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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 mechanisms for this activation have 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 gamma 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* (18/20 cases; 90%) and *DLL4* (10/20 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 pharmacological 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 pre-treatment of pancreatic cancer cells with GSI-18 resulted in depletion in the proportion of tumor-initiating aldehyde dehydrogenase (ALDH)-expressing subpopulation, and was associated with inhibition of colony formation *in vitro* and xenograft engraftment *in vivo*, underscoring a requirement for the Notch-dependent ALDH-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.

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Keywords

Pancreatic cancer; Notch; NOTCH-1; gamma secretase inhibitor; aldehyde dehydrogenase; tumor initiating cells

Statement of 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 demonstrate this to be ligand-driven rather than mutationally activated. Second, we demonstrate the requirement of sustained Notch signaling for pancreatic cancer maintenance using genetic and pharmacological approaches towards 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 comprised of tumor-initiating "stem cells" and the "bulk" tumor population, and therapeutic success will be engendered by dual targeting of both compartments.

Introduction

Pancreatic cancer is an almost uniformly lethal disease with an overall five-year survival of approximately 5%, and this dire prognosis has not markedly improved over the last few decades (1). In the United States, approximately 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 impact in ameliorating survival. Identification of aberrant signalling pathways that can also form the substrate for targeted therapies has thus become an area of foremost priority.

The re-activation 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 (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 non-invasive precursor lesions of this malignancy (13). In a series of elegant studies, Sarkar and colleagues have demonstrated a requirement for active Notch signalling for tumor maintenance in pancreatic cancer, with downregulation of Notch-1 contributing to growth inhibition and apoptosis of cancer cells through inhibition of key survival pathways like nuclear factor kappa B (NFκB) (14–17). 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*, appears 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 hematological malignancies like T-cell leukemia (18), mutational activation of Notch is rare to absent in pancreatic cancer. Our studies also demonstrate that sustained Notch signalling 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 non-malignant 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 amongst the 20 cancer cell lines.

RNAi-mediated transcript knockdown

For knockdown of *NOTCH1* transcripts, PANC-1 and CAPAN-1 cells were transiently transfected with gene specific or scrambled siRNA using Oligofectamine (Invitrogen) following the standard procedure recommended by the manufacturer. Efficacy of knockdown was confirmed by qRT-PCR, as described below. The sequences for the synthetic siRNAs against NOTCH1 (Dharmacon, Lafayette, CO, USA) have been previously described (21). Similarly, RNAi against *DLL3* was performed 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 NICD in PANC-1 cells

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

Notch pathway inhibitor GSI-18

Synthesis of the gamma-secretase inhibitor [11-endo]-N-(5,6,7,8,9,10-hexahydro-6,9-methanobenzo[a][8]annulen-11-yl)-thiophene-2-sulfonamide (a.k.a. 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 performed 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® A_{queous} Cell Proliferation Assay (Promega, Madison, WI, USA), which relies on the conversion of a tetrazolium compound (MTS) to a colored formazan product by the activity of living cells. Briefly, 2000 cells/well were plated in 96 well plates, and were treated with 2, 5 and 10 μM concentrations of GSI-18, for 96 hours, at which point the assay was terminated, and relative growth inhibition compared to vehicle-treated cells measured using the CellTiter 96® reagent, as described in the manufacturer's protocol. A panel of six human pancreatic cancer cell lines were examined

(PANC-1, CAPAN-1, BxPC-3, MIAPaca-2, PANC-8.13, PANC-3.27) in the MTS assays. Cell viability assays were also performed 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 standard deviations.

Anchorage independent growth

Anchorage independent growth was assessed in PANC-1 and CAPAN-1 cells following either genetic inhibition (*NOTCH1* siRNA) or with pharmacological inhibition of Notch signaling with GSI-18 (5 μM). Soft agar assays were set up in 6-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 pharmacological inhibition experiments, mixtures in each well were supplemented with GSI-18 at the respective concentration or solvent only, and the plates were incubated for three weeks. An independent series of colony assays was performed 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 was 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 (BioRad, Hercules, California, USA).

Evaluation of aldehyde dehydrogenase (ALDH) activity

ALDH expression was determined at baseline and after pharmacological Notch inhibition in two pancreatic cancer cell lines, E3LZ10.7 and CAPAN-1, where we have previously demonstrated that inhibition of Hedgehog signaling selectively depletes the ALDH "bright" subpopulation (5,6). After incubation with either vehicle or GSI-18 (5μM) for 24 hours, E3LZ10.7 and CAPAN-1 cells were stained for ALDH expression using the Aldefluor reagent (StemCell Technologies, Vancouver, Canada) 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 to a control staining reaction containing the ALDH inhibitor diethylamino-benzaldehyde (DEAB).

