority of cases at >50-fold elevation), and to a lesser extent with *DLL4*, with 10 of 20 lines (50%) showing mRNA overexpression compared with hTERT-HPNE cells (Fig. 1B). *JAGGED1* and *DLL1* transcripts were expressed at more attenuated levels (no higher than 10-fold relative overexpression compared with hTERT-HPNE cells) and were up-regulated in fewer cell lines within the panel (Supplementary Fig. S1). Consistent with Notch pathway activation, striking overexpression of the Notch target genes *HES1* and *HEY2* (*HERP1*) was seen in 16 of 20 (80%) and 13 of 20 (65%) of pancreatic cancer cell lines, respectively (Fig. 1C). In contrast, overexpression of the remaining Notch gene targets, *HEY1* (*HERP2*) and *HEYL*, was observed in only a minority of cancer cell lines when compared with corresponding transcript levels in hTERT-HPNE cells. Comparable expression results were obtained when *GUSB* was used as housekeeping control instead of *PGK1* (data not shown). On correlating Notch ligand levels with that of target genes, *JAGGED2* mRNA expression was most closely and significantly correlated with that of *HES1* transcripts ($P = 0.045$, Pearson correlation), further underscoring the importance of this basic helix-loop-helix transcription factor in the context of pancreatic neoplasia (13, 25). On the contrary, there was highly significant correlation between the patterns of expression of *DLL4* and the Notch target gene *HEYL* in pancreatic cancer cell lines ($P = 0.003$, Pearson correlation), reiterating previous observations that despite the commonalities within the pathway, individual ligands have disparate effects on target genes (21).

Amplification of DLL3 is an uncommon "driver" for Notch signaling in pancreatic cancer. Previously published genomic copy number analyses of pancreatic cancer cell lines and xenografts by our group and others have shown that the *DLL3* locus on chromosome 19q13 is included in a recurrent amplicon in this malignancy (19, 26, 27). Therefore, we assessed *DLL3* gene dosage in 22 pancreatic cancer cell lines and found two lines, PANC-1 and SU86.86, which showed 3-fold or greater copy number by genomic quantitative PCR compared with hTERT-HPNE cells (Fig. 2A). Transcript profiling confirmed that PANC-1 and SU86.86 had strikingly high expression of DLL3 mRNA, ~200-fold that of hTERT-HPNE cells (Fig. 2B). *DLL3* was down-regulated by transient RNAi in both cell lines, and effects on *in vitro* growth and anchorage independence were determined following validation of gene-specific knockdown. No significant effects were observed on either phenotype in PANC-1 cells with *DLL3* RNAi (data not shown), suggesting redundant mechanisms for Notch pathway activation in this cell line. In contrast, knockdown of *DLL3* in SU86.86 resulted in significant growth inhibition by MTS assay ($P = 0.0005$; Fig. 2C) as well as significant inhibition of anchorage-independent growth in soft agar ($P = 0.0016$; Fig. 2D). Thus, in a minor subset of pancreatic cancers, Notch pathway activation is likely to be driven by increased *DLL3* copy number and resulting endogenous overexpression of the ligand protein.

