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Down Regulation of CSL Activity Inhibits Cell Proliferation in Prostate and Breast Cancer Cells

Thomas Yong,[1] Amanda Sun,[1] Michael D. Henry,[2] Shari Meyers,[3] and J. Nathan Davis[3*]

[1] *Department of Medicine, Feist-Weiller Cancer Center, Louisiana State University Health Sciences Center-Shreveport, Shreveport, Louisiana*

[2] *Department of Molecular Physiology and Biophysics, Holden Comprehensive Cancer Center, University of Iowa, Carver College of Medicine, Iowa City, Iowa*

[3] *Department of Biochemistry and Molecular Biology, Feist-Weiller Cancer Center, Louisiana State University Health Sciences Center-Shreveport, Shreveport, Louisiana*

ABSTRACT

The Notch receptor pathway provides a paradigm for juxtacrine signaling pathways and controls stem cell function, developmental cell fate decisions, and cellular differentiation. The Notch pathway is constitutively activated in human cancers by chromosomal rearrangements, activating point mutations, or altered expression patterns. Therefore, the Notch pathway is the subject of chemotherapeutic intervention in a variety of human cancers. Notch receptor activation results in the gamma-secretase dependent proteolytic cleavage of the receptor to liberate the Notch intracellular domain that acts to mediate co-activator recruitment to the DNA binding transcription factor, CSL (CBF-1/RBP-Jκ, Su(H), Lag-1). Therapeutic targeting of the Notch pathway by gamma-secretase inhibitors prevents NICD production and regulates CSL-dependent transcriptional activity. To interrogate the loss of CSL activity in breast and prostate cancer cells, we used lentiviral-based shRNA knockdown of CSL. Knockdown of CSL expression was assessed by decreased DNA binding activity and resulted in decreased cell proliferation. In contrast, gamma-secretase inhibitor (GSI) treatment of these prostate and breast cancer cell lines resulted in minimal growth effects. PCR profiling of Notch pathway genes identified expression changes in few genes (Delta-like-1, Deltex-1, LMO2, and SH2D1A) after CSL knockdown. Consistent with differential effects of GSI on cell survival, GSI treatment failed to recapitulate the gene expression changes observed after CSL knockdown. Thus, CSL inhibition may provide a more effective mechanism to inhibit Notch-pathway dependent cancer cell proliferation as compared to GSI treatment. J. Cell. Biochem. 112: 2340–2351, 2011.

KEY WORDS: NOTCH; CSL; CBF1; RBP-JK; TRANSCRIPTION; SIGNAL TRANSDUCTION; PROSTATE CANCER; BREAST CANCER

The CSL (CBF-1/RBP-Jκ, Su(H), Lag-1) transcription factor lies at the heart of the canonical Notch receptor signaling pathway [Bray, 2006; Kopan and Ilagan, 2009]. Engagement of the Notch family of receptors (Notch-1, Notch-2, Notch-3, or Notch-4) with their ligands, DSL (Delta, Serrate, and Lag-2) proteins, results in the gamma-secretase dependent proteolytic liberation of the Notch intracellular domain (NICD). Following nuclear transport, the NICD binds CSL, displaces transcriptional repressive factors and recruits transcriptional activators through the mastermind family (MAML, mastermind-like) of co-activators [Kovall, 2008; Borggrefe and Oswald, 2009]. In the absence of Notch receptor activation, CSL nucleates transcriptional repressive complexes via recruitment of histone deactylases through interactions with SMRT and HDAC associated repressor (SHARP) and co-repressors like SMRT/NcoR, CtIP/CtBP, or ETO family members [Oswald et al., 2002; Kuroda et al., 2003; Salat et al., 2008]. Thus, CSL binding to its cognate DNA binding sequence in the regulatory regions of Notch responsive genes mediates dynamic transcriptional control through a Notch receptor dependent co-repressor–co-activator exchange.

Since both the Notch receptors and ligands are single pass transmembrane proteins, the Notch pathway is a paradigm for juxtacrine signaling, requiring direct contact between the ligand expressing cells and the receptor expressing cells. Genetic studies on Notch receptors, DSL ligands, and CSL in Drosophila, zebrafish, *C. elegans*, and mice have demonstrated a requirement for the canonical Notch signaling pathway in development through control

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*Correspondence to: Dr. J. Nathan Davis, Department of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center-Shreveport, 1501 Kings Highway, Shreveport, LA 71130.
E-mail: ndavis@lsuhsc.edu

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of stem cell function, cell fate determinations, differentiation programs, and cellular proliferation in neuronal, hematopoietic, and other tissues [Artavanis-Tsakonas et al., 1995; Bray, 2006]. Like other important regulators of stem cell proliferation and differentiation, dysregulation of the Notch pathway is associated with human disease and Notch exhibits both oncogenic and tumor suppressor activities depending on cell context. Notch pathways display tumor suppressive function in cells or tissues, where Notch predominately promotes differentiation and has been best illustrated in carcinoma of the skin, where keratinocyte specific inactivation of Notch1, Delta-like 1 (Dl1), or gamma-secretase treatment accentuates tumor formation in chemical carcinogenesis models of mouse skin [Nicolas et al., 2003]. Alternatively, Notch pathways are oncogenic in cells or tissues where the Notch pathway acts primarily to maintain stemness and proliferative capacity. The paradigm of Notch-dependent oncogenesis occurs in T-cell acute lymphocytic leukemia (T-ALL) where Notch-1 is the target of the t(7;9) [Ellisen et al., 1991] and activating point mutations [Weng et al., 2004]. Subsequent to the identification of Notch-1 activation in T-ALL, activated Notch signaling was identified in other leukemias [Lee et al., 2009; Rosati et al., 2009] as well as a variety of human solid cancers including breast [Weijzen et al., 2002; Pece et al., 2004; Reedijk et al., 2005], ovarian [Hopfer et al., 2005; Park et al., 2006], prostate [Santagata et al., 2004; Zayzafoon et al., 2004], colorectal [Fernandez-Majada et al., 2007; Reedijk et al., 2008], and pancreatic [Miyamoto et al., 2003]. Inherent to the oncogenic activation of the Notch pathway is the constitutive production of the NICD leading to CSL-dependent transcriptional activation, suggesting that transcriptional control through CSL is central to the malignant phenotype.

Since Notch signaling is a major contributor to the malignant status of diverse cancers, the chemotherapeutic targeting of the Notch pathway has stimulated much interest [Nickoloff et al., 2003; Miele et al., 2006]. Targeting of Notch receptor activity is often achieved using gamma-secretase inhibitors (GSIs), which were designed primarily for the study of Alzheimer's disease. Ablation of gamma-secretase activity inhibits the generation of the NICD and maintains CSL in repressive complexes [De Strooper et al., 1999; Berezovska et al., 2000]. While GSIs developed by Merck, Novartis, Pfizer, and Roche are currently in clinical trials for a number of malignancies including T-ALL, lymphoma, breast, colorectal, brain, pancreatic, and non-small cell lung carcinoma, preclincial studies examining GSI function in vitro have been problematic. With the notable exception of GSI-1, GSIs have not displayed strong inhibitory effects on cell growth or survival in vitro. Additionally, while these drugs do inhibit Notch signaling, their specificity is not limited to the Notch pathway. For example, GSI-1 inhibition of survival in breast cancer cell lines was associated with inhibition of the proteosome [Han et al., 2009]. Thus, inhibition of the Notch pathway through more direct mechanisms should have additional benefits of increased specificity and efficacy.