Pre-treatment with GSI-18

We have previously shown that transient *ex vivo* pre-treatment with Hedgehog antagonists inhibits both anchorage independent growth and *in vivo* tumorigenicity of pancreatic cancer and glioblastoma cell lines (6,24). In order to determine the effects of Notch antagonism on tumor initiation, pancreatic cancer cell lines E3LZ10.7 and CAPAN-1 cells were pre-treated with either vehicle or GSI-18 for 24 hours (2 and 5 μM), and allowed to recover in full serum for 24 hours. 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. Pre-treated 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/PI-3-kinase pathway inhibito

In order to confirm the specificity of Notch inhibition against the tumor initiating component and exclude the potential for artefact, we performed a series of experiments using CAPAN-1 cells treated with LY294002, a small molecule inhibitor of the oncogenic Akt/PI-3-kinase pathway. Two parallel sets of anchorage independent assays were performed: first, a "*pre-treatment*" experiment mirroring the GSI-18 study, with two doses of LY294002 (5 and 10 μM). In this experiment, CAPAN-1 cells were exposed to the drug for 24 hours, followed by full serum recovery and plating in soft agar. The second set of experiments, with the same dosages, utilized a "*continuous*" (conventional) approach, where the cells were incubated in

soft agar with continuous exposure to LY294002 for two weeks. Colony counts were performed as described above.

Generation of murine subcutaneous 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, pre-treated with either vehicle or with GSI-18 at 5μM, and allowed to recover in full serum for 24 hours, were injected subcutaneously into male CD1 nu/nu athymic mice (Charles River). Tumor volumes (V) were determined after measuring the larger (a) and smaller (b) diameters as $V=\frac{a \cdot b^2}{2}$, as previously described (5,6).

Statistical analysis

Kruskal-Wallis analysis was performed using SPSS version 15.0.1 for Microsoft Windows, two-tailed t-test, one way ANOVA and linear regression analysis (Pearson's test) were performed using GraphPad Prism for Windows version 5. $P<0.05$ was regarded as statistically significant. Results in bar diagrams are plotted as means and standard deviations 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 *4* transcripts, with most cell lines not demonstrating any evidence of receptor mRNA overexpression. Thus, compared to 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 (Figure 1A). Curiously, *NOTCH3* mRNA expression was lower than hTERT-HPNE cells in all 20 cancer cell lines. In contrast to receptor mRNA levels, marked upregulation 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%) demonstrating mRNA overexpression compared to hTERT-HPNE cells (Figure 1B). *JAGGED1* and *DLL1* transcripts were expressed at more attenuated levels (no higher than 10-fold relative overexpression compared to hTERT-HPNE cells), and were upregulated in fewer cell lines within the panel (Supplementary Figure 1). 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 PC cell lines, respectively (Figure 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*).Upon 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 (bHLH) 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 a panel of 20 cell lines, and found two lines – PANC-1 and SU86.86 that demonstrated 3-fold or greater copy number by genomic quantitative PCR (qPCR), compared to hTERT-HPNE cells (Figure 2A). Transcript profiling confirmed that PANC-1 andSU86.86 had strikingly high expression of DLL3 mRNA, ~200-fold that of hTERT-HPNE cells (Figure 2B). *DLL3* was downregulated by transient RNA interference (RNAi) in both cells 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 (Figure 2C, P=0.0005), as well as significant inhibition of anchorage independent growth in soft agar (Figure 2D, P=0.0016). 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.

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 performed by direct Sanger sequencing of the coding regions. All sequence variations from RefSeq (http://www.ncbl.nlm.nih.gov/RefSeq) were first confirmed by replicate PCR, and subsequently cross-matched against the single nucleotide polymorphism database dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP). A previously undescribed heterozygous L2458V alteration was identified in the C-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 Figure 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 downregulate *NOTCH1* transcript levels in PANC-1 and CAPAN-1 cancer cell lines; efficacy of RNAi was confirmed by real-time PCR demonstrating downregulation of *NOTCH1* transcripts, as well as multiple Notch target genes (Figure 3A). Both cell lines transfected with *NOTCH1* siRNA demonstrated a significant reduction in the number of colonies formed in soft agar compared to scrambled siRNA transfected controls, confirming a requirement of active Notch signaling for anchorage independent growth (P=0.0341 and P=0.0126, respectively; Figure 3B).

To complement the RNAi findings, we also studied the effects of pharmacological blockade of Notch signaling in pancreatic cancer cells on *in vitro* growth in monolayer and anchorage

independent growth in soft agar. GSI-18 is a previously described gamma 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 CBF1-binding site luciferase reporter assays (Figure 4A). A panel of six pancreatic cancer cell lines were used for *in vitro* growth (MTS) assays. As shown in Figure 4B, only modest growth inhibition was observed with GSI-18 at the highest dose (10 μM), and cell viability was largely unaffected at 2 and 5μM 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 5μM GSI-18 (P=0.0034 and P=0.0010, respectively; Figure 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 Notch 1 Intracellular Domain (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* as compared to empty vector transfected cells was confirmed by qRT-PCR (Figure 5A). Of note, enforced *N1ICD* expression *per se* markedly enhanced anchorage independent growth of PANC-1 cells in soft agar assays (P=0.001). Treatment with GSI-18 at a concentration of 5μM led to a more than seven-fold reduction in colony numbers in empty vector-transfected PANC-1 cells (P<0.001), while only minimal reduction in colony formation was observed in PANC-1-N1ICD cells (Figures 5B and C). 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 aldehyde dehydrogenase (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 pre-treatment with Hedgehog inhibitors inhibits subsequent tumor initiation (engraftment) in xenograft models (6). In order 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 (Figure 6A). Upon subsequent plating in soft agar, these transiently pre-treated cells also demonstrated profound inhibition of anchorage independent growth (Figure 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 subcutaneously in athymic mice, a significant blockade of xenograft engraftment was observed in both sets of treated cell lines, during 5 weeks of follow up (Figure 6C). These findings underscore the importance of sustained Notch signaling in maintaining the viability of tumor-initiating ALDH "bright" cells in pancreatic cancer, and demonstrate that even transient exposure to Notch antagonists has deleterious effects on tumor engraftment *in vivo*.

