Notch1-Induced Transformation of RKE-1 Cells Requires Up-regulation of Cyclin D1

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Abstract

RKE-1 cells induced to overexpress activated Notch1 (RKE-ER-N^{ic}) exhibit increased cyclin D1 transcripts and become transformed. However, the oncogenic pathway of Notch1-induced transformation is not known. Here, we use mutational analysis to functionally identify the sole region of the cyclin D1 promoter that responds to activated Notch1. The same region responds to activated Notch4 as well as to physiologic Notch ligand-induced Notch receptor signaling. The cyclin D1 gene was subsequently found to be a physiologic target of Notch signaling in *Pofut1*$^{-/-}$ mouse embryos defective in canonical Notch signaling and in embryos with an inactivating mutation in *Notch1*. To determine if Notch1-induced cyclin D1 expression in RKE-ER-N^{ic} cells plays a direct role in transformation, cyclin D1 up-regulation was inhibited using a cyclin D1 antisense cDNA. We report here that transformation of RKE-ER-N^{ic} cells is dependent on increased expression of cyclin D1 protein, which represents a new mechanism of Notch1-induced transformation. (Cancer Res 2006; 66(15): 7562-70)

Introduction

Cyclin D1 overexpression is now established as a common feature of many human tumors (1–3). In most cancers, cyclin D1 overexpression is not the result of a genetic lesion but is due to induced expression by oncogenic signals (4). Thus, cyclin D1 transcription is up-regulated in Notch1-induced murine mammary tumors that arise during lactation (5). Notch1 and the Notch ligand Jagged1 overexpression are also associated with poor clinical outcomes in human breast cancer (6). In fact, overexpression of activated forms of any of the four mammalian Notch receptors induces cellular transformation and oncogenesis in mouse models (7–10), and Notch signaling may also cause cell proliferation (11, 12). However, few downstream mechanisms by which transformation or proliferation are induced by Notch signaling have been defined.

In rat kidney epithelial cells containing an estrogen-inducible activated Notch1 (RKE-ER-N^{ic}), cyclin D1 mRNA levels increase when the Notch1 intracellular domain (ICD) is overexpressed and the cells are transformed (13). The cyclin D1 promoter contains a binding site identified *in vitro* by electrophoretic mobility shift assay (EMSA) for the Notch pathway transcription factor CSL (CBF-1/RBP-Jκ; ref. 12). This site was predicted to be responsible for the up-regulation of cyclin D1 transcripts in cells constitutively expressing an activated Notch1 receptor, but reporter assays showing a functional response of the CSL site to Notch signaling were not done (12). Thus, cyclin D1 transcription might have been up-regulated in RKE-ER-N^{ic} by Notch1 ICD or by a mitogen-activated signal transduction pathway (14, 15). For example, ablation of the cyclin D1 gene in mice confers resistance to mammary tumors induced by Ras and ErbB2 (16). On the other hand, removal of cyclin D1 does not affect tumorigenesis induced by c-myc or activated β-catenin (16). Therefore, cyclin D1 is a necessary component of some, but not all, oncogenic pathways. Cyclin D1 is also important for normal physiology and development. Cyclin D1–null mice exhibit retinal hypoplasia and apoptosis (17, 18), have defects in the migration of macrophages (19), and do not lactate due to the failure of the mammary epithelium to differentiate (18). Highly coordinated cyclin D1 expression is also critical for the expansion and differentiation of precursor cell populations in the gut (20) and the central nervous system (21). Therefore, identifying signaling pathways that regulate cyclin D1 expression is important in understanding its function in both tumorigenesis and development.

The combined data implicate cyclin D1 as a Notch target gene that is up-regulated on nonphysiologic overexpression of an activated Notch ICD. However, it is not known whether increased cyclin D1 expression could be a necessary component of Notch1-induced cellular transformation. It is also unknown whether the previously identified CSL binding site in the cyclin D1 promoter is functional *in vivo* or if other sites in the cyclin D1 promoter may respond to activated Notch receptors. Finally, it is not known if cyclin D1 may be a target of Notch signaling induced by physiologic Notch ligands. Here, we address these questions by mutational analysis of the cyclin D1 promoter, by examining cyclin D1 gene expression in mouse embryos lacking global Notch signaling or Notch1, and by inhibiting cyclin D1 up-regulation during the transformation of RKE-ER-N^{ic} cells.