In the simplest canonical Notch signaling model, CSL DNA binding nucleates transcriptionally repressive complexes that are replaced by transcriptionally activating complexes upon NICD binding to CSL. Thus, CSL is actively regulating gene expression under both Notch-activated and Notch-quiescent conditions while inhibition of Notch activation by gamma-secretase or other inhibitors of receptor activation specifically target the Notch activated state. Moreover, CSL inhibition would target Notch signaling irrespective of the receptor or ligand isoforms, since all activated Notch receptor signaling converge at CSL regulation. Additionally, CSL transcriptional regulation extends beyond the canonical pathway and Notch-independent CSL activities cannot be defined through inhibition of gamma-secretase. Herein, we demonstrate that CSL is required for the survival of prostate and breast cancer cell lines. Lentiviral delivery of shRNA's targeting CSL resulted in the loss of CSL DNA binding activity and decreased cell survival. In contrast, GSI treatment of these prostate and breast cancer cell lines resulted in minimal growth effects. Interestingly, an examination of the mRNA levels of Notch pathway genes identified expression changes in few genes (Delta-like-1 [DLL1], Deltex-1 [DTX1], LIM domain only 2 [LMO2], and SH2 domain protein 1A [SH2D1A]) after CSL knockdown. Consistent with differential effects of GSI on cell survival, GSI treatment failed to recapitulate the gene expression changes observed after CSL knockdown. Thus, CSL inhibition may provide a more effective mechanism to inhibit Notch-pathway dependent cancer cell proliferation as compared to GSI treatment.

MATERIALS AND METHODS

CELL CULTURE

The prostate cancer cell line PC3-CMVluc expressing luciferase [Svensson et al., 2007] was cultured in RPMI-1640. The breast cancer cell line MDA-MB-231 was obtained from American Type Culture Collection (Manassas, VA). MDA-MB-231 cells were cultured in RMPI-1640, and the HEK293T and NIH/3T3 cell lines were cultured in DMEM. RPMI-1640 and DMEM were obtained from Lonza (Walkersville, MD) and CellGro (Manassas, VA), respectively, and all media were supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (supplements were purchased from Gibco, Carlsbad, CA). Media for the PC3-CMVluc cell line was also supplemented with 400 μg/ml G418 (Sigma–Aldrich, St. Louis, MO).

LENTIVIRUS ENCODING shRNA PREPARATION AND CELL TRANSDUCTION

The puromycin-resistant pLKO.1 lentiviral vectors expressing the five shRNAs specific for human CSL and a non-target (NT) shRNA (with no known matching sequence in the GenBank human database) were obtained from Open Biosystems (Huntsville, AL) and Sigma–Aldrich, respectively. Lentivirus production was performed by co-transfecting the pLKO.1 vectors and packaging plasmids (Viropower Kit; Invitrogen, Carlsbad, CA) into HEK293T cells with Lipofectamine 2000 (Invitrogen) following manufacturer's instructions, and supernatants containing the lentiviral particles were harvested at 48 h post-transfection. Virus titer was determined in NIH/3T3 cells and a multiplicity of infection (MOI) of approximately 10 was used to transfer shRNA expression. Infections were performed with viral supernatant adjusted to 8 μg/ml polybrene (Sigma–Aldrich). After 16 h fresh media containing

puromycin (Sigma–Aldrich) was added. PC3-CMVluc and MDA-MB-231 cells selected for 4 days with 1 and 0.75 μg/ml of puromycin, respectively, were used for subsequent studies. The CSL-specific shRNA sequences were TRCN0000016203 (CSL-KD1): GCTGGAATACAAGTTGAACAA; TRCN0000016204 (CSL-KD2): CCCTAACGAATCAAACACAAA; TRCN0000016205 (CSL-KD3): GCACAGATAAGGCAGAGTATA; TRCN0000016206 (CSL-KD4): GCAGCTAAACTTGGAAGGAAA; TRCN0000016207 (CSL-KD5): CCAGACAGTTAGTACCAGATA; and the NT shRNA sequence was SHC002: CAACAAGATGAAGAGCACCAA.

CELL PROLIFERATION AND TREATMENT ASSAY

For cell proliferation studies following CSL knockdown, cells infected with lentiviral particles for 16 h were seeded in 12-well plates in media supplemented with puromycin. Cell counts were performed everyday for 6 days with media replacement every 2 days. All experiments were performed in triplicate. For drug treatment studies, cells seeded in 12-well plates were treated with the Notch gamma-secretase inhibitor, DAPT (*N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester; Sigma–Aldrich), for 3 days (with media replaced everyday) and counted. Vehicle control cells were cultured in media containing 0.1% DMSO (Sigma–Aldrich). All experiments were performed in triplicate.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Electrophoretic mobility shift assay (EMSA) analysis was performed as described [Meyers et al., 1993]. For preparation of whole cell extracts, PC3-CMVluc and MDA-MB-231 cells were viral infected and selected in puromycin for 4 days. The monolayers were washed with phosphate-buffered saline (PBS), scraped into microextraction buffer (20 mM HEPES pH 7.4, 450 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 25% glycerol) containing protease inhibitors (Sigma–Aldrich), lysed by sonication and the lysates were clarified by high-speed centrifugation. Equal amounts of protein were used in each DNA binding reaction. The double-stranded oligonucleotide containing a CSL-specific binding site was prepared by annealing single-stranded oligonucleotides in G50 buffer (100 mM Tris–HCl pH 7.4, 100 mM NaCl, and 2 mM EDTA) at 90°C for 5 min and slowly cooled to room temperature (RT) over 30 min. The annealed oligomer was labeled with α-^{32}P dATP (6000 Ci/mmol; MP Biomedicals, Solon, OH) using Klenow DNA polymerase (Promega, Madison, WI), and the probe was incubated with cell lysates at RT in binding buffer containing 20 mM HEPES pH 7.8, 1 mM $MgCl_2$, 0.1 mM EGTA, 0.4 mM dithiothreitol, 40 mM KCl, 4% Ficoll-400, and 60 μg/ml poly[d(I-C)] (Sigma–Aldrich). Unlabeled CSL-specific double stranded oligomer and a control double stranded oligomer containing base substitutions in the CSL binding site were used at 100-fold excess in competition reactions. The binding reactions were resolved in 4% non-denaturing polyacrylamide gels, dried and then exposed to X-ray film (FujiFilm, Stamford, CT) at −80°C for signal detection. The sequence of the CSL-specific and its mutant binding site probes were previously reported [Cheng et al., 2003] and their sequences were 5′-AATTCTGGTGTAAACACGCCGTGGGAAAAAATTTAG-3′ (WT) and 5′-AATTCTGGTGTAAACACGCCGTTGGAAAAAATTTAG-3′ (MT), respectively. To perform EMSA using recombinant CSL, pcDNA3.1-myc/HisB encoding human CSL (GenBank Accession NP_203284) was transfected into HEK293T using Lipofectamine 2000 and 48 h post-transfection the cells were used to make whole cell lysates. Ten micrograms of the whole cell lysates containing myc/His-tagged CSL was used for CSL-specific DNA binding assays containing double stranded oligonucleotides encompassing putative CSL DNA binding sites from the promoters of LMO2 and SH2D1A. As a control, cells were transfected with pcDNA3.0(+) and used to make whole cell extracts for use in EMSA. The oligonucleotide sequences for the putative CSL DNA binding sites within LMO2 and SH2D1A promoters were LMO2 (−2,018 bp): 5′-AATTCTTCCCCTTTTTCCCACTTTTGGATTG-3′ (WT) and 5′-AATTCTTCCCCTTTTTACAGCTTTTGGATTG-3′ (MT) at −2,018 to −2,012 bp from the transcription start site (TSS) based on LMO2 variant 1 (GenBank Accession NM_005574); SH2D1A (+172 bp): 5′-AATTCAACTGAAGTGTGAGAAGGAGGTTTAG-3′ (WT) and 5′-AATTCAACTGAAGTGTTAGAAGGAGGTTTAG (MT) at +172 to +178 bp from TSS, SH2D1A (−1,527 bp): 5′-AATTCCACTGAATGTTCTCACAGTTCACCTG-3′ (WT) and 5′-AATTCCACTGAATGTTCTAACAGTTCACCTG-3′ (MT) at −1,527 to −1,521 bp from TSS, both putative CSL DNA binding sites were based on SH2D1A variant 1 transcript (GenBank Accession NM_002351).