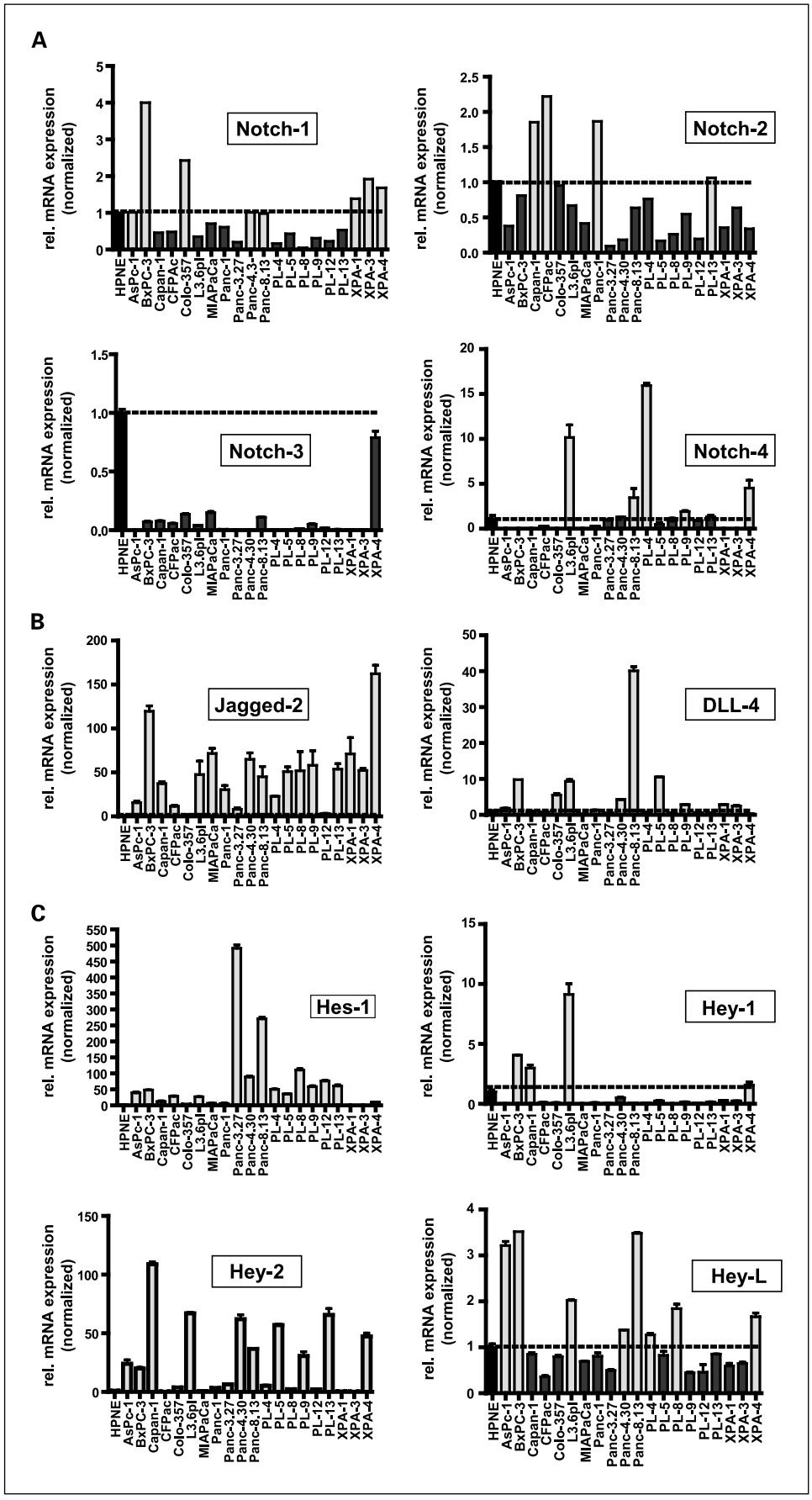

Fig. 1. Profiling the Notch pathway in pancreatic cancer cell lines. RNA from a panel of 20 pancreatic cancer cell lines was assessed for expression of Notch receptors *NOTCH1, NOTCH2, NOTCH3*, and *NOTCH4* (*A*), Notch ligands *JAGGED2* and *DLL4* (*B*), and Notch gene targets *HES1, HEY1, HEY2*, and *HEYL* (*C*), and relative fold levels compared with immortalized hTERT-HPNE cells. Horizontal line, normalized ratio of 1 in hTERT-HPNE cells. *X* axis, individual cell line samples; *Y* axis, relative fold level of expression. Light gray columns, cancer cell lines with overexpression of corresponding mRNA compared with hTERT-HPNE cells; dark gray columns, cell lines with equal or lesser expression. All assays were done in triplicate using *PGK1* as housekeeping control, and an independent set of assays was done using *GUSB* as housekeeping control (data not shown).