One potential pitfall of the "pre-treatment" strategy is the possibility that overall cellular function is sufficiently compromised by the transient exposure to GSI-18 that colony formation and engraftment in nude mice are inhibited, irrespective of any specific impact on the tumorigenic population of cells. To exclude this possibility, we performed a parallel series of

colony assays in soft agar, wherein CAPAN-1 cells were either “pre-treated” transiently with LY294002, an antagonist of Akt/PI-3-kinase pathway, prior to plating, or exposed continuously to the drug in a more conventional colony assay format. In contrast to our observations with GSI-18, transient pre-treatment has no effect on anchorage independent growth, while the conventional colony assays demonstrate the expected reduction in colonies at 2 weeks (Figure 6D). This provides additional confirmation that the loss of tumorigenic phenotype observed with transient Notch inhibition is unlikely to be a non-specific deleterious effect on cellular function.

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, while 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 (bHLH) transcription factor Hes-1 is a prototypal Notch gene target (36), and Hes-1 upregulation is observed at the earliest, non-invasive stages of human and mouse pancreatic cancer (13,35).

In mammalian cells, the canonical Notch pathway includes four distinct Notch receptors, NOTCH1-4. 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), while loss of NOTCH1 function promotes tumorigenesis in medulloblastoma and in skin cancers (21,38). In medulloblastoma, by contrast, NOTCH2 appears to be the dominant oncogenic receptor (21). Sarkar et al have demonstrated the primacy of NOTCH1 as the Notch pathway receptor responsible for tumor maintenance in pancreatic cancer (14–17). Genetic or pharmacological inhibition of NOTCH1 activity in pancreatic cancer has profound deleterious effects on cell growth, cell survival, and invasion, through downregulation of critical signaling moieties like NFκB, vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs) (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 approximately 50% of T-cell leukemias (18), and in a minor subset (<5% by conservative estimates) of other solid cancers like breast, lung and colon cancer (39). The Catalog of Somatic Mutations in Cancer (COSMIC) database (http://www.sanger.ac.uk/genetics/CGP/cosmic/) 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 non-synonymous 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 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 (JAGGED-1, JAGGED-2, DLL-1, DLL-3, and DLL-4) initiate Notch signaling upon binding to the cognate receptors. We found evidence for striking overexpression of Notch ligand transcripts, especially *JAGGED-2* and *DLL-4*, in the majority of pancreatic cancer cell lines. Thus, as many as 18 of 20 (90%) of the cell lines examined in our panel demonstrated *JAGGED-2* upregulation compared to hTERT-HPNE cells, with the majority having >50-fold relative expression levels. *JAGGED-2* expression was mirrored by, and correlated with, mRNA expression of the *Hairy enhance of split* family of bHLH 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 (for example, *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 upregulates the transcription of endogenous 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 Miele and colleagues pertaining to the absolute requirement of Notch signaling for maintaining the neoplastic phenotype of human *Ras*-transformed cells (45), 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 appears 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 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 Sarkar 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 anti-cancer effects of Notch inhibition, either by genetic or pharmacological means, are evident. Further, our findings confirm that NOTCH1 is possibly the dominant oncogenic Notch receptor in this malignancy, and that gamma secretase inhibitors like 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 gamma secretase inhibitors are likely to have a more limited repertoire of targeted intracellular effects. Gamma secretase inhibitors are currently in advanced phase clinical trials for Alzheimer disease, having demonstrated favorable toxicity profiles in healthy volunteers (47,48), and therefore, the transition to being utilized as an anti-cancer 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* pharmacological 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 upon systemic Hedgehog inhibitor therapy, and correlate with abrogation of metastases in orthotopic xenograft models of pancreatic cancer (5,6). Here we have demonstrated 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 perform 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 anti-metabolite like gemcitabine, as well as a stem cell directed therapy like Notch or Hedgehog inhibitor to eliminate the cells responsible for metastases and disease recurrence.