RT-PCR

Total RNA was extracted from transduced and treated cells using RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. First strand cDNA synthesis was performed using 2.5 μg of total RNA in a 20 μl reaction using the SuperScript III Reverse Transcriptase kit (Invitrogen) following the manufacturer's instructions. The reverse transcription reaction was diluted to 100 μl with water, and 10 μl was used for PCR using the *Taq* DNA polymerase kit (Invitrogen). PCR amplification was performed in 20 μl reaction using a MasterCycler Gradient machine (Eppendorf, Hauppauge, NY) as follows: 1 cycle at 94°C for 2 min, either 25, 30, or 35 cycles at 94°C for 45 s, 55°C for 30 s, and 72°C for 30 s, and followed by a final elongation at 72°C for 10 min. The amplified products were resolved using a 3% agarose gel, stained with ethidium bromide and visualized using ultraviolet light (UV). Gene expression levels were determined by PCR amplification at 25 cycles for CSL and HES1, 30 cycles for LMO2, and 35 cycles for DLL1, DTX1, and SH2D1A. The primer sequences were: CSL (171 bp): 5′-CGCATTATTGGATGCAGATG-3′ and 5′-CAGGAAGCGCCATCATTTAT-3′; HES1 (102 bp): 5′-AGGCGGACATTCTGGAAATG-3′ and 5′-GGTACTTCCCCAGCACACTT-3′; DLL1 (110 bp): 5′-TGCCTGGATGTGATGAGCAG-3′ and 5′-ACAGCCTGGATAGCGGATAC-3′; DTX1 (104 bp): 5′-AATCCCGAGGATGTGGTTCG-3′ and 5′-TCGTAGCCTGATGCTGTGAC-3′; LMO2 (105 bp): 5′-GCCTCTACTACAAACTGGGC-3′ and 5′-CATAGGCACGAATCCGCTTG-3′; SH2D1A (127 bp): 5′-GCTGGATGGCAGCTATTTGC-3′ and 5′-AGCACTCCAAGAACCTGTTTC-3′. To prevent amplification of potential contaminating genomic DNA, the primer pairs were designed to anneal to different exons. As a control, GAPDH (145 bp) was amplified using the following primer pair: 5′-GTCCACTGGCGTCTTCAC-3′ and 5′-CTTGAGGCTGTTGTCATACTTC-3′.

NOTCH SIGNALING PATHWAY PCR ARRAY

Three independent infections of PC3-CMVluc cells were performed with NT shRNA (SHC002) and CSL-specific shRNA (TRCN0000016203, CSL-KD1) expressing viruses and selected for 4 days in puromycin. Total cellular RNA was prepared and subjected to PCR array analysis for genes related to the Notch signaling pathway using the RT^2 Profiler^{TM} PCR Array System (PAHS-059A; SABiosciences, a QIAGEN company, Frederick, MD) following the manufacturer's instructions. In brief, 1.0 μg of total RNA was treated with RNase-free DNase I followed by first strand cDNA synthesis using the RT^2 First Strand kit (C-03; SABiosciences). Real-time PCR was performed using the RT^2 SYBR Green/Fluorescein qPCR Master Mix (PA-011; SABiosciences) in the iCycler® machine (Bio-Rad, Hercules, CA). Data analysis was performed using the web-based RT^2 Profiler^{TM} PCR Array Data Analysis program (SABiosciences) and genes with greater than threefold change were confirmed using RT-PCR. Putative CSL DNA binding sites within 3,000 bp 5′ from the TSS of the promoter of these genes were analyzed and identified based on sequence homology to the consensus sequence 5′-T/C GTG T/A GAA C/A-3′.

RESULTS

In the canonical Notch signaling pathway, transcriptional activation of Notch target genes is mediated by NICD binding to CSL, suggesting that CSL is a major mediator of Notch-dependent signaling [Bray, 2006; Kopan and Ilagan, 2009]. Notch-regulated pathways are required for prostatic growth and development and for cell fate specification within the prostate [Wang et al., 2004, 2006]. Moreover, recent data link Notch signaling to prostate cancer development and progression [Santagata et al., 2004; Wang et al., 2010, 2011]. To address the role of CSL in Notch-dependent signaling in prostate cancer cell lines, lentiviral mediated transfer of shRNA specific for CSL was used to knockdown expression of CSL in the prostate cancer derived cell line PC3-CMVluc. Five independent CSL-specific shRNAs designated CSL-KD1-5 and a control NT shRNA lacking homology to any mammalian mRNA were tested for their ability to knockdown CSL expression. After transduction, CSL mRNA was analyzed by RT-PCR and CSL DNA binding activity was determined by using EMSA (Fig. 1A,B). CSL expression was performed using total RNA isolated from NT-shRNA and CSL-shRNA infected PC3-CMVluc cells following infection and puromycin selection. As shown in Figure 1A, several of the CSL-shRNA infected PC3-CMVluc cultures displayed decreased CSL mRNA levels with the most efficient knockdown achieved using the CSL-KD1 shRNA. In order to relate knockdown of CSL mRNA to CSL function, CSL DNA binding activity was examined by EMSA. An oligonucleotide containing the consensus CSL binding site was ^{32}P-labelled and mixed with extracts prepared from the parental PC3-CMVluc, PC3-CMVluc infected with NT and the PC3-CMVluc infected with the CSL knockdown shRNAs. Migration of the CSL protein/DNA complex is indicated by the arrow on the left side (Fig. 1B) and corresponds to the only DNA binding activity that is specifically inhibited by addition of excess unlabelled competitor oligonucleotide (+WT competitor) but unaltered by the addition of excess oligonucleotide in which the CSL consensus site was changed (+MT competitor). The CSL/DNA complex migrates in the gel just above the non-specific protein/DNA complex indicated by the double asterisks (**). The CSL/DNA complex is clearly observed in the parental PC3-CMVluc cell line and the NT infected PC3-CMVluc cell line but minimal residual CSL binding activity was evident in the CSL-specific shRNA infected PC3-CMVluc cell line. Thus, CSL DNA binding activity was severely inhibited in the CSL-specific shRNA infected cells.

The CSL-specific shRNA that displayed greatest activity both by RT-PCR and EMSA analysis (CSL-KD1) was used to determine the effect of CSL knockdown on the proliferation of the PC3-CMVluc cell line. Attempts to generate stably infected cell lines after infection with the CSL-specific shRNA failed (Fig. 1C,D). The NT virus did, however, successfully produce a cell line, suggesting that CSL-KD was not compatible with either cell proliferation or cell survival (Fig. 1D). By 48 h post-infection, the CSL-KD cells displayed fewer cells and began to lose attachment as compared to the NT infected cells (Fig. 1C). By contrast, the NT cell line was near confluence at 96 h.

To extend the analysis of the Notch pathway in prostate cancer cells lines, expression of the Notch pathway genes in the PC3-CMVluc cell line was determined by using an RT-PCR array and compared to that in the CSL-KD cells. The NT infected cells express both Notch receptors and ligands (Fig. 2A), indicating that the Notch pathway is constitutively activated via an autocrine loop, as previously reported [Santagata et al., 2004; Zhang et al., 2006]. Notch activation in this cell line is consistent with the expression of the Notch-regulated genes, HES1, and HEY1 (Fig. 2A). Thus, the Notch pathway genes were expressed in the PC3-CMVluc cells and the transcriptional profile was consistent with active Notch signaling.

Of the 84 Notch pathway genes contained in the array, a total of 8 genes displayed gene expression changes in CSL knockdown cells that were both twofold or larger and statistically significant (P-value ≤ 0.05) (Fig. 2B). Six genes displayed increased expression: CDKN1 (Cyclin-dependent kinase inhibitor 1A, p21$^{Cip1/Waf1}$), DLL1 (Notch ligand), DTX1, FZD6 (Frizzled homolog 6), LMO2, and POFUT1 (Protein O-fucosyltransferase 1). Two genes displayed reduced expression: FZD3 (Frizzled homolog 3) and SH2D1A. The well-characterized CSL targets HES1 and HEY1 did not display significant changes in mRNA expression levels in the CSL knockdown cells suggesting that CSL was dispensable for the transcriptional activation of HES1 and HEY1. Expression changes for the genes exhibiting the most dramatic changes in mRNA levels after CSL knockdown (Fig. 2C) were confirmed by using RT-PCR. DLL1, DTX1, and LMO2 expression was increased with CSL knockdown while the expression of SH2D1A was decreased (Fig. 2D).