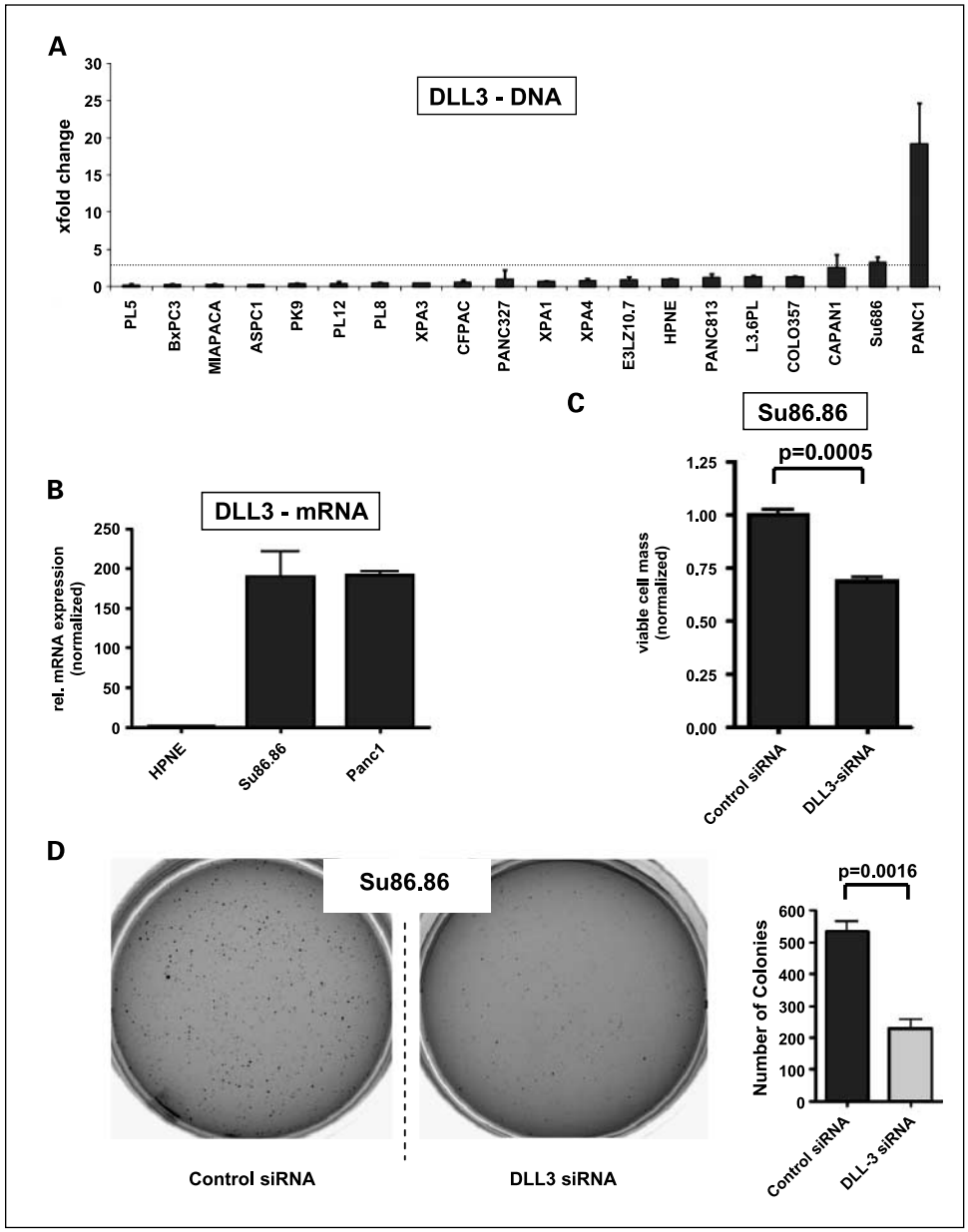

Fig. 2. Copy number alteration of *DLL3* in a subset of pancreatic cancer cell lines. *A,* genomic quantitative PCR for *DLL3* copy number shows two cell lines (PANC-1 and SU86.86) with an average gene dosage ratio of greater than three. The chromosome 19q13 gene *KCL3* was used as a reference control. Genomic quantitative PCR was done in triplicate. Columns, average; bars, SD. *B,* qRT-PCR for *DLL3* mRNA in PANC-1 and SU86.86 cells shows striking overexpression (~200-fold) relative to levels in hTERT-HPNE cells. *C,* knockdown of DLL3 by synthetic siRNA significantly inhibits *in vitro* growth of SU86.86 cells, as measured by an MTS cell viability assay at 96 h, compared with control (scrambled siRNA transfected) cells ($P = 0.0005$). *D,* knockdown of *DLL3* by siRNA significantly inhibits anchorage-independent growth in SU86.86 cells, as assessed by colony formation in soft agar, compared with control (scrambled siRNA transfected) cells ($P = 0.0016$). Colony assays were done in triplicate. Columns, average; bars, SD.

NOTCH1 ***or*** **NOTCH2** ***mutations are rare to absent in pancreatic cancer.*** Activating mutations of the *NOTCH* receptors have been suggested to be the underlying driving force of Notch pathway activation in several malignancies, particularly in T-cell leukemias, wherein activating *NOTCH1* mutations are found in as many as 50% of cases (18). To determine whether such coding sequence mutations of *NOTCH1* or *NOTCH2* exist in the setting of pancreatic cancer, mutational analysis of 20 pancreatic cancer cell lines, as well as 22 patient-derived pancreatic cancer xenografts, was done by direct Sanger sequencing of the coding regions. All sequence variations from RefSeq[5] were first confirmed by replicate PCR and subsequently cross-matched against the single nucleotide polymorphism database dbSNP.[6] A previously undescribed heterozygous L2458V alteration was identified in the COOH-terminal PEST domain of *NOTCH1* in the MIAPaCa-2 pancreatic cancer cell line (data not shown). However, gauged by the low expression levels of Notch pathway target genes in this line (see Fig. 1C), the functional significance of this alteration was uncertain. We failed to find any evidence of activating mutations in any of the 42 cancer samples within the *NOTCH1* and *NOTCH2* coding regions.

Sustained Notch signaling is required for pancreatic cancer maintenance. In light of the evidence suggesting ligand-dependent Notch activation in the majority of human pancreatic cancer cell lines, we then evaluated whether sustained Notch signaling is required for the maintenance of pancreatic cancer and, in particular, for anchorage-independent growth, a property of transformed cells. We first used RNAi to down-regulate *NOTCH1* transcript levels in CAPAN-1 and PANC-1cancer cell lines; efficacy of RNAi was confirmed by

[5] http://www.ncbl.nlm.nih.gov/RefSeq
[6] http://www.ncbi.nlm.nih.gov/projects/SNP

real-time PCR showing down-regulation of *NOTCH1* transcripts, as well as multiple Notch target genes (Fig. 3A). Both cell lines transfected with *NOTCH1* siRNA showed a significant reduction in the number of colonies formed in soft agar compared with scrambled siRNA – transfected controls, confirming a requirement of active Notch signaling for anchorage-independent growth (Fig. 3B).

To complement the RNAi findings, we also studied the effects of pharmacologic blockade of Notch signaling in pancreatic cancer cells on *in vitro* growth in monolayers and anchorage-independent growth in soft agar. GSI-18 is a previously described γ-secretase inhibitor with potent inhibitory effects on Notch signaling (21 – 23). We first established that exposure of PANC-1 cells to GSI-18 leads to significant down-regulation of Notch activity, as observed using CBF-1 – binding site luciferase reporter assays (Fig. 4A). A panel of six pancreatic cancer cell lines was used for *in vitro* growth (MTS) assays. As shown in Fig. 4B, only modest growth inhibition was observed with GSI-18 at the highest dose (10 μmol/L), and cell viability was largely unaffected at 2 and 5 μmol/L doses. In contrast, significant reduction in colony formation in soft agar was observed in both CAPAN-1 and PANC-1 cell lines when exposed to 5 μmol/L GSI-18 (Fig. 4C). Thus, based on the combined results of *NOTCH1* RNAi and GSI-18 treatment, we conclude that continuous blockade of Notch signaling is deleterious for the anchorage-independent growth of pancreatic cancer cells.

Overexpression of N1ICD rescues GSI-18-mediated inhibition of anchorage-independent growth in PANC-1 cells. A mammalian expression vector encoding *N1ICD* was stably transfected in PANC-1 cells ("PANC-1-N1ICD"), and overexpression of *N1ICD* compared with empty vector – transfected cells was confirmed by qRT-PCR (Fig. 5A). Of note, enforced *N1ICD* expression per se markedly enhanced anchorage-independent growth of PANC-1 cells in soft agar assays (Fig. 5B and C). Treatment with GSI-18 at a concentration of 5 μmol/L led to a >7-fold reduction in colony numbers in empty vector – transfected