In conclusion, we demonstrate that ligand-dependent activation of the Notch signaling pathway is common in pancreatic cancer. Pharmacological 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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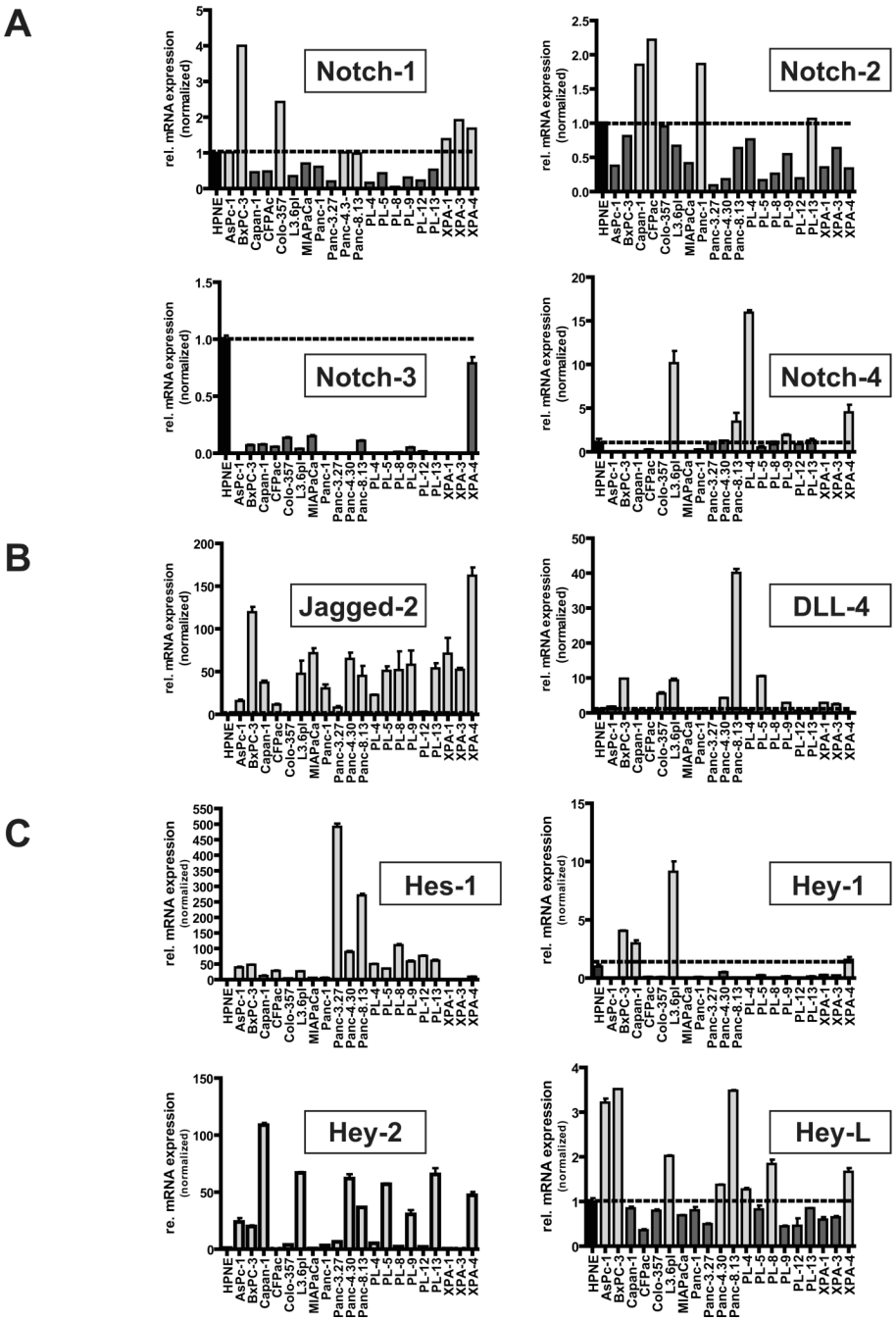

Figure 1. Profiling the Notch pathway in pancreatic cancer cell lines

RNA from a panel of 20 pancreatic cancer cell lines was assessed for expression of Notch receptors *NOTCH1*,*NOTCH2*, *NOTCH3* and *NOTCH4* (**panel A**), Notch ligands *JAGGED2* and *DLL4* (**panel B**) and Notch gene targets *HES1*, *HEY1*, *HEY2* and *HEYL* (**panel C**), and relative fold levels compared to immortalized hTERT-HPNE cells. Horizontal line indicates normalized ratio of 1 in hTERT-HPNE cells. X-axis corresponds to individual cell line samples, and Y-axis to relative fold level of expression. Light grey bars indicate cancer cell lines with overexpression of corresponding mRNA compared to hTERT-HPNE cells, while dark grey bars indicate cell lines with lesser expression. All assays were performed in triplicate, using

PGK1 as housekeeping control, and an independent set of assays was performed using *GUSB* as housekeeping control (data not shown).