The alterations in gene expression may reflect direct or indirect regulation by CSL. To begin to identify genes directly regulated by CSL, we performed a computer-assisted search of the upstream regions of genes whose expression was altered after CSL knockdown. Putative CSL binding sites were found in the upstream regulatory regions of LMO2 and SH2D1A (Fig. 3A). The ability of CSL to bind to these sites was evaluated in vitro by using

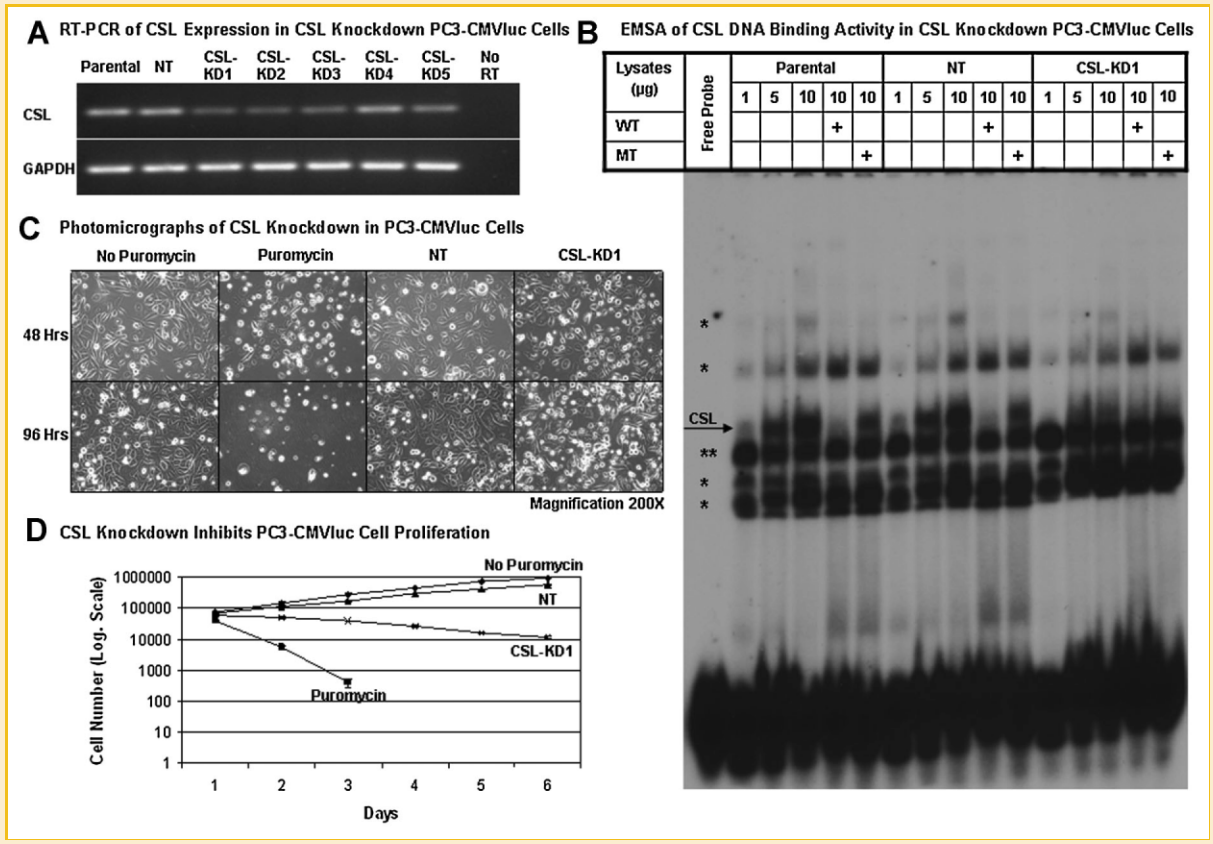

Fig. 1. Knockdown of CSL in the PC3-CMVluc cell line. A: Reduction of CSL mRNA levels as measured by RT-PCR. Photograph of gel electrophoresis of RT-PCR products of mRNA isolated from PC3-CMVluc cells either mock-infected (Control), or infected with the non-target (NT) shRNA, or the five CSL-specific (CSL-KD1–CSL-KD5) shRNAs as indicated in the panel. The right lane (No RT) shows the products of RT-PCR reaction of the NT shRNA-infected cells when the reverse transcriptase was not included as a control for contamination. The mRNA was isolated 120 h after infection and 96 h selection in puromycin. B: Reduced CSL DNA binding activity measured by electrophorectic mobility shift assays (EMSA). Autoradiogram of EMSA gel performed with a P32-labelled oligonucleotide probe containing a consensus CSL binding site and 1, 5, or 10 µg of whole cell extracts prepared from PC3-CMVluc cells infected with lentiviruses expressing a NT shRNA, or one of five different CSL-specific shRNA (CSL-KD1) as indicated above the autoradiogram. Specific CSL-containing protein/DNA complexes were identified by incorporation of 100× excess unlabelled oligonucleotide containing the wild type (WT) CSL consensus binding site or a mutant version (MT) of the binding site, as indicated. The protein/DNA complex containing CSL is indicated by the arrow and migrates just slower than a major non-specific DNA binding protein (double asterisks). Single asterisks denote other non-specific DNA binding complexes. C: Photomicrographs of the CSL-specific shRNA. PC3-CMVluc cells un-infected without puromycin (No Puromycin), un-infected with puromycin (Puromycin), infected with the non-target shRNA with puromycin (NT), infected with the CSL-specific shRNA with puromycin (CSL-KD1). D: Knockdown of CSL inhibits proliferation of the PC3-CMVluc cells. Growth curves of PC3-CMVluc cells mock-infected without puromycin (No Puromycin) and with puromycin (Puromycin), non-target shRNA with puromycin (NT) and the CSL-specific shRNA with puromycin (CSL-KD1).

oligonucleotides containing the genomic sequences as competitors in EMSA employing a consensus CSL binding site (Fig. 3B). Site, −1,527 bp from the SH2D1A gene displayed the highest degree of competition, while site +172 bp from the SH2D1A gene showed a lower degree of competition. The putative CSL site, −2,018 bp derived from LMO2 competed poorly or not at all for the consensus CSL binding site, suggesting a lower affinity for the sequence in the LMO2 gene as compared to the consensus site. While this analysis does not identify any of these genes as direct CSL targets, the presence of CSL binding sites within the SH2D1A upstream regulatory region suggests the possibility of direct regulation. Regardless, CDKN1A, DLL1, DTX1, FZD6, LMO2, POFUT1, FZD3, and SH2D1A should be considered part of a CSL-dependent pathway in the PC3-CMVluc cells.

Constitutive activation of the Notch pathway is consistent with the expression of the Notch receptors (Notch-1, Notch-2, Notch-3, and Notch-4), and the ligands (DLL1, Jagged-1, and Jagged-2) (Fig. 2A). Moreover, constitutive Notch signaling has been defined in prostate carcinomas cell lines using GSI treatment [Zayzafoon et al., 2004; Scorey et al., 2006]. To fully evaluate the CSL responsive genes defined by CSL knockdown, the expression of DLL1, DTX1, LMO2, and SH2D1A was assayed after inhibition of the Notch pathway using the GSI, DAPT (Fig. 4A, Table I). The increased expression displayed by DLL1 in response to DAPT treatment was similar to that observed in the CSL knockdown cells. DAPT also resulted in increased SH2D1A expression in contrast to the decrease observed with CSL knockdown. DTX1 gene expression was decreased by DAPT, while it was increased in the CSL knockdown cells. The expression of LMO2 was slightly increased by DAPT treatment. The fact is that, these Notch pathway genes were altered by both CSL knockdown and DAPT treatment strongly argues that the Notch pathway regulates the expression of these genes. DAPT