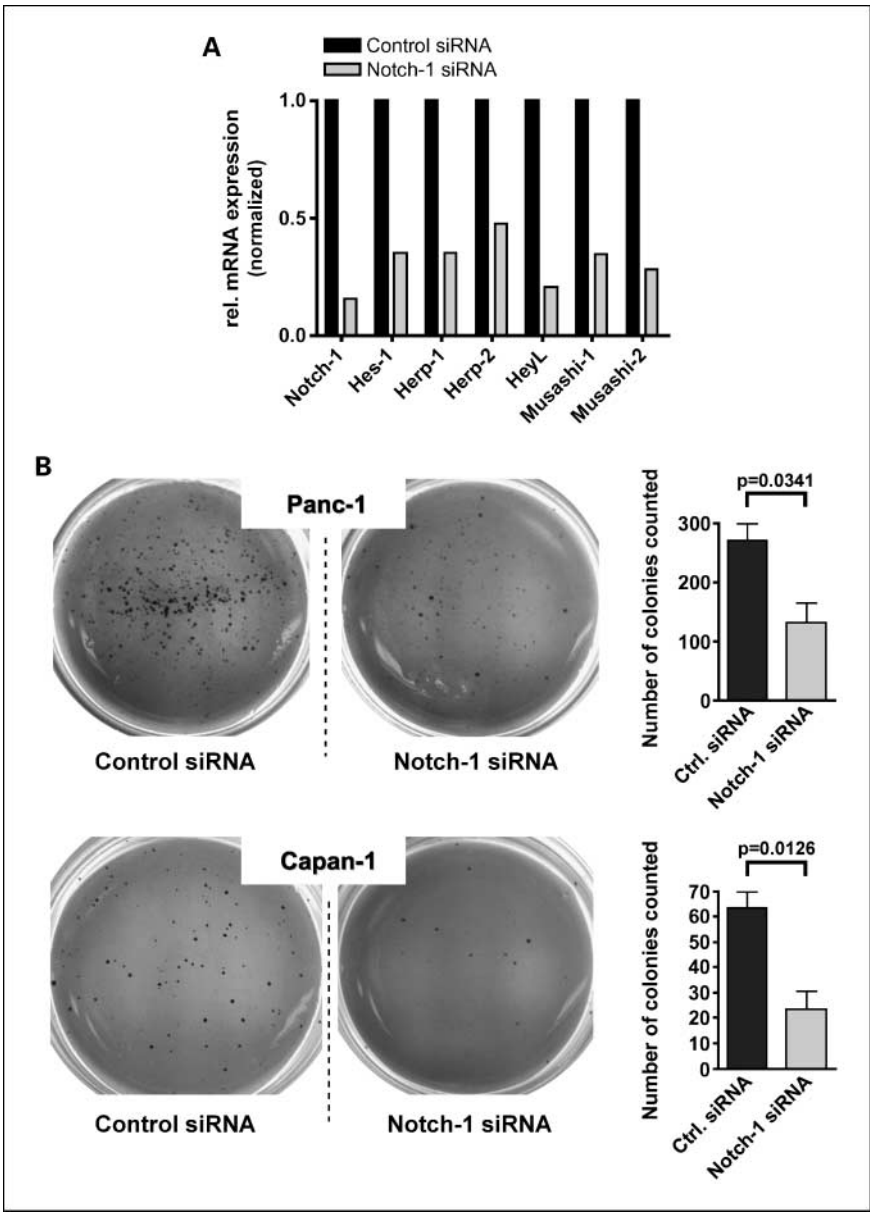

Fig. 3. Genetic knockdown of *NOTCH1* function in pancreatic cancer cells inhibits anchorage-independent growth. *A, NOTCH1* RNAi in CAPAN-1 cells leads to >80% down-regulation of gene-specific transcript levels compared with scrambled siRNA – transfected control cells. In addition, efficacy of functional knockdown is confirmed by reduced transcript levels for Notch gene targets, including *HES1, HEY1* (*HERP2*), *HEY2* (*HERP1*), *HEYL, MUSASHI1*, and *MUSASHI2*, compared with scrambled siRNA – transfected control cells. *B,* NOTCH1 siRNA significantly inhibits anchorage-independent growth in CAPAN-1 and PANC-1 cells, as assessed by colony formation in soft agar, compared with control (scrambled siRNA transfected) cells ($P < 0.05$). Colony assays were done in triplicate. Columns, average; bars, SD.