Materials and Methods

Cell culture. Clonal RKE-ER-N^{ic} and vector control cells were described previously (13). Lec1.3C Chinese hamster ovary (CHO) cells containing the vector pMIRB were shown previously to be competent in Notch signaling (22). Ligand cells for coculture were L cells expressing Jagged1 (J1 cells; ref. 23) and control L cells. The tumor cell line 5505 was derived from a mammary tumor in transgenic mice expressing the mouse mammary tumor virus (MMTV) long terminal repeat inserted into the Notch4 locus and shown to express Notch4 ICD (24). Cells were cultured in α-MEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Gemini, West Sacramento, CA) unless otherwise indicated.

Cyclin D1 reporter plasmids. The cyclin D1 promoter luciferase reporter constructs in Fig. 1 were described previously (14, 25). A CSL site 5′-GCTGAGAT-3′ at position −513 in the human cyclin D1 promoter was identified previously (12). The cyclin D1 binding site reporter (CSL-BSwt; Fig. 1) contained three copies of the previously identified CSL binding

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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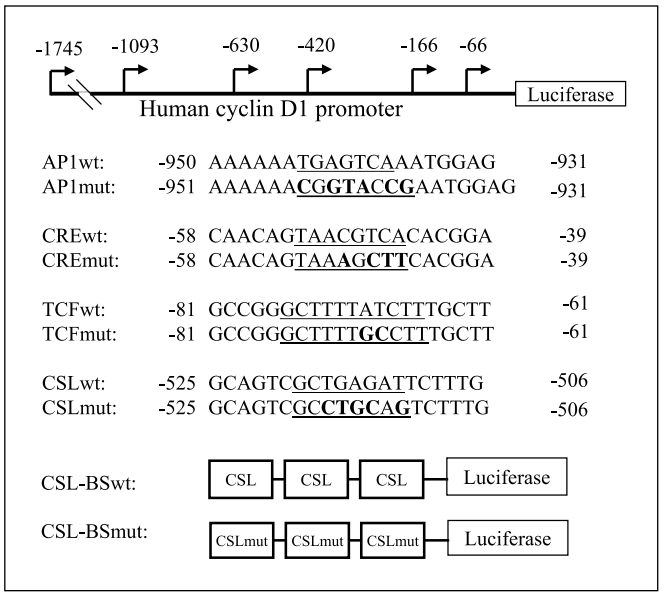

Figure 1. Schematic diagram of the human cyclin D1 promoter −1745CD1 with deletion and mutant constructs.

sequence and flanking sequences from −528 to −506 of the human cyclin D1 promoter (5′-GATCCATGCAGTC*GCTGAGAT*TCTTTGGATGC-AGTC*GCTGAGAT*TCTTTGGATGCAGTC*GCTGAGAT*TCTTTGGATGCA-3′) cloned into the pGa50-7 luciferase plasmid (26) between *Bam*HI and *Nsi*I sites (CSL sites italicized). The 3× cyclin D1 mutant binding site reporter (CSL-BSmut; Fig. 1) contained three copies of a mutated CSL site shown by EMSA not to bind CSL (12) with the sequence 5′-GATCCATGCAGTC*GC**CTGCAG***TCTTTGGATGCAGTC*GC**CTGC-AG***TCTTTGGATGCAGTC*GC**CTGCAG***TCTTTGGATGCA-3′ (mutant nucleotides in bold). The CSL point mutant reporter (CSLmut; Fig. 1) was made on the −1745CD1Luc plasmid using the QuikChange II mutagenesis kit (Stratagene, La Jolla, CA) with the primers 5′-AAAATGAAAGAAGATG-CAGTC*GC**CTGCAG***TCTTTGGCCGTCTGTCCGC-3′ (mutant nucleotides in bold) and 5′-GCGGACAGACGGCCAAAGACTGCAGGCGACTG-CATCTTCTTTCATTTT-3′.