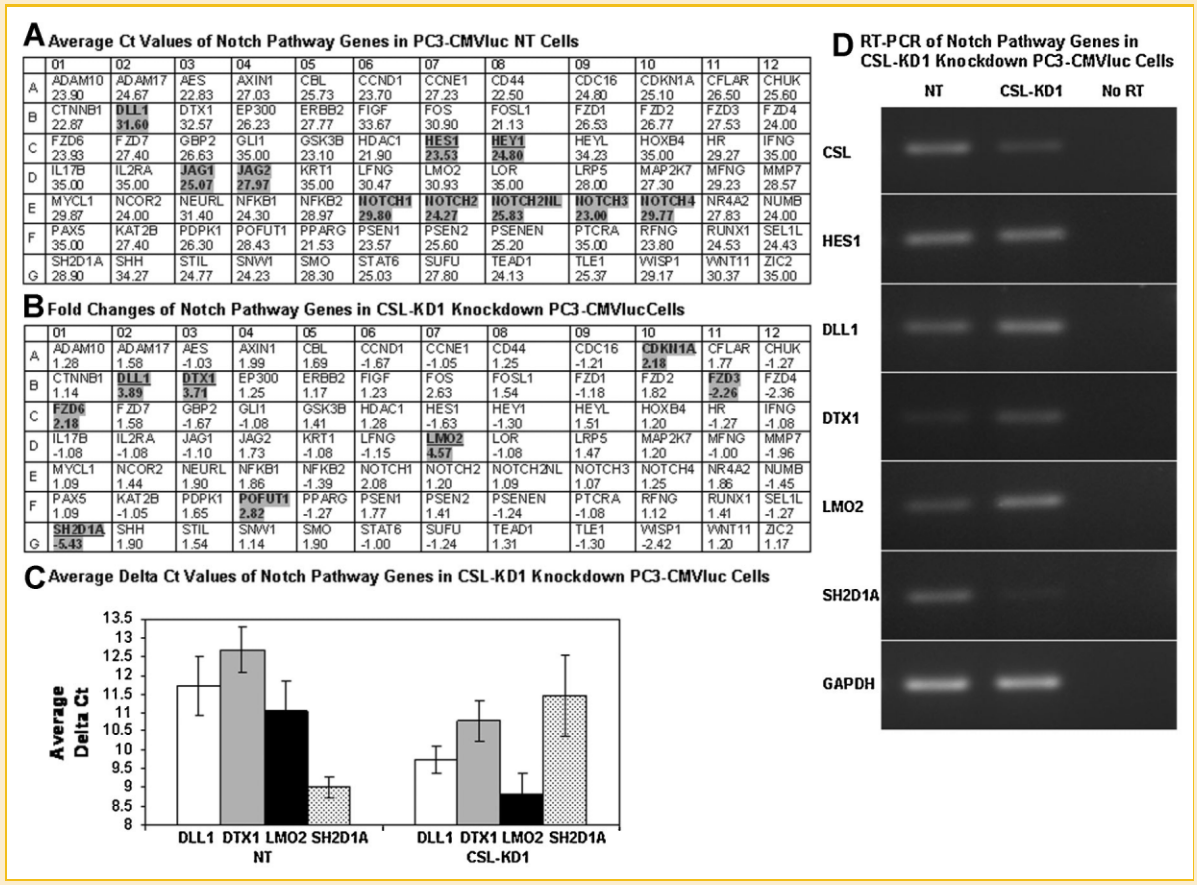

Fig. 2. Notch pathway gene expression in control and CSL-KD PC3-CMVluc cells. A: Notch pathway gene expression in the PC3-CMVluc cells infected with the non-target shRNA. The average Ct value for the Notch pathway genes is shown and the expression of the Notch receptors (Notch-1, Notch-2, Notch-3, and Notch-4) and ligands (Delta-like-1, Jagged-1, and Jagged-2) are shaded. The Notch responsive genes are shaded and underlined (HES1 and HEY1). B: Gene expression changes within the Notch pathway after CSL knockdown. The Notch PCR array was used to quantitate the expression of the 84 Notch pathway genes in the NT and CSL-KD PC3-Luc cells. Genes with significant fold-changes are shaded. C: Fold change of Notch pathway genes. Plot of the average delta Ct of the genes (DLL1, DTX1, LMO2, and SH2D1A) with greater than threefold change in expression following CSL knockdown of the PC3-CMVluc cells (P-value < 0.05). D: RT-PCR confirms expression changes for DLL1, DTX1, LMO2, and SH2D1A. Photograph of ethidium bromide stained agarose gel showing the RT-PCR products using mRNA isolated from NTshRNA infected (NT, first lane of lane of each panel) and CSL knockdown shRNA (CSL-KD1, second lane of each panel). As a control for contamination the mRNA was subjected to PCR amplification without reverse transcription (No RT). The gene specific primers are designated at the left of each panel.

inhibition of Notch signaling was confirmed by examination of HES1 expression. Decreased HES1 expression following DAPT treatment but not after CSL knockdown agrees with the hypothesis that transcriptional repression is the primary mechanism of CSL regulation of these genes.

Clinical trials are underway to examine the efficacy of GSI in the treatment of breast cancer. However, GSI treatment of breast carcinoma cell lines has either failed to produce dramatic growth effects or the GSI growth effects have been linked to the inhibition of other pathways [Han et al., 2009]. Since knockdown of CSL provides an additional method to define the requirement of the Notch pathway for breast carcinomas, the effect of CSL knockdown on the growth of the breast carcinoma cell line MDA-MB-231 was interrogated. Infection of MDA-MB-231 cells with virus encoding the CSL-shRNA eliminated CSL-dependent DNA binding while DNA binding was retained in cells infected with NT virus or mock-infected cells (Fig. 5A). CSL knockdown resulted in an inhibition of MDA-MB-231 cell proliferation similar to that observed for the PC3-Luc cells (Fig. 5B,C). Microscopic examination of the CSL knockdown cultures documented fewer cells and increased cell rounding as compared to untreated or NT infected cultures. Infection of the MDA-MB-231 cells with the CSL-specific shRNA resulted in loss of proliferation and the cells slowly died over the time course of the experiment. Similar to the PC3-CMVluc cells, MDA-MB-231 cells infected with the lentivirus expressing the CSL-specific shRNA failed to establish a cell line. This was not due to poor viral infection, as evidenced by the ability of the virus to knockdown CSL expression and confer short-term puromycin resistance as compared to the mock-infected and puromycin selected control (Fig. 5C) These findings suggest that MDA-MB-231 cells are dependent upon a CSL-regulated pathway for survival.

To compare CSL knockdown in the MDA-MB-231 cells with GSI treatment, the expression of the CSL-regulated genes that were defined in the PC3-CMVluc cells were examined after CSL