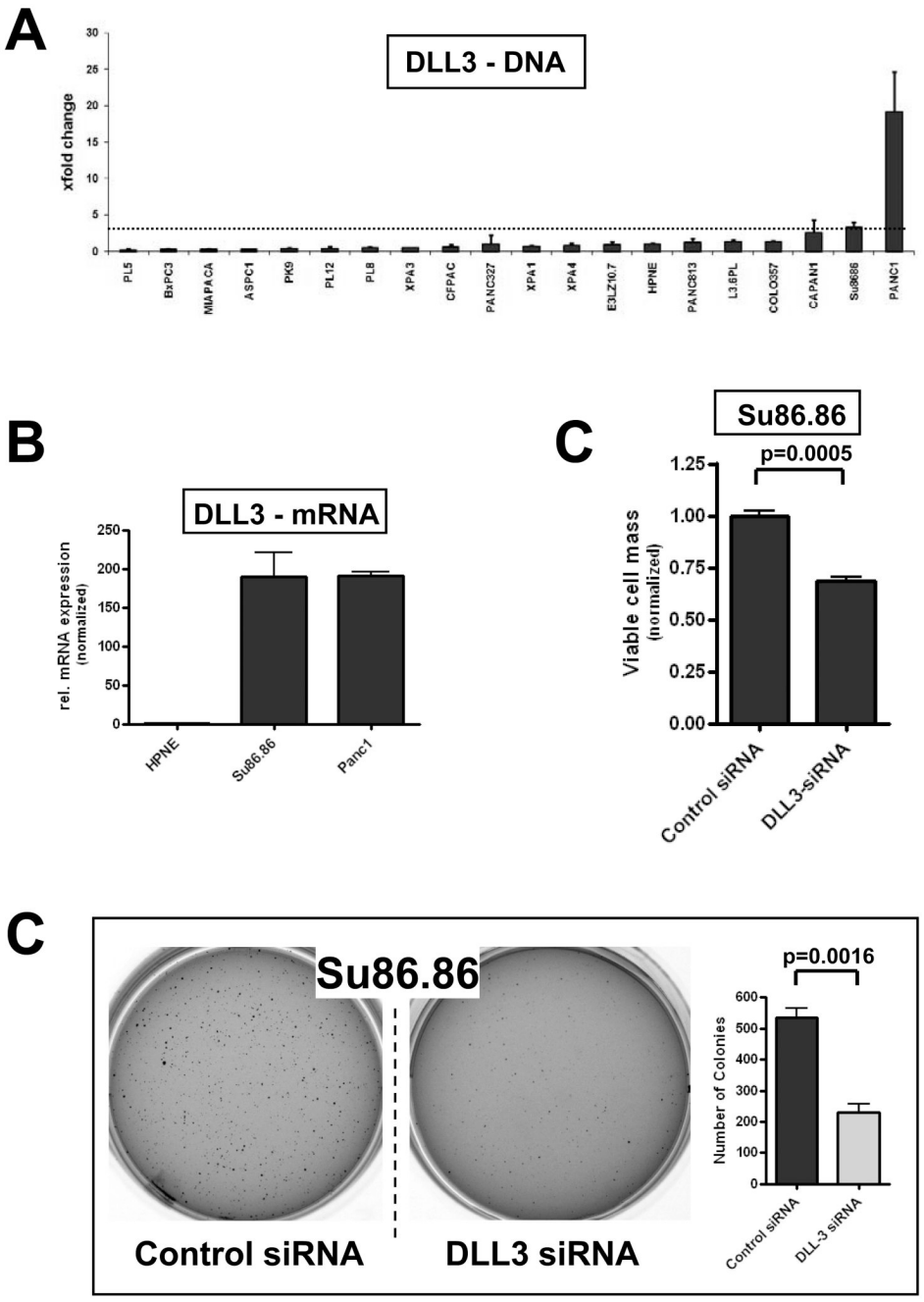

Figure 2. Copy number alteration of *DLL3* in a subset of pancreatic cancer cell lines

(**A**) Genomic qPCR for *DLL3* copy number demonstrates 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 qPCR was performed in triplicate, and average ± SD calculated, with the latter expressed as error bars.

(**B**) Quantitative reverse transcription PCR (qRT-PCR) for *DLL3* mRNA in PANC-1 and SU86.86 cells demonstrates striking overexpression (~200-fold), relative to levels in hTERT-HPNE cells.

(**C**) Knockdown of DLL3 by synthetic small interfering RNA (siRNA) significantly inhibits *in vitro* growth of SU86.86 cells, as measured by an MTS cell viability assay at 96 hours, compared to 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 to control (scrambled siRNA transfected) cells; (P =0.0016). Colony assays were performed in triplicate, and average ± SD calculated, with the latter expressed as error bars.