Signaling assays using cyclin D1 promoter luciferase reporters. Hormone-induced activated Notch1 signaling was assayed by luciferase reporter activity in RKE-ER-N^{ic} cells. Duplicate or triplicate cultures of RKE-ER-N^{ic} cells growing in phenol red–free DMEM and 10% charcoal-stripped FBS on six-well plates were transfected with 1,800 ng cyclin D1 promoter reporter construct (Fig. 1) and 200 ng *Renilla* luciferase plasmid pRL-TK (Promega, Madison, WI) using LipofectAMINE 2000 (Invitrogen). After 16 hours at 37°C, the cells were treated with 2 μmol/L 4-hydroxytamoxifen (OHT; Sigma, St. Louis, MO) or ethanol vehicle. After another 8 hours, dual luciferase activities were measured. Activity is expressed as the ratio of *Renilla*-normalized luciferase units in OHT-treated compared with vehicle-treated cultures with SD.

For 5505 (Notch4 ICD) tumor cells, triplicate cultures were seeded in 60-mm dishes and transfected with 1,500 ng cyclin D1 reporter construct (Fig. 1) and 100 ng pCMV-β-galactosidase control plasmid (Clontech, Mountain View, CA) using LipofectAMINE 2000. After 24 hours, luciferase and β-galactosidase activities were assayed. Activity is expressed as a ratio of β-galactosidase-normalized luciferase units from each construct compared with the −66CD1 promoter with SD.

Signaling assays in CHO cells with constitutive Notch activation used a Notch1 construct lacking the extracellular ligand-binding domain (ZED N1; ref. 27). Duplicate cultures of Lec1 pMIRB CHO cells in a six-well plate were cotransfected with 1,800 ng of the indicated cyclin D1 reporter plasmid (Fig. 1), 200 ng pRL-TK, and 200 ng ZED N1 or control vector using FuGENE 6 (Roche, Basel, Switzerland). Firefly and *Renilla* luciferase activities were measured after 32 hours. Cyclin D1 promoter activation is expressed as fold induction in cultures transfected with ZED N1 over vector-transfected cells with SD.

The coculture Notch signaling assay was done as described (22). Duplicate cultures of Lec1 pMIRB CHO cells or Lec1 cell expressing Lunatic fringe (Lfng; ref. 22) were cotransfected on six-well plates with 1,800 ng of the indicated cyclin D1 reporter plasmid (Fig. 1) and 200 ng pRL-TK using FuGENE 6. After 16 hours at 37°C, 2×10^6 Jagged1/L (J1/L) or parental L cells were overlaid. After another 32 hours, firefly and *Renilla* luciferase activities were measured. Activation of cyclin D1 promoter is expressed as a ratio of *Renilla*-normalized luciferase units in J1/L versus L cell cocultures with SD. All experiments were done in duplicate at least twice.

Notch pathway mutant mice. The generation of embryos lacking protein *O*-fucosyltransferase 1 (*Pofut1*$^{-/-}$) was described previously (28). The generation and characterization of *Notch1*$^{-/-}$ embryos will be described in detail elsewhere.[3] Briefly, a *Notch1* mutant construct was generated with *loxP* sites flanking exons 6 to 8 and targeted to the *Notch1* locus in WW6 embryonic stem cells (29) and mice with a floxed *Notch1* allele were generated. Homozygous *Notch1*$^{F/F}$ mice were crossed with mice carrying a ZP3*Cre* transgene (30) to produce *Notch1*$^{+/-}$ heterozygotes. *Notch1*$^{-/-}$ embryos died at ~E10.5 with similar developmental defects to those described previously for *Notch1* null embryos (31, 32). Staged embryos from E8.5 or E9.5 were obtained by placing mice heterozygous for either mutation together at ~4:00 p.m. Noon of the next day was considered E0.5. All animal experiments were approved by the Animal Institute Committee of the Albert Einstein College of Medicine.