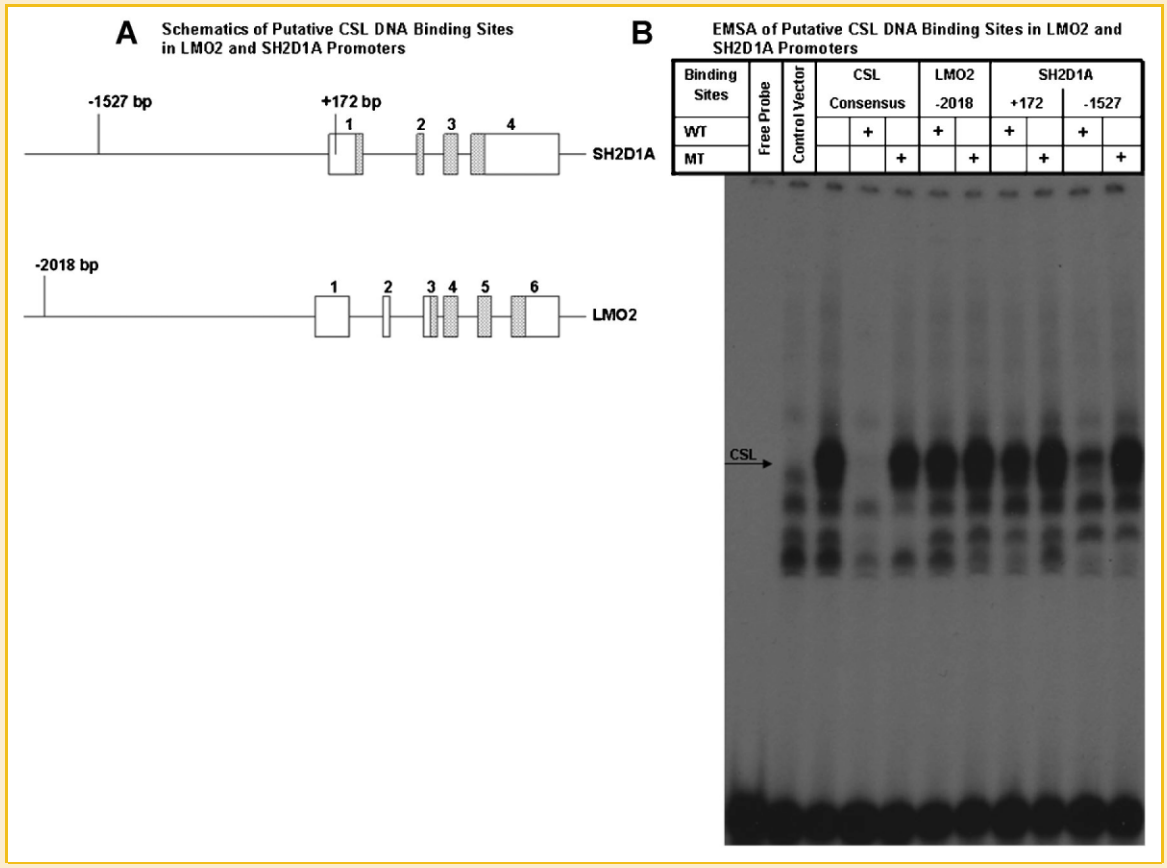

Fig. 3. Putative CSL binding sites in the upstream regulatory region of SH2D1A. A: Schematic representation of putative CSL DNA binding sites in the upstream regulatory regions of LMO2 and SH2D1A. Putative CSL binding sites were identified within the upstream regulatory regions of LMO2 (−2,018 to −2,012 bp from transcription start site (TSS) of variant 1 transcript; NM_005574) and SH2D1A (+172 to +178 bp and −1,527 to −1,521 bp from TSS of variant 1 transcript; NM_002351). The exons are represented as boxes, numbered numerically, and the dotted areas indicate the open reading frame (ORF). B: EMSA of over-expressed myc/His-tagged CSL from HEK293T cells for DNA binding competition assay with double-stranded oligonucleotides (ds-oligos) encoding putative CSL DNA binding site within the upstream regulatory regions of LMO2 and SH2D1A. The plasmid pcDNA3.1-myc/HisB encoding CSL was transfected into HEK293T cells for 48 h using Lipofectamine 2000 and 10 μg of the cell lysate was used for EMSA of CSL using a radiolabelled ds-oligo containing the CSL consensus binding site, GTGGGAA, with the addition of 100× excess of unlabelled ds-oligo (WT) and unlabelled mutant ds-oligo (MT). Competition assay was performed by the addition of 100× excess of unlabelled ds-oligo (WT) and unlabelled mutant ds-oligo (MT) encoding LMO2 and SH2D1A putative CSL DNA binding sites. Control vector was pcDNA3.0(+). CSL-DNA binding complex is indicated by arrow. Significant competition for the bound complex was observed for the ds-oligo encoding the SH2D1A putative CSL DNA binding site −1,527 bp, with weak competition for the SH2D1A putative CSL DNA binding site +172 bp. The LMO2 putative CSL DNA binding site −2,018 bp did not exhibit any competition for the consensus CSL DNA binding site. The putative CSL DNA binding site sequences were: LMO2 (−2,018 bp: 5′-AATTCTTCCCCTTTTTCCCACTTTTGGATTG-3′) and SH2D1A (+172 bp: 5′-AATTCAACTGAAGTGTGAGAAGGAGGTTTAG-3′; and −1,527 bp: 5′-AATTCCACTGAATGTTCTCACAGTTCACCTG-3′).

knockdown or DAPT treatment. Expression of both DLL1 and LMO2 increased in the CSL knockdown cells but not in mock-infected or NT expressing cells (Fig. 6A). Unfortunately, DTX1 and SH2D1A were not detected in any of the MDA-MB-231 cells regardless of treatment, suggesting that these genes are not active in the breast cancer cell line. Unlike the PC3-CMVluc cells, DLL1 expression was slightly decreased in the DAPT treated MDA-MB-231 cells, while LMO2 expression similar to PC3-CMVluc cells was slightly increased (Fig. 6B, Table I). We expected that, inhibition of the Notch pathway in MDA-MB-231 cells using DAPT or CSL would identify genes commonly regulated by both treatments, LMO2 is such a gene. However, DAPT treatment did not result in a dramatic inhibition of cell proliferation (unlike CSL knockdown) for both PC3-CMVluc (Fig. 4B) and MDA-MB-231 (Fig. 6C).

DISCUSSION

While integral to TALL, evidence that Notch signaling is activated in diverse cancers is accumulating through expression and functional studies of the Notch pathway. Many of these studies have exploited the action of GSIs to inhibit Notch receptor cleavage to block the subsequent generation of the CSL-co-activator, NICD. However, GSI sensitivity as a surrogate marker for Notch pathway dependence has limited utility for in vitro studies. For example, in the case for breast carcinoma most GSIs fail to show strong activity in vitro. Additionally, Notch-independent effects of GSI, such as alternative gamma-secretase targets or off-target effects of the drugs, complicate the experimental interpretation. The recent demonstration of proteosome inhibition by GSI-1 is an example of such

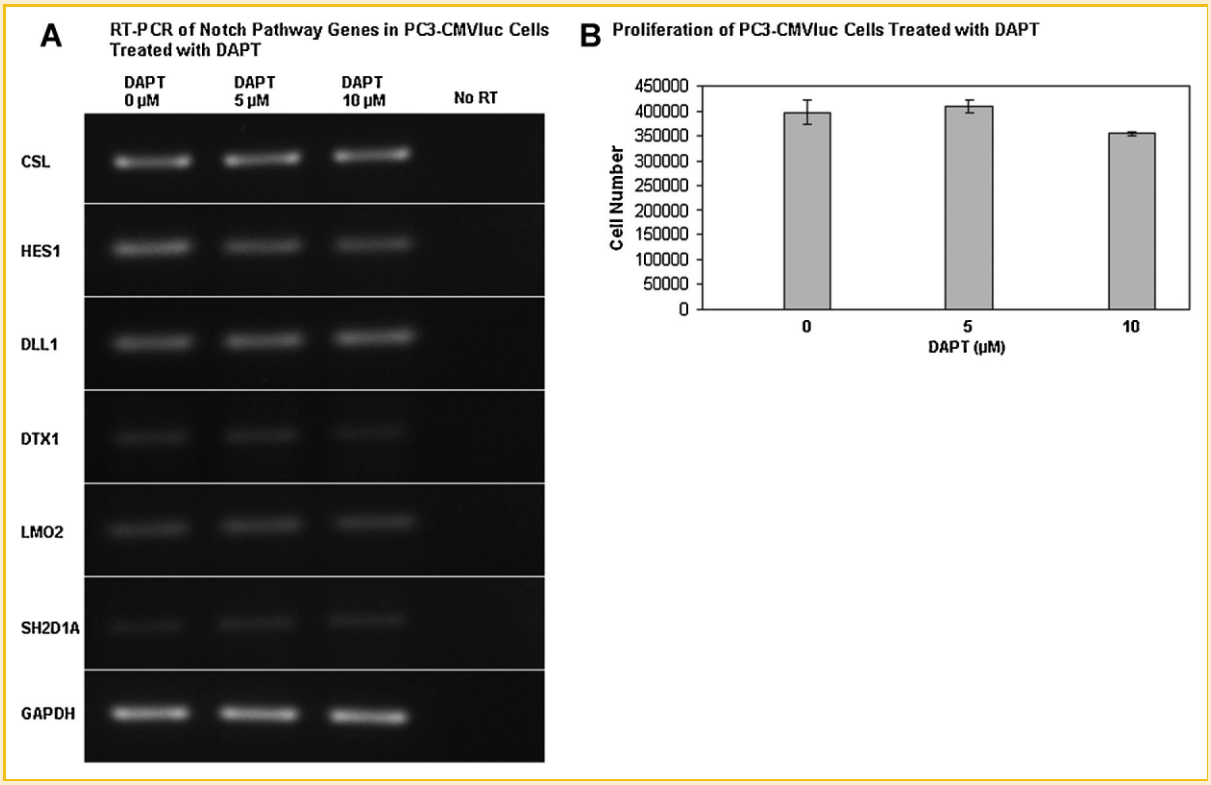

Fig. 4. DLL1, DTX1, LMO2, and SH2D1A expression in PC3-CMVluc cells is regulated by the gamma-secretase inhibitor, DAPT. A: RT-PCR analysis of DLL1, DTX1, LMO2, and SH2D1A expression after DAPT treatment. Photograph of the ethidium bromide stained agarose gel after electrophoretic separation of the PCR amplified DNA. Amplification targets are indicated to the left of each panel with the concentration of DAPT used above the panels. B: Survival of the PC3-CMVluc cell lines after DAPT treatment. Cell survival was determined by trypan-blue exclusion of the treated cells. Control and DAPT treated cells are indicated below the bars.