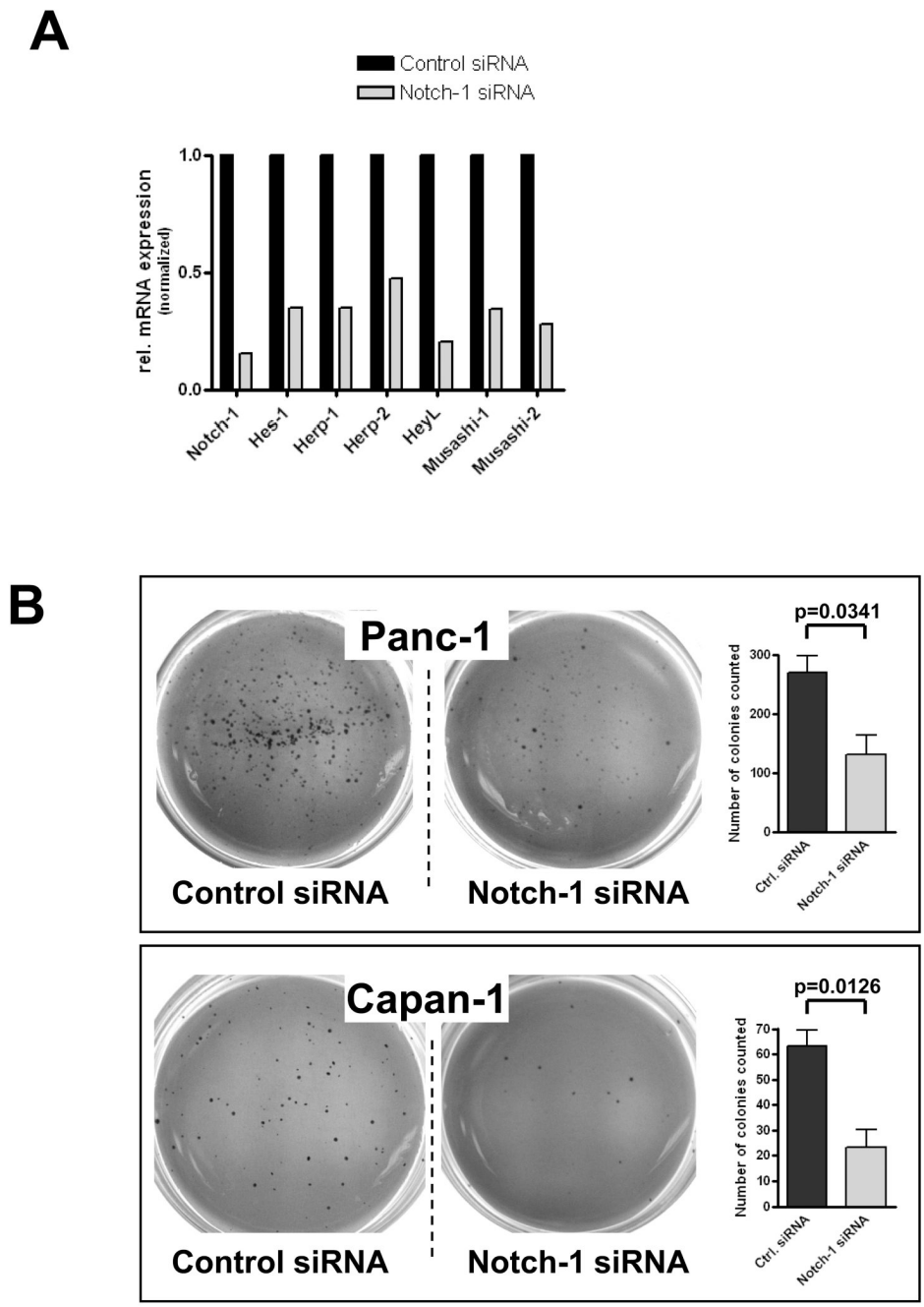

Figure 3. Genetic knockdown of *NOTCH1* function in pancreatic cancer cells inhibits anchorage independent growth

(A) *NOTCH1* RNAi in CAPAN-1 cells leads to >80% downregulation of gene specific transcript levels compared to 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 to 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 to control (scrambled

siRNA transfected) cells; (P <0.05). Colony assays were performed in triplicate, and average ± SD calculated, with the latter expressed as error bars.

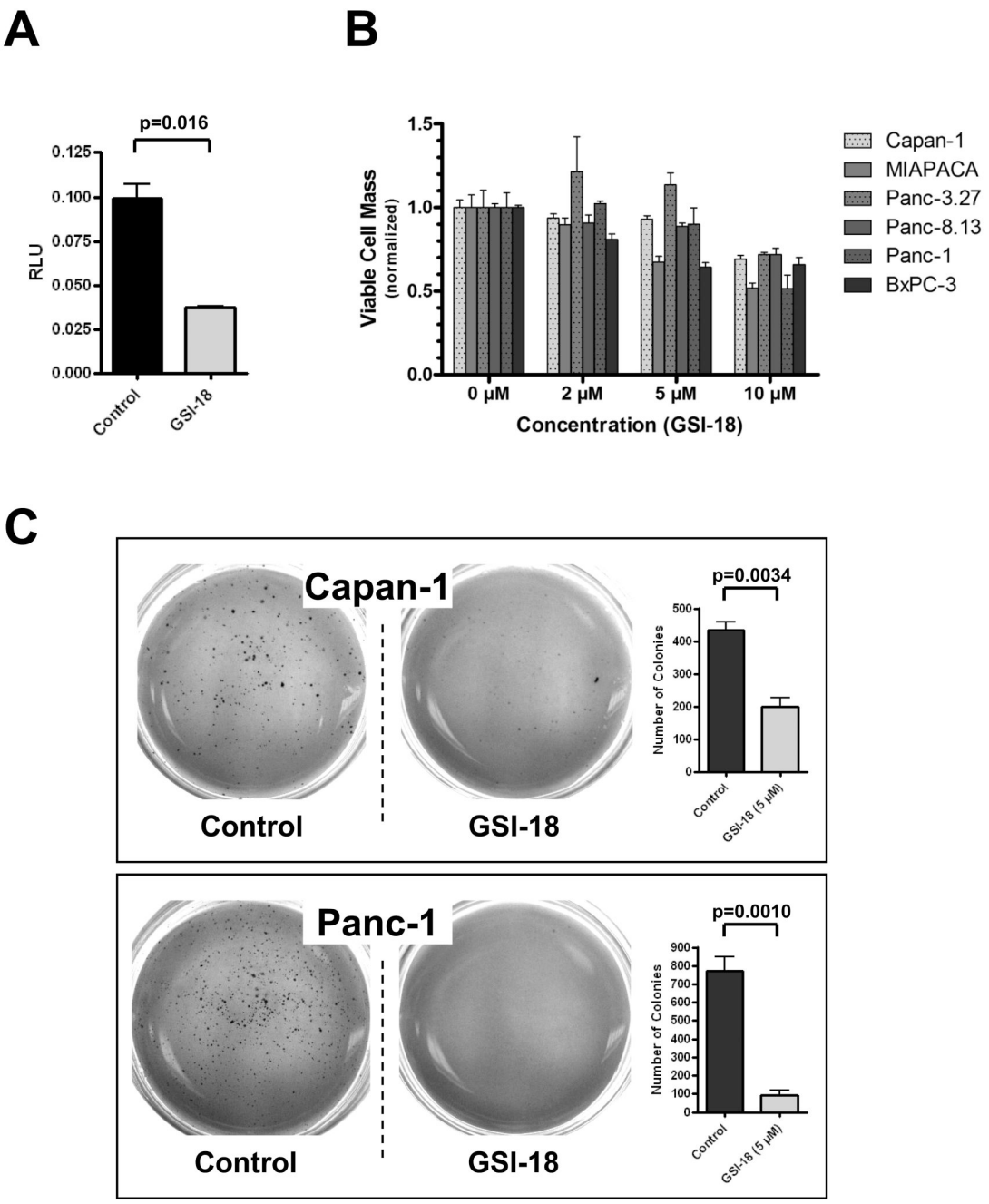

Figure 4. Pharmacological knockdown of Notch function in pancreatic cancer cells inhibits anchorage independent growth

(**A**) Gamma secretase inhibitor GSI-18 (2μM) significantly downregulates 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 depicts relative luciferase activity (RLU).