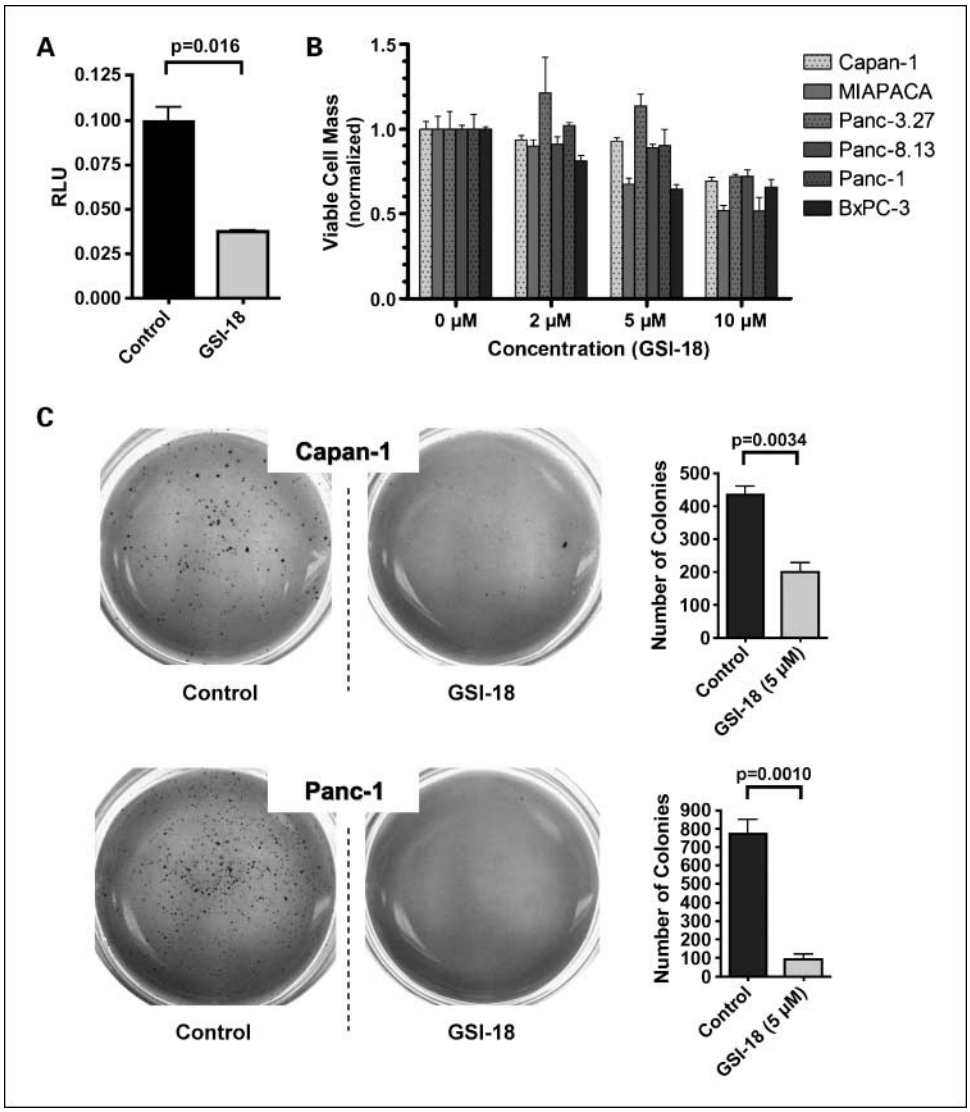

Fig. 4. Pharmacologic knockdown of Notch function in pancreatic cancer cells inhibits anchorage-independent growth. *A,* γ-secretase inhibitor GSI-18 (2 μmol/L) significantly down-regulates CBF-1 – binding site luciferase reporter activity ($P = 0.016$) in PANC-1 cells, consistent with inhibition of intracellular Notch function. Control cells are treated with DMSO vehicle. *Y* axis, relative luciferase activity (*RLU*). *B,* modest dose-dependent inhibition of *in vitro* cell growth (assessed by MTS cell viability assay at 96 h) is observed in a panel of six pancreatic cancer cell lines (CAPAN-1, PANC-1, MIAPaCa-2, BxPC-3, Panc-8.13, and Panc-3.27) on GSI-18 treatment. Three independent doses (2, 5, and 10 μmol/L) are used, and cell viability is normalized to DMSO vehicle-treated cells (0 μmol/L column). All MTS assays are done in triplicate. Columns, average; bars, SD. *C,* GSI-18 significantly inhibits anchorage-independent growth in CAPAN-1 and PANC-1 cells, as assessed by colony formation in soft agar, compared with control (DMSO treated) cells ($P < 0.005$). Colony assays were done in triplicate. Columns, average; bars, SD.

PANC-1 cells, whereas no significant reduction in colony formation was observed in PANC-1-N1ICD cells. Thus, enforced expression of N1ICD is able to rescue PANC-1 cells from the effects of GSI-18, underscoring the relative "on-target" effects of this small-molecule inhibitor.

Transient Notch pathway inhibition eliminates a subpopulation of ALDH bright cells with tumor-initiating properties in pancreatic cancer. Emerging lines of evidence in solid cancers suggest that a subpopulation of cells with tumor-initiating properties (so-called cancer stem cells) can be identified by elevated expression of the enzyme ALDH (5, 24, 28). We have recently identified ALDH bright cells in pancreatic cancer that are highly sensitive to Hedgehog pathway blockade with cyclopamine or related small-molecule inhibitors (5, 6). We have also shown that selective elimination of these ALDH bright cells by transient pretreatment with Hedgehog inhibitors inhibits subsequent tumor initiation (engraftment) in xenograft models (6). To determine whether this putative tumor-initiating population is also Notch pathway dependent, we treated CAPAN-1 and E3LZ10.7 cells with GSI-18 *in vitro* for 24 hours. These two cell lines have been documented to have robust ALDH bright cells detectable by the Aldefluor assay (6). We observed a selective depletion of this subpopulation with transient GSI-18 exposure in both CAPAN-1 and E3LZ10.7 cells (Fig. 6A). On subsequent plating in soft agar, these transiently pretreated cells also showed profound inhibition of anchorage-independent growth (Fig. 6B). Further, when equal numbers of viable E3LZ10.7 or PANC-1 cells, which had been transiently exposed to either GSI-18 or vehicle for 24 hours, respectively, were injected s.c. in athymic mice, a significant blockade of xenograft engraftment was observed in both sets of treated cell lines at 5 weeks of follow-up (Fig. 6C). These findings underscore the importance of sustained Notch signaling in maintaining the viability of tumor-initiating ALDH bright cells in pancreatic cancer and show that even transient exposure to Notch antagonists has deleterious effects on tumor engraftment *in vivo*.

One potential pitfall of the pretreatment strategy is the possibility that overall cellular function is sufficiently compromised by the transient exposure to GSI-18 so that colony formation and engraftment in nude mice are inhibited, irrespective of any specific effect on the tumorigenic population of cells. To exclude this possibility, we did a parallel series of colony assays in soft agar, wherein CAPAN-1 cells were

either "pretreated" transiently with LY294002, an antagonist of Akt/phosphatidylinositol 3-kinase pathway, before plating or exposed continuously to the drug in a more conventional colony assay format. In contrast to our observations with GSI-18, transient pretreatment has no effect on anchorage-independent growth, whereas the conventional colony assays show the expected reduction in colonies at 2 weeks (Fig. 6D). This provides additional confirmation that the loss of tumorigenic phenotype observed with transient Notch inhibition is unlikely to be a nonspecific deleterious effect on cellular function.