off-target effects [Han et al., 2009]. In order to circumvent the problems associated with using GSIs, we hypothesized that Notch-dependent cancers would require the function of CSL and we tested this idea by targeting CSL for shRNA-dependent knockdown. To do this, lentiviral expressed CSL-specific shRNAs were used to infect cell lines derived from prostate and breast carcinomas. The knockdown of the CSL mRNA was associated with a loss of CSL DNA binding activity, an inhibition of cell growth and changes in the expression of Notch pathway genes. These data demonstrate that some prostate and breast cancer cell lines required CSL for growth and/or survival. Sensitivity to CSL knockdown likely reflects a requirement for Notch signaling in these cells, although Notch-independent functions of CSL cannot be ruled out.

While the gene expression analysis presented here is limited to the Notch signaling pathway PCR array, the analysis provides some insight into the transcriptional regulation by CSL and the Notch pathway. Interestingly, CSL knockdown did not significantly alter HES1 expression, one of the best-characterized Notch target genes. Thus, CSL-dependent transcriptional activation of HES1 may be dispensable in these cell types and HES1 expression is likely regulated via multiple pathways [Bolos et al., 2007]. While HES1 expression was not significantly altered by CSL knockdown, other Notch pathway genes showed expression changes. One such gene, DTX1 is thought to regulate Notch signaling either by targeting the NICD for ubiquitination and degradation or by altering NICD transcriptional functions, possibly by competing for co-activator

TABLE I. Summary of Gene Expression Changes by RT-PCR in PC3-CMVluc and MDA-MB-231 Cells Following CSL Knockdown and DAPT Treatment

	PC3-CMVluc		MDA-MB-231	
Genes	CSL-KD1	DAPT	CSK-KD1	DAPT
CSL	Decreased	No change	Decreased	No change
HES1	No change	Decreased	Increased	Decreased
DLL1	Increased	Increased	Increased	Slightly decreased
DTX1	Increased	Decreased	Not detected	Not detected
LMO2	Increased	Slightly increased	Increased	Slightly increased
SH2D1A	Decreased	Increased	Not detected	Not detected

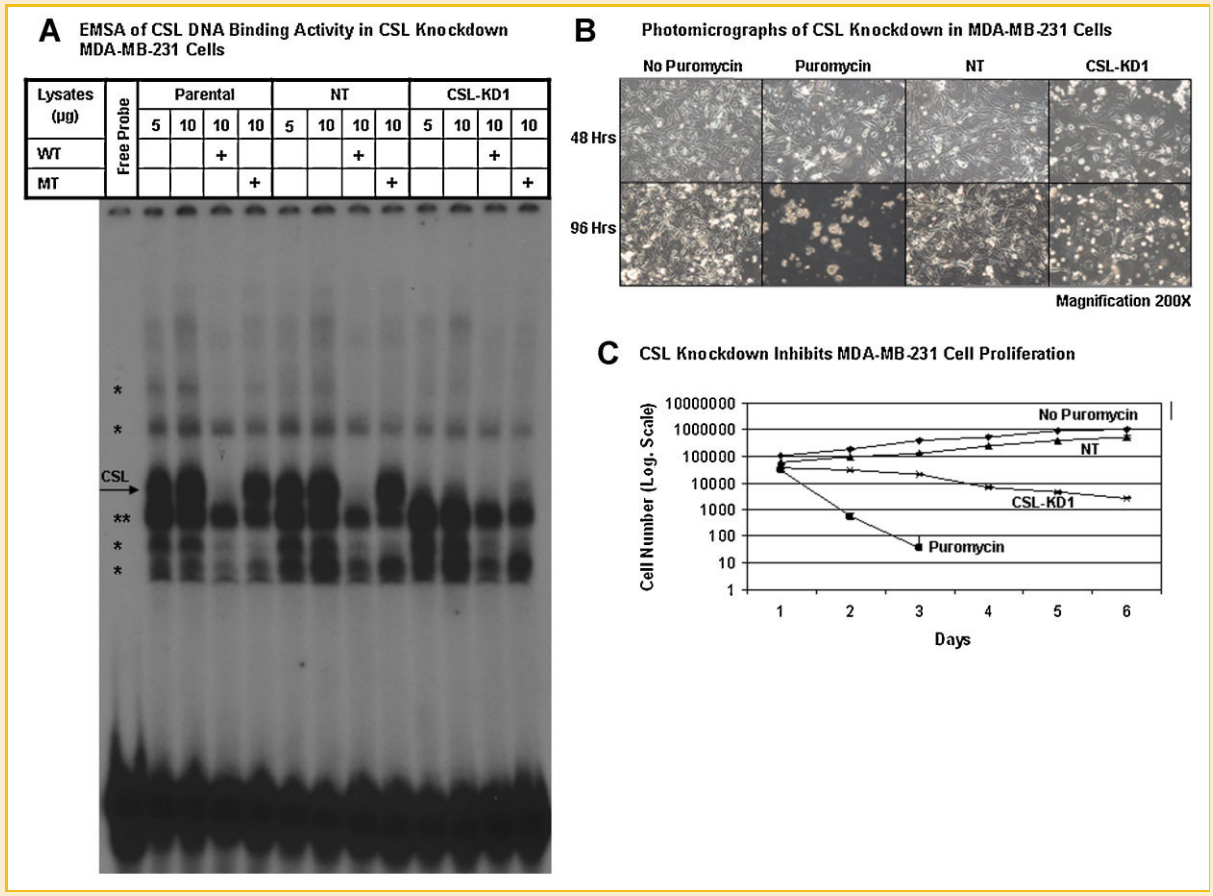

Fig. 5. Knockdown of CSL in the MBA-MB-231 cell line. A: CSL DNA binding activity after knockdown in the MBA-MB-231 cells measured by EMSA. Autoradiogram of EMSA gel performed with extracts prepared from MBA-MB-231 cells (Control), MBA-MB-231 cells infected with the NT shRNA expressing virus (NT), MBA-MB-231 cells infected with the CSL-specific shRNA (CSLKD1) and P32-labelled oligonucleotide probe containing a consensus CSL binding site. The EMSA was performed with either 5 or 10 µg of total cell lysate (as indicated) and the presence of either cold wild type (WT) or mutant (MT) oligonucleotide as indicated above the autoradiogram. The protein/DNA complex containing CSL is indicated by the arrow and migrates just slower than a major non-specific DNA binding protein (double asterisks). Single asterisks denote other non-specific DNA binding complexes. B: Photomicrograph of CSL Knockdown in MDA-MB-231 cells. Photomicrograph of MBA-MB-231 cells un-infected without puromycin (No Puromycin), uninfected with puromycin (Puromycin), infected with the non-target shRNA with puromycin (NT), and infected with the CSL-specific shRNA with puromycin (CSL-KD1). C: Knockdown of CSL inhibits proliferation of the MBA-MB-231 cells. Growth curves of MDA-MB-231 cells mock control infected without puromycin (No Puromycin) and with puromycin (Puromycin), non-target shRNA with puromycin (NT), and the CSL-specific shRNA with puromycin (CSL-KD1).

recruitment to CSL [Matsuno et al., 1998, 2002; Yamamoto et al., 2001; Izon et al., 2002]. Although, not mutually exclusive, neither activity is consistent with genetic experiments that suggest that Deltex plays a positive role in Notch signaling in Drosophilia [Xu and Artavanis-Tsakonas, 1990; Gorman and Girton, 1992]. These conflicting results may reflect the function of Deltex as an adaptor for recruitment of cell type-specific or developmental regulated activities that modulate Notch pathway signaling. In either case, DTX1 expression is increased in the CSL knockdown, implicating CSL-dependent repressor functions for the regulation of DTX1.