(**B**) Modest dose dependent inhibition of *in vitro* cell growth (assessed by MTS cell viability assay at 96 hours) is observed in a panel of six pancreatic cancer cell lines (CAPAN-1, PANC-1, MIAPACA-2, BxPC-3, PANCA-8.13, and PANC-3.27) upon GSI-18 treatment. Three independent doses (2, 5, and 10μM) are used, and cell viability is normalized to DMSO vehicle

treated cells (0μM column). All MTS assays are performed in triplicate, and average ± SD calculated, with the latter expressed as error bars.

(**C**) GSI-18 significantly inhibits anchorage independent growth in CAPAN-1 and PANC-1 cells, as assessed by colony formation in soft agar, compared to control (DMSO-treated) cells; (P <0.005). Colony assays were performed in triplicate, and average ± SD calculated, with the latter expressed as error bars.

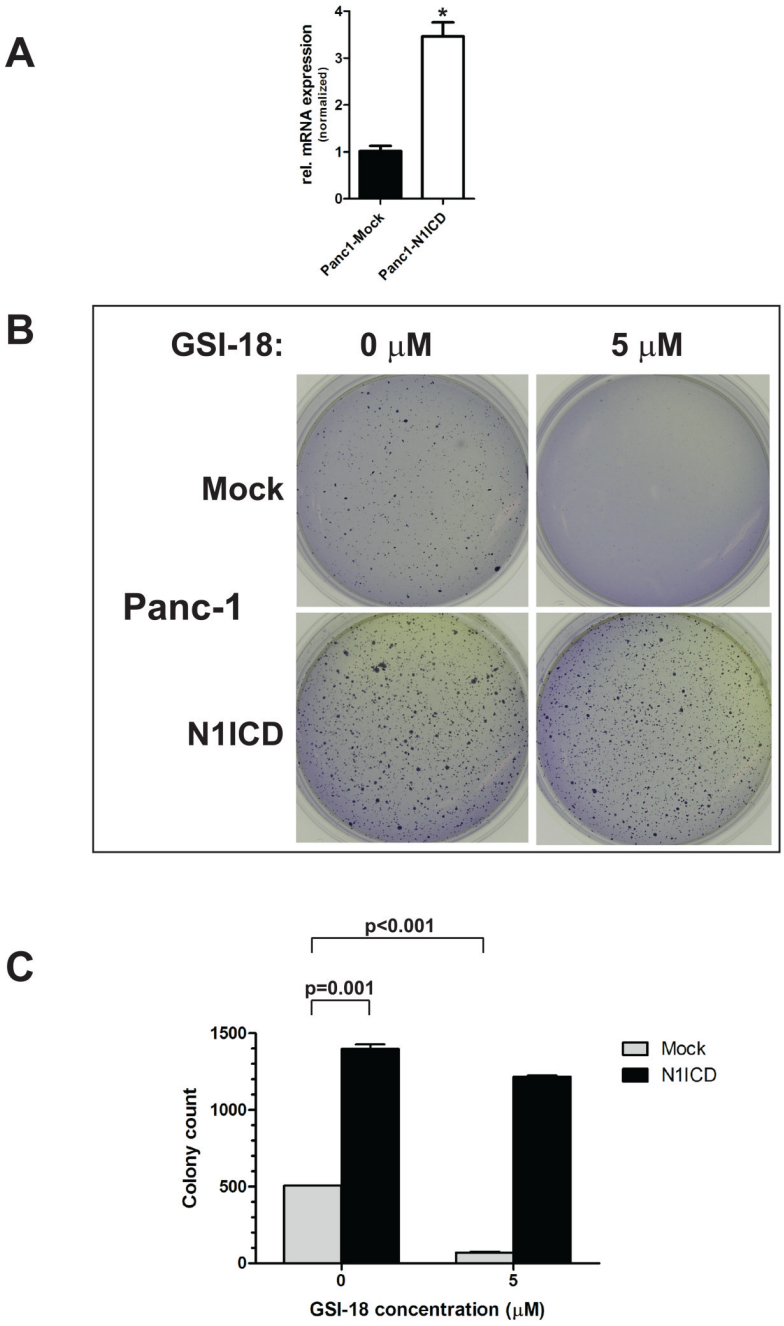

Figure 5. Enforced expression of Notch 1 intracytoplasmic domain (N1ICD) rescues anchorage independent growth phenotype of PANC-1 cells treated with GSI-18

Enforced expression of N1ICD in PANC-1 cells (**A**) led to increased colony formation and anchorage independent growth in soft agar as compared to empty vector transfected cells. Treatment with GSI-18 (5 μM) 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)**.