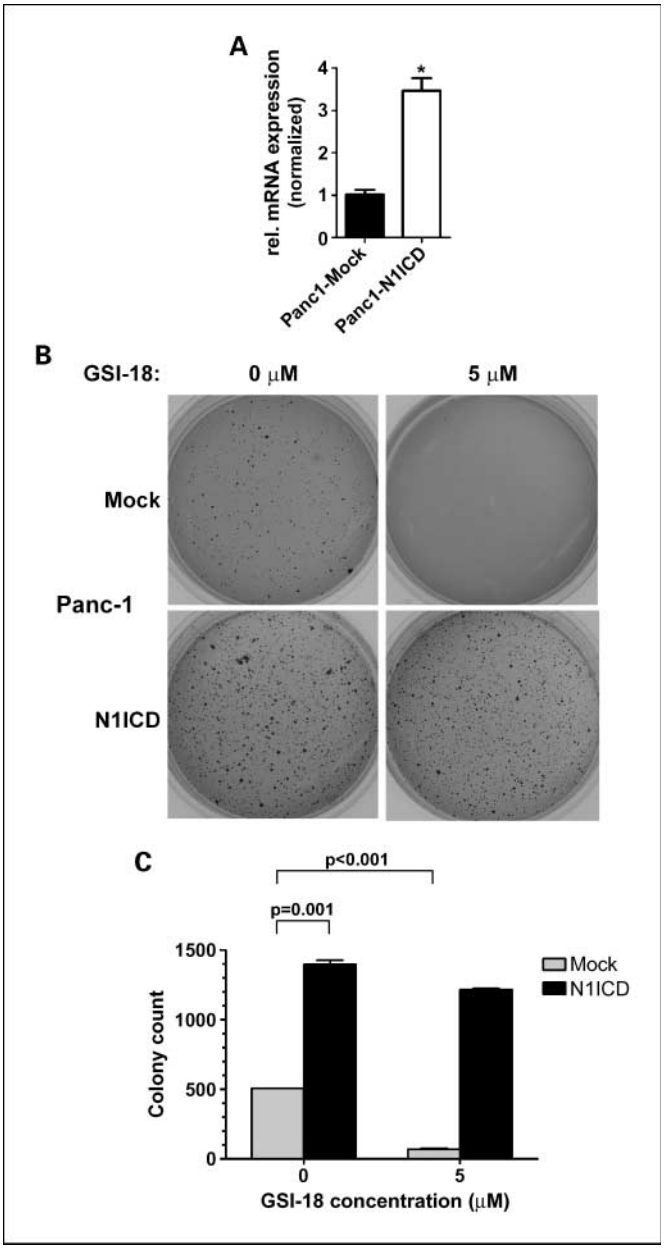

Fig. 5. Enforced expression of N1ICD rescues anchorage-independent growth phenotype of PANC-1 cells treated with GSI-18. Enforced expression of N1ICD in PANC-1 cells (*A*) led to increased colony formation and anchorage-independent growth in soft agar compared with empty vector – transfected cells. Treatment with GSI-18 (5 μmol/L) significantly reduced colony formation of empty vector – transfected cells, but no significant effects were observed in the N1ICD-overexpressing cells (*B*). Colony counts are provided in *C*.

Discussion

The Notch signaling pathway plays a critical role in pancreatic development and in the homeostasis of mature pancreatic tissues (9, 29, 30). In the adult pancreas, we and others have shown that Notch activation is predominantly restricted to the centroacinar cells within the exocrine compartment (13, 31). It is believed that these cells represent a persistent pool of progenitor-type cells in the adult pancreas and that the Notch pathway is a sine qua non for maintaining the undifferentiated state of these cells. An abnormal expansion of Notch-expressing cells is observed in states of exocrine injury, whereas abrogation of Notch signaling impairs subsequent epithelial regeneration, underscoring the importance of this pathway to tissue homeostasis in the pancreas (32 – 34). A role for aberrant Notch signaling in pancreatic cancer has emerged from studies conducted in human and animal models of this disease (13 – 17, 35). For example, the basic helix-loop-helix transcription factor Hes-1 is a prototypal Notch gene target (36), and Hes-1 up-regulation is observed at the earliest, noninvasive stages of human and mouse pancreatic cancer (13, 35).

In mammalian cells, the canonical Notch pathway includes four distinct Notch receptors: NOTCH1, NOTCH2, NOTCH3, and NOTCH4. Previous reports have elucidated context-dependent and cancer type – specific effects of the Notch receptors on carcinogenesis. Thus, NOTCH1 is oncogenic in T-cell leukemia and in breast cancers (18, 37), whereas loss of NOTCH1 function promotes tumorigenesis in medulloblastoma and in skin cancers (21, 38). In medulloblastoma, by contrast, NOTCH2 seems to be the dominant oncogenic receptor (21). Wang et al. (14 – 17) have shown the primacy of NOTCH1 as the Notch pathway receptor responsible for tumor maintenance in pancreatic cancer. Genetic or pharmacologic inhibition of NOTCH1 activity in pancreatic cancer has profound deleterious effects on cell growth, cell survival, and invasion through down-regulation of critical signaling moieties, such as nuclear factor-κB, vascular endothelial growth factor, and matrix metalloproteinases (14 – 17). These existing reports provided the seedbed for our current study exploring the mechanisms of Notch activation in pancreatic cancer and an assessment of the effects of Notch inhibition on the putative tumor-initiating compartment in this malignancy.

The Notch pathway is activated through somatic mutations of *NOTCH1* in ~50% of T-cell leukemias (18) and in a minor subset (<5% by conservative estimates) of other solid cancers, such as breast, lung, and colon cancer (39). The Catalog of Somatic Mutations in Cancer database[7] provides an online compendium of these mutations. Sequencing the complete coding regions of *NOTCH1* and *NOTCH2* genes in 42 pancreatic cancer samples (20 cell lines and 22 xenografts) failed to elicit evidence of activating nonsynonymous alterations. A single hemizygous L2458V PEST domain alteration was identified in the MIAPaCa-2 cell line; however, in the absence of functional correlates of pathway activation (as gauged by Notch reporter and target gene analysis), the significance of this change remains uncertain. Of note, a recent large-scale sequencing effort of the pancreatic cancer genome

[7] http://www.sanger.ac.uk/genetics/CGP/cosmic/

also failed to identify somatic point mutations in *NOTCH1* or *NOTCH2* as well as within any of the genes encoding Notch ligands (40). These results reiterate that mutational activation of the Notch pathway in pancreatic cancer is rare.