***In situ* hybridization.** A pcDNA3 plasmid containing the mouse cyclin D1 gene coding sequence (ref. 33; a gift from Dr. C. Sherr, Howard Hughes Medical Institute, St. Jude Children's Research Hospital, Memphis, TN) was linearized with *Bam*HI. Sense and antisense probes were transcribed using T7 and SP6 polymerase, respectively, and labeled using the digoxigenin RNA labeling kit (Roche). Purified probe was hybridized to fixed embryos as described (28). For detection with antidigoxigenin antibody, mutant and control embryos were developed together with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (Roche) until the signal was developed in control embryos. Embryos were photographed in 70% glycerol at ×40 (individual embryos) or ×25 (multiple embryos) magnification through a Leica (Wetzlar, Germany) Wild M3Z dissecting microscope using a Canon (Tokyo, Japan) PowerShot S40 digital camera.

Whole mount immunohistochemistry. Embryos were fixed in 4% paraformaldehyde in PBS, dehydrated, and treated for 4 hours in 5% H_2O_2-methanol at room temperature. After rehydration, embryos were washed in PBS containing 3% nonfat milk powder and 0.1% Triton X-100 (PBSMT) and incubated at 4°C overnight in primary antibody (anti–cyclin D1 DCS-6; Neomarkers, Fremont, CA) at 10 μg/mL. After five washes with PBSMT, embryos were incubated with horseradish peroxidase (HRP)–conjugated anti-mouse IgG secondary antibody (Zymed, Invitrogen), washed, and developed with 3′,3′-diaminobenzidine (Sigma) until signal was readily visible in control embryos. Embryos were postfixed in 2% paraformaldehyde-0.1% glutaraldehyde and photographed as above.

Western analysis after serum starvation. RKE-ER-N^{ic} cells were grown to ~90% confluence in DMEM containing 10% FBS before being serum starved in phenol red–free DMEM containing 0.1% charcoal-stripped FBS for 48 hours. Cells were treated with 1 μmol/L OHT or ethanol in phenol red–free DMEM containing 2% charcoal-stripped FBS for 12 hours. Cells were washed in PBS, and lysates were prepared in radioimmunoprecipitation assay buffer [10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA, 1% SDS, 1% NP40] containing phosphatase inhibitors (50 mmol/L NaF, 50 mmol/L β-glycerophosphate, 1 mmol/L orthovanadate) and protease inhibitors (1× complete protease inhibitor cocktail; Roche). Protein (~50 μg) was separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Blocking was done in TBST [25 mmol/L Tris-HCl, 125 mmol/L NaCl, 0.1% Tween 20 (pH 7.4)] containing 5% nonfat dry milk. Primary and secondary antibodies were diluted in TBST containing 5% nonfat dry milk

[3] C. Ge and P. Stanley, unpublished observations.

and incubated with the blot for 2 hours and 30 minutes, respectively. Proteins were visualized using enhanced chemiluminescence (Amersham, General Electric Healthcare, Fairfield, CT). Primary antibodies were cyclin D1 (DCS-6), cyclin E (sc-481; Santa Cruz Biotechnology), phospho-Rb (Ser^{795}) (sc-7986; Santa Cruz Biotechnology), and guanine nucleotide dissociation inhibitor (GDI) antibody (a gift from Dr. Perry Bickel, Washington University School of Medicine, St. Louis, MO). Anti-mouse IgG HRP-conjugated secondary antibody was used for cyclin D1 detection (Pierce, Rockford, IL) and anti-rabbit HRP-conjugated secondary antibody (Pierce) for cyclin E, phospho-Rb, and GDI. NIH ImageJ software was used to perform densitometric analysis of western blots.

Stable transfectants expressing antisense cyclin D1 cDNA. The pBABE vector expressing a previously characterized human cyclin D1 antisense cDNA (15) was cotransfected with pcDNA3.1/Hygro(+) (Invitrogen) into RKE-ER-N^{ic} cells using LipofectAMINE 2000. Colonies that arose in α-MEM supplemented with 10% FBS and 500 μg/mL hygromycin (Calbiochem, San Diego, CA) were expanded and analyzed by western blot with anti–cyclin D1 antibody DCS-6. Cell lines with a low cyclin D1 protein level (lines 1 and 20), a control line carrying the plasmid but with no apparent reduction in cyclin D1 protein (line 25), and control RKE-ER-N^{ic} cells were compared in subsequent assays.