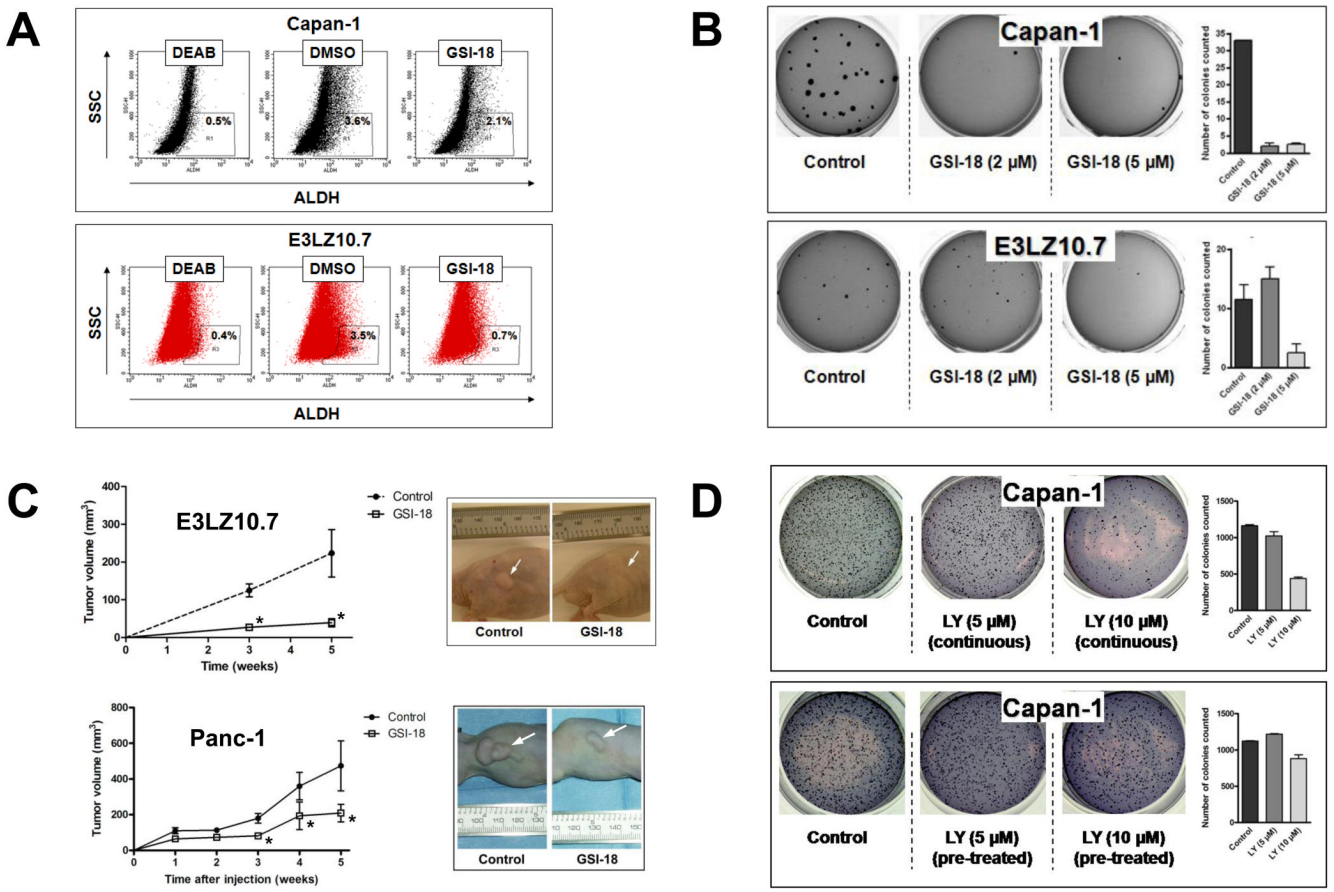

Figure 6. Transient *ex vivo* exposure of pancreatic cancer cells to Notch inhibitors depletes ALDH "bright" cells and impedes tumor initiation *in vivo*

(**A**) Transient incubation of CAPAN-1 and E3LZ10.7 cells with GSI-18 (5 μM) for 24 hours reproducibly diminished the fraction of ALDH "bright" cells as determined by the Aldefluor assay (3.6% to 2.1% for CAPAN-1 and 3.5% to 0.7% for E3LZ10.7). The ALDH inhibitor DEAB was used as negative control in the assay.

(**B**) CAPAN-1 and E3LZ10.7 cells were incubated with 2 and 5μM doses of GSI-18 for 24 hours, followed by full serum recovery for an additional 24 hours, and plating in soft agar for colony assays. No further GSI-18 exposure was administered to the plated cells. Compared to equal numbers of viable plated cells in the DMSO-treated group, reduction in colony formation is observed at 2 and 5μM doses for CAPAN-1 cells, and at 5μM dose for E3LZ10.7 cells.

(**C**) E3LZ10.7 or PANC-1 cells were incubated with GSI-18 at a concentration of 5 μM for 24 hours, followed by full serum recovery for an additional 24 hours, and injection of 5×10^6 cells in the subcutaneous milieu of athymic mice. No further *in vivo* treatment was performed. Compared to 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 weeks post-injection and persisting at 5 weeks (*asterisks*).

(**D**) In contrast to the phenotype observed with GSI-18 pre-treatment, 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 10μM) 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 "pre-treatment", mimicking the transient pre-treatment exposure for 24 hours performed with GSI-18.