In mammalian cells, at least five distinct ligands (JAGGED1, JAGGED2, DLL1, DLL3, and DLL4) initiate Notch signaling on binding to the cognate receptors. We found evidence for striking overexpression of Notch ligand transcripts, especially *JAGGED2* and *DLL4*, in the majority of pancreatic cancer cell lines. Thus, as many as 18 of 20 (90%) of the cell lines examined in our panel showed *JAGGED2* up-regulation compared with hTERT-HPNE cells, with the majority having >50-fold relative expression levels. *JAGGED2* expression was mirrored by, and correlated with, mRNA expression of the *Hairy enhance of split* family of basic helix-loop-helix transcription factors recognized as Notch gene targets, underscoring the functional relevance of ligand-dependent activation. Ligand-dependent activation of embryonic signaling pathways is not unique to Notch, as we and others have described the existence of an analogous mechanism for both Hedgehog and *wnt* pathways, respectively, in pancreatic cancer (41, 42). In both instances, somatic mutations in downstream components (e.g., *GLI1* or *CTNNB1*) are rare to absent, accompanied by endogenous overexpression of stimulatory ligand. An enigmatic question pertains to the upstream genetic influence(s) promoting such profound Notch ligand expression in pancreatic cancer cells. In the context of Hedgehog signaling, we and others have shown that mutant Kras up-regulates the transcription of endogenous