DLL1, LMO2, and SH2D1A also displayed altered expression in the prostate cell line. DLL1 and LMO2 expression was increased in both the prostate and breast carcinoma derived cell lines. Unfortunately, DTX1 and SH2D1A expression was only observed in the prostate cells and CSL-dependent regulation of these genes could not be confirmed in the breast cancer cell line. However, the observation that SH2D1A expression is dependent upon the Notch pathway is intriguing. Both Notch and SH2D1A are important in the development of natural killer (NK) cell development. SH2D1A, which encodes SAP (SLAM-associated protein, CD2 family of receptors) acts as an adaptor protein for downstream signaling pathways and is mutated in X-linked lymphoproliferative disease type-1 (XLP) [Hare et al., 2006]. XLP is exemplified by defects in NK T-cell development and function that underlie the pathophysiology of the disorder. Interestingly, a search of the GEO Database indicates that SH2D1A expression was increased with GSI treatment of a T-cell line [Dohda et al., 2007]. The requirement for the Notch pathway in normal T-lymphocyte and NK cell development, the increase of SH2D1A expression resulting from GSI treatment and reduction following CSL knockdown together with the presence of consensus CSL binding sites in the upstream regulatory region of SH2D1A are all consistent with Notch regulation.

The expression profile of Notch-regulated genes varies between the CSL knockdown cells and those cells treated with GSI. The

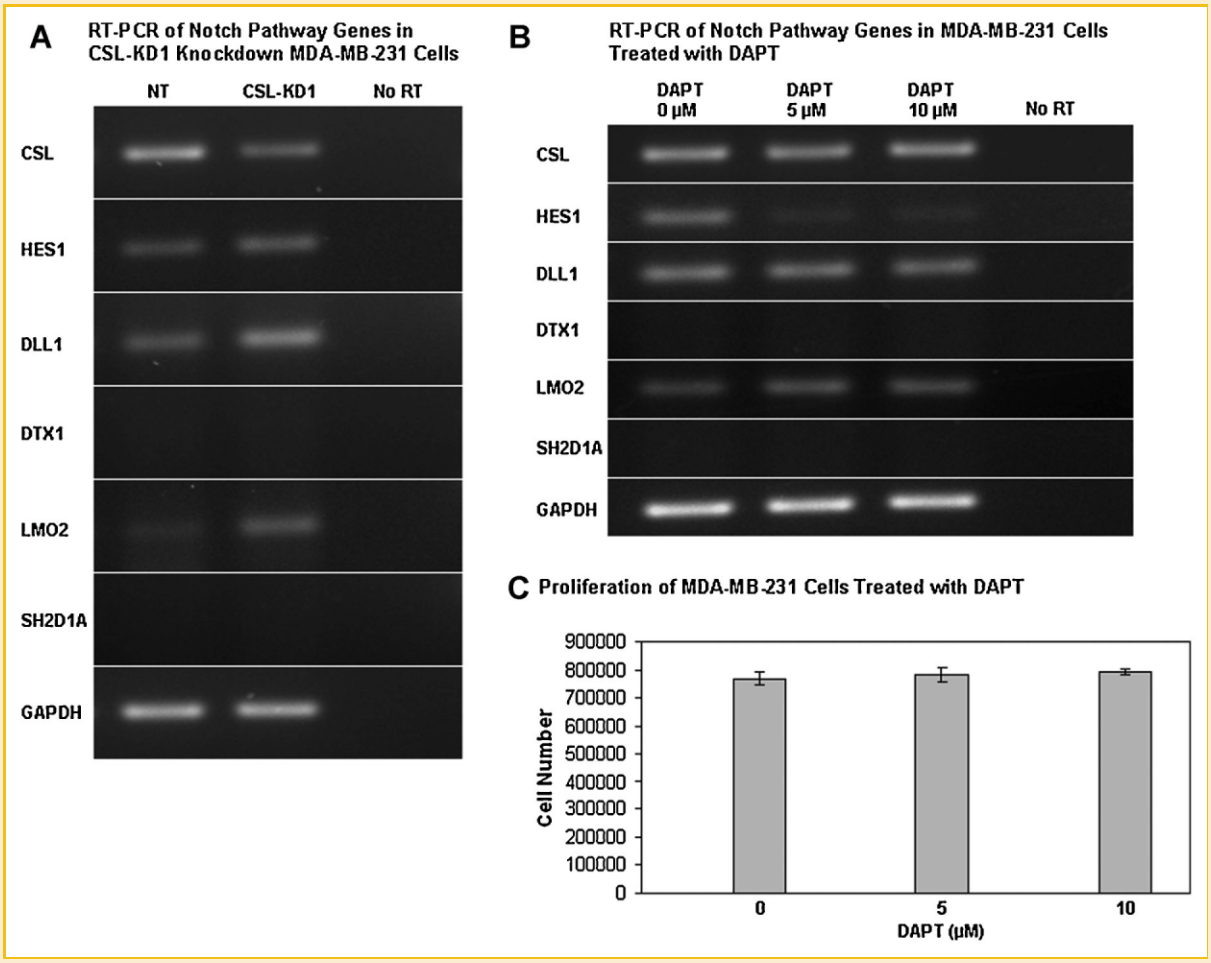

Fig. 6. CSL and GSI regulation of DLL1 and LMO2 mRNA levels in the MBA-MB-231 cells. A: DLL1 and LMO2 expression were elevated in the CSL knockdown cell line. Photograph of PCR products amplified from cDNA prepared from RNA extracted from non-target (NT) and CSL-specific (CSL-KD1) shRNA infected MBA-MB-231 cells (as indicated above the panels). Gene specific primers used in the RT-PCR reactions are indicated to the left of the panels. B: RT-PCR analysis of DLL1, DTX1, LMO2, and SH2D1A expression after DAPT treatment. Photograph of the ethidium bromide stained agarose gel after electrophoretic separation of the PCR amplified DNA. Amplification targets are indicated to the left of each panel with the concentration of DAPT used above the panels. C: Survival of the MBA-MB-231 cell lines after DAPT treatment. Cell survival was determined by trypan-blue exclusion of the treated cells. Control and DAPT treated cells are indicated below the bars.

differential changes in gene expression between CSL knockdown and inhibition of Notch by DAPT is expected since cell survival and growth are sensitive to CSL knockdown but not DAPT. Interestingly, CSL knockdown appears to result in complex gene expression changes; some Notch targets exhibited little or no expression changes (HES1), while other genes showed increased (DTX1) or decreased expression (SH2D1A). To understand the CSL-dependent changes in gene expression that must underlie the loss of proliferation observed in prostate and breast cancer cells, an analysis of the transcriptome associated with CSL knockdown must be performed. Comparison of the CSL-dependent gene expression changes to those changes mediated by GSI treatment should identify Notch-regulated genes that are critical for cellular growth in these cells. Such a gene expression profile could provide a surrogate marker for Notch-dependent cancers and might serve to predict clinical response to Notch pathway inhibitors.

The Notch pathway is a critical mediator of the malignant phenotype in a variety of cancers and the chemotherapeutic targeting of this pathway is actively being pursued. This report provides a strong evidence that CSL, a major Notch pathway effector, is required for cell growth in both prostate and breast carcinoma derived cell lines. Thus, CSL represents a novel chemotherapeutic target for cancers that rely upon the Notch pathway.

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