Cell proliferation assay. RKE-ER-N^{ic} transfectants (10^5 cells) with reduced cyclin D1 protein (lines 1 and 20) and control lines 25 and RKE-ER-N^{ic} cells were seeded in triplicate into a 12-well plate in α-MEM containing 10% FBS and 2 μmol/L OHT or ethanol vehicle. Each day for 6 days, triplicate wells were washed, trypsinized, and counted in a Coulter Z1 particle counter (Beckman Coulter, Fullerton, CA). The medium was changed on days 3 and 5. The experiment was done twice with all lines, and the average cell number for the six wells is presented with SD.

Cell transformation assay. Anchorage-independent growth was assessed by colony formation in soft agar. RKE-ER-N^{ic} transfectants with reduced cyclin D1 (lines 1 and 20) and controls (lines 25 and RKE-ER-N^{ic}) were suspended in 10 mL phenol red–free DMEM with 10% charcoal-stripped FBS and 0.1% low melting temperature (LMT) agarose containing 2 μmol/L OHT or ethanol vehicle and seeded at 2×10^5 cells per 100-mm dish. On a base of 1 mL medium containing 0.5% LMT agarose, 1 mL of the same medium containing 2 μmol/L OHT or ethanol was added every 5 days. After ~26 days, the number of colonies per field was determined by counting six random fields at ×10 magnification. Colony size was determined by measuring the surface area of 20 colonies in random ×40 fields using NIH Image, and the mean and SD were determined.

Results

Functional response element of the cyclin D1 promoter to Notch signaling. The human cyclin D1 promoter spanning 1,745 bp upstream of the transcriptional start site contains a CSL binding site located at position −513. This site binds CSL protein *in vitro* by EMSA (12), but functional responsiveness to Notch signaling *in vivo* was not shown previously. To determine whether this site responds to Notch signaling and whether it is the only functional site, a series of luciferase reporter plasmids containing different deletions and point mutations of the cyclin D1 promoter region were tested (Fig. 1). Some of these were described previously (14) and the remainder were generated for this work. Deletion constructs containing the cyclin D1 promoter ranging from full-length (−1745CD1) to minimal (−66CD1) were transfected into RKE-ER-N^{ic} cells and Notch1 ICD was induced by treatment with 2 μmol/L OHT. Reporter constructs containing the cyclin D1 promoter region between −630 and −420 had reporter activity similar to the full-length promoter, whereas deletion mutants lacking the region between −630 and −420 had virtually no promoter activity (Fig. 2*A*). The full-length cyclin D1 promoter containing an inactivating mutation in the binding site for AP1, CRE, AP1 plus CRE, or TCF had activity similar to the wild-type cyclin D1 promoter (Fig. 2*A*). The slightly decreased signal in the TCF mutant is consistent with endogenous Notch signaling being a target of Wnt signaling (34). Cyclin D1 full-length and mutated promoter constructs gave similar results in the cell line 5505 that was derived from mammary tumors expressing activated Notch4 (Fig. 2*B*), showing that the same cyclin D1 promoter elements function in response to at least two Notch receptor ICDs.

To further examine the functional significance of the CSL site, mutations that inactivate CSL binding to the cyclin D1 promoter *in vitro* by EMSA (12) were introduced into the −1745CD1 reporter plasmid (Fig. 1). The CSL site at −513 was changed from GCTGAGAT in the −1745CD1 construct to GC**CTGC**A**G** to generate −1745CSLmut (altered nucleotides in bold). The −1745CSLmut had reduced activity similar to the −66CD1 minimal cyclin D1 promoter (Fig. 2*A* and *D*), in contrast to the reporter constructs with an AP1, CRE, or TCF mutation that responded similarly to the full-length promoter (Fig. 2*A* and *B*). To confirm that overexpressed Notch1 ICD is capable of activating transcription through the CSL site, three copies of the cyclin D1 CSL binding site with a small amount of flanking sequence (CSL BSwt) or a similar mutant sequence (CSL-BSmut) were placed upstream of a minimal β-globin promoter coupled to a luciferase reporter gene and transfected into RKE-ER-N^{ic} cells. Hormone treatment stimulated luciferase expression ~10-fold from the CSL-BSwt reporter, whereas the CSL-BSmut reporter was unresponsive (Fig. 2*A*). The ~3-fold higher activity of the CSL-BSwt reporter compared with the −1745CD1 reporter with its single CSL site suggests that this site is solely responsible for the observed Notch1-induced activity of the cyclin D1 promoter.

Cyclin D1 promoter constructs were also activated in CHO cells expressing a constitutively active form of Notch1 that lacks the extracellular ligand binding region (ZED N1; ref. 27). Following cotransfection with ZED N1, the full-length −1745CD1 reporter was induced ~7-fold and the CSL-BSwt reporter was induced ~18-fold, whereas the CSL-BSmut mutant had little activity and was similar to the −66CD1 minimal promoter (Fig. 2*C*). A more physiologic test of cyclin D1 as a Notch target gene was done using a CHO coculture assay in which endogenously expressed Notch receptors are activated ectopically by the Notch ligand Jagged1 (22). In the coculture assay, the full-length −1745CD1 cyclin D1 promoter was induced ~5-fold, whereas neither the −66CD1 minimal reporter lacking the CSL site nor the −1745CSLmut reporter was induced significantly above background (Fig. 2*D*). It was also found that Jagged1-induced Notch signaling activated the CSL-BSwt reporter ~10-fold and did not activate the CSL-BSmut reporter or the −1745CSLmut reporter (Fig. 2*D*). The lack of activity in the −1745CSLmut reporter again shows the absence of cryptic Notch-responsive elements in the cyclin D1 promoter. Notch signaling was also responsive to modulation by Lunatic fringe, which functions to inhibit Notch signaling induced by Jagged1 (Fig. 2*D*; refs. 15, 35), confirming that cyclin D1 promoter activation was taking place via the canonical Notch signaling pathway. Notch signaling thus activates the cyclin D1 promoter through a single CSL site in multiple cell types when an activated Notch ICD is overexpressed and in response to an ectopic signal from a Notch ligand.

Cyclin D1 is an *in vivo* target of Notch signaling. The fact that cyclin D1 expression was induced by Notch ligand (Fig. 2*D*) suggested that it may be a physiologic target of Notch signaling, an important point not examined previously. To investigate, we did whole mount *in situ* hybridization with cyclin D1 probes in

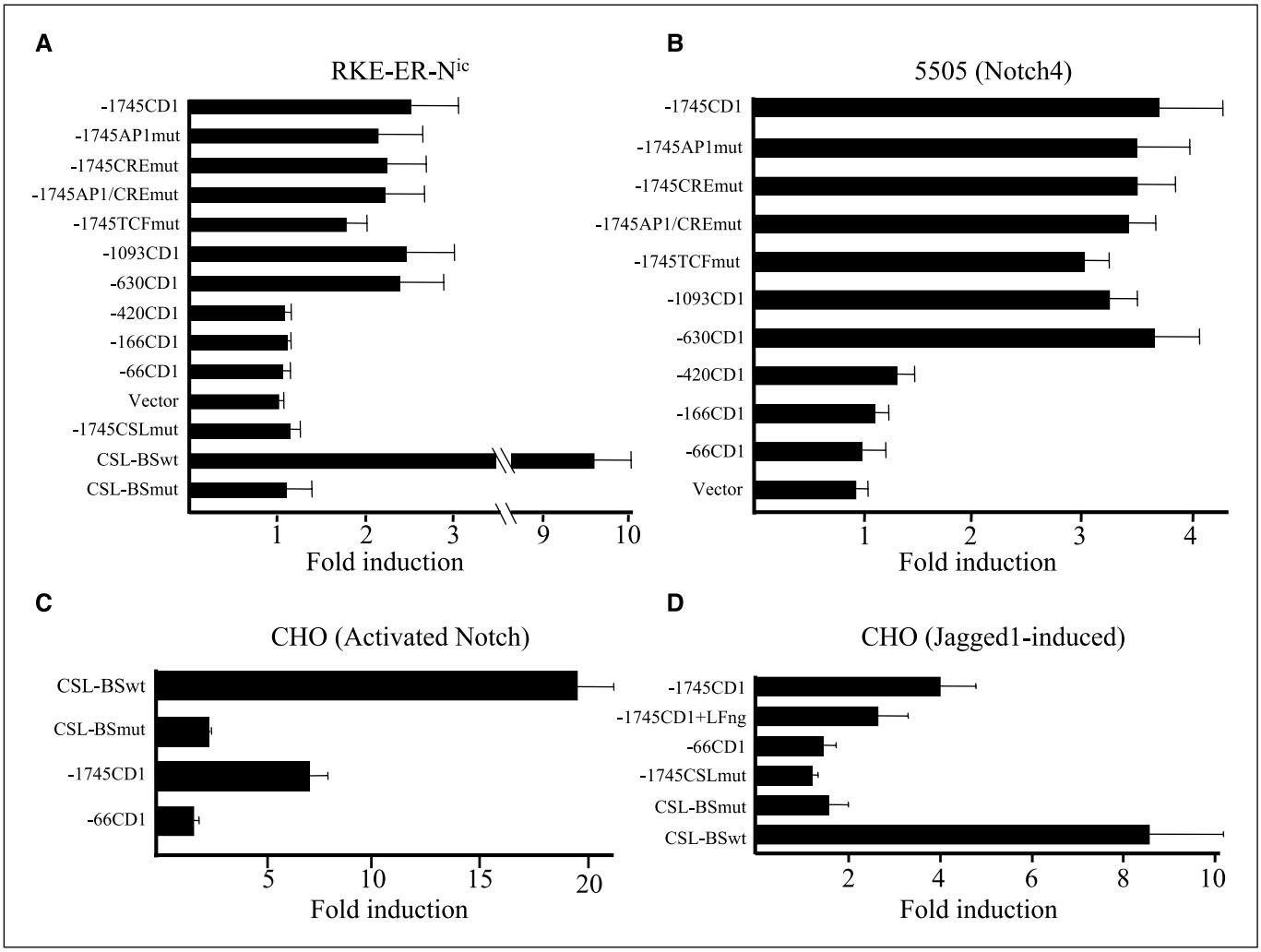

Figure 2. The cyclin D1 promoter CSL site is a functional Notch response element. *A,* RKE-ER-N^{ic} cells were transfected with the indicated cyclin D1 promoter construct and a *Renilla* luciferase control. Replicate cultures were treated with 2 μmol/L OHT or ethanol, luciferase activities were normalized, and fold induction for +OHT:−OHT was calculated ($n \geq 4$). *B,* cells derived from a Notch4 ICD-induced mouse mammary tumor (line 5505) were transfected with the indicated cyclin D1 promoter construct and a β-galactosidase control cDNA, luciferase and β-galactosidase activities were normalized, and fold induction was calculated compared with the −66CD1 construct ($n = 6$). *C,* CHO cells were cotransfected with the indicated cyclin D1 reporter, the constitutively active Notch construct ZED N1 or control vector, and *Renilla* luciferase control, luciferase activities were normalized, and fold induction for ZED N1:vector was calculated ($n \geq 4$). *D,* CHO cells or CHO cells expressing Lfng were transfected with the cyclin D1 reporter indicated and *Renilla* luciferase control. Notch signaling was activated by coculture with Jagged1-expressing L cells or control L cells, luciferase activities were assayed and normalized, and the fold induction for J1/L:L was calculated ($n \geq 4$). In the experiments with Lfng, the combined data reflect signaling that was reduced by ∼20% when transfection was for 12 hours and ∼52% when transfection was for 16 hours.

embryos defective in Notch signaling. This has long been a strategy for identifying Notch target genes (36). We previously used this approach to identify Pofut1 as an essential component of the canonical Notch signaling pathway (28). *Pofut1*$^{-/-}$ embryos die at ∼E9.5 (28), with a phenotype typical of embryos lacking downstream effectors of Notch signaling, such as RBP-Jκ (CSL) or presenilins 1 and 2 (37–39). We also examined cyclin D1 expression in embryos with inactive Notch1 receptors. The targeted mutation in the *Notch1* locus deleted epidermal growth factor–like