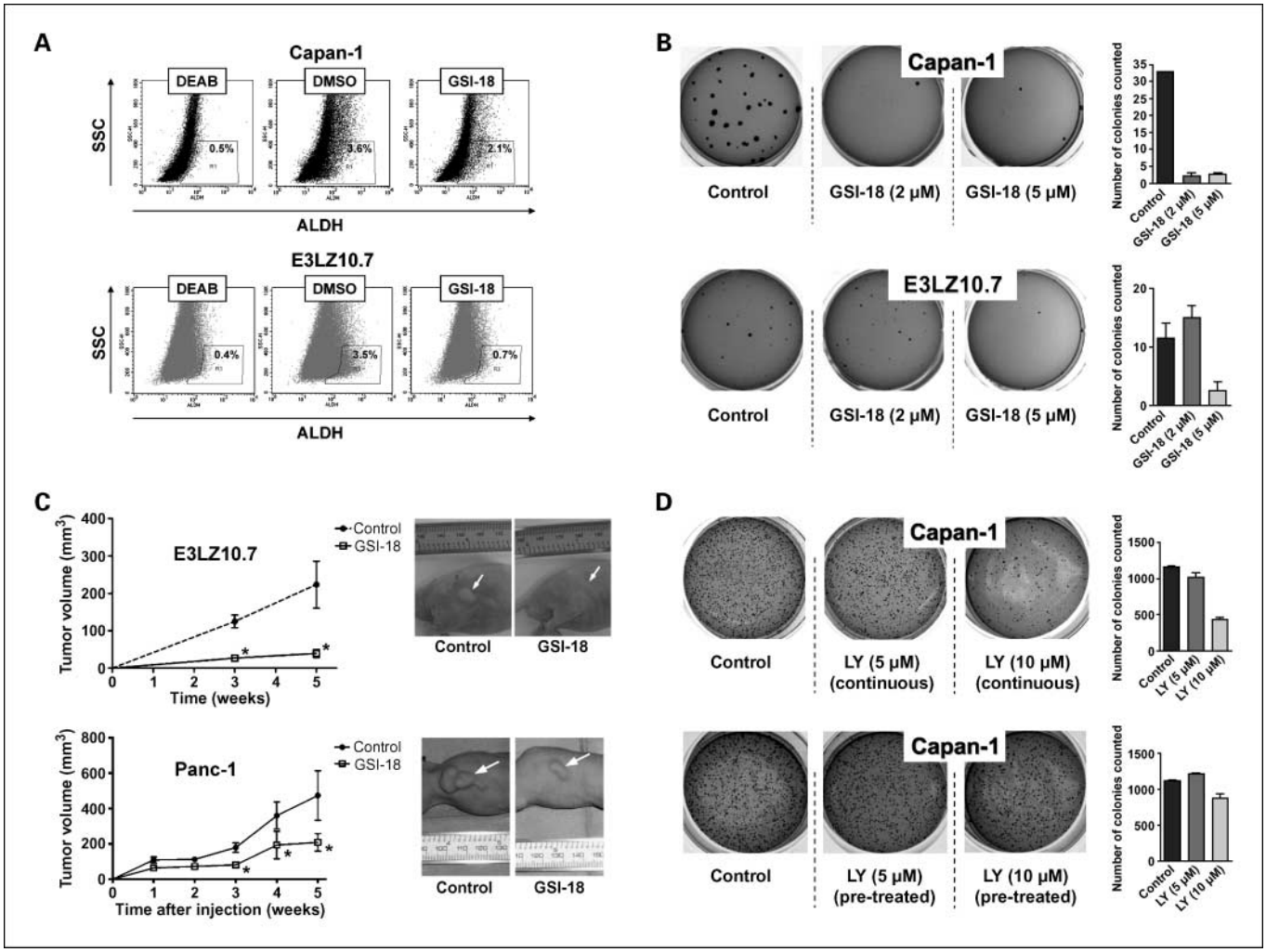

Fig. 6. Transient *ex vivo* exposure of pancreatic cancer cells to Notch inhibitors depletes ALDH bright cells and impedes tumor initiation *in vivo*. *A,* transient incubation of CAPAN-1 and E3LZ10.7 cells with GSI-18 (5 μmol/L) for 24 h reproducibly diminished the fraction of ALDH bright cells as determined by the Aldefluor assay (3.6-2.1% for CAPAN-1 and 3.5-0.7% for E3LZ10.7). The ALDH inhibitor diethylaminobenzaldehyde (*DEAB*) was used as negative control in the assay. *B,* CAPAN-1 and E3LZ10.7 cells were incubated with 2 and 5 μmol/L doses of GSI-18 for 24 h followed by full serum recovery for an additional 24 h and plating in soft agar for colony assays. No further GSI-18 exposure was administered to the plated cells. Compared with equal numbers of viable plated cells in the DMSO-treated group, reduction in colony formation is observed at 2 and 5 μmol/L doses for CAPAN-1 cells and at 5 μmol/L dose for E3LZ10.7 cells. *C,* E3LZ10.7 or PANC-1 cells were incubated with GSI-18 at a concentration of 5 μmol/L for 24 h followed by full serum recovery for an additional 24 h and injection of 5×10^6 cells in the s.c. milieu of athymic mice. No further *in vivo* treatment was done. Compared with equal numbers of viable injected cells in the DMSO-treated group, significant reduction in size of the engrafted tumors is seen with transient GSI-18 exposure in both sets of cell lines, beginning at 3 wk after injection and persisting at 5 wk (*asterisks*). *D,* in contrast to the phenotype observed with GSI-18 pretreatment, no effects of transient exposure are seen with LY294002, a small-molecule inhibitor of the Akt signaling pathway, in CAPAN-1 cells. Specifically, CAPAN-1 cells were exposed to two doses (5 and 10 μmol/L) of LY294002 in one of two modes: continuous, wherein cells were incubated with continuous exposure to the drug, as in a conventional colony assay, and pretreatment, mimicking the transient pretreatment exposure for 24 h done with GSI-18.

Hedgehog ligand in pancreatic cancer cells (43, 44). Whether parallel mechanisms are in place for the Notch pathway remains a matter of investigation. In light of the prior observations by Weijzen and colleagues (45) pertaining to the absolute requirement of Notch signaling for maintaining the neoplastic phenotype of human *Ras*-transformed cells, and the demonstration of Notch activation in pancreatic ductal lesions arising in Kras-driven genetically engineered mouse models of pancreatic cancer (35), the existence of such an axis is not beyond the realm of speculation. In passing, we should add that in a minority of instances, Notch activation seems to be a consequence of genomic copy number alterations at chromosome 19q13, the *DLL3* gene locus (19, 26, 27). We have confirmed the existence of increased gene dosage, and associated *DLL3* transcript overexpression, in two cell lines and have shown that knockdown of *DLL3* by RNAi can inhibit anchorage-independent growth in the SU86.86 cell line. Curiously, *DLL3* RNAi in PANC-1 cells did not exhibit a discernible growth phenotype, suggesting that redundant ligand-driven activation can bypass the blockade of any one single Notch ligand, and underscores the need for targeting downstream elements of this pathway in cancer therapy.

In addition to exploring the mechanisms of Notch activation in pancreatic cancer, we also assessed the potential of Notch as a therapeutic target in pancreatic cancer and, in particular, whether Notch inhibition has a preferential deleterious effect on the tumor-initiating (cancer stem cell) compartment. In light of the prior series of studies by Wang and colleagues (14–17), our findings on Notch inhibition and pancreatic cancer maintenance are mainly confirmatory in nature. Nevertheless, these studies expand the repertoire of pancreatic cancer cell line models in which the anticancer effects of Notch inhibition, either by genetic or pharmacologic means, are evident. Further, our findings confirm that NOTCH1 is possibly the dominant oncogenic Notch receptor in this malignancy and that γ-secretase inhibitors such as GSI-18, or other comparable small molecules (7, 46), warrant further preclinical evaluation in pancreatic cancer. In contrast to genistein and curcumin, two previously reported Notch inhibitors that are natural plant polyphenols (14, 16, 17), the synthetic γ-secretase inhibitors are likely to have a more limited repertoire of targeted intracellular effects. γ-Secretase inhibitors are currently in advanced-phase clinical trials for Alzheimer disease, having shown favorable toxicity profiles in healthy volunteers (47, 48), and therefore, the transition to being used as an anticancer agent may be an option in not too distant a future. Besides pancreatic cancer maintenance, however, a novel finding of our study has been the demonstration that even transient *ex vivo* pharmacologic Notch inhibition depletes the putative tumor-initiating population in pancreatic cancer. We and others have recently identified ALDH bright cells detectable by Aldefluor assay as an enriched cancer stem cell compartment in a variety of solid cancers, including pancreatic cancer (5, 6, 24, 28). The ALDH bright cells are eliminated on systemic Hedgehog inhibitor therapy and correlate with abrogation of metastases in orthotopic xenograft models of pancreatic cancer (5, 6). Here, we have shown that transient *ex vivo* treatment with GSI-18 depletes the ALDH bright population in pancreatic cancer cell lines, and this is paralleled by a significant reduction in anchorage-independent growth and xenograft engraftment in athymic mice. Due to limited drug availability, we were unable to do systemic trials in orthotopic xenograft models, but our results lay the groundwork for such analyses in the future. The observation that the ALDH bright cells are both Hedgehog and Notch dependent for their viability suggests that combinatorial therapy with small-molecule inhibitors against both pathways might have even more potent effects *in vivo* than single-agent treatment. Further, our findings reiterate our previously stated postulate that effective therapy of pancreatic cancer will likely require targeting both the "bulk" tumor cells with a conventional antimetabolite, such as gemcitabine, as well as a stem cell–directed therapy, such as Notch or Hedgehog inhibitor, to eliminate the cells responsible for metastases and disease recurrence.

In conclusion, we show that ligand-dependent activation of the Notch signaling pathway is common in pancreatic cancer. Pharmacologic inhibition of Notch signaling is a valid therapeutic strategy in this malignancy based on the requirement of sustained Notch activation for tumor initiation as well as for tumor maintenance of pancreatic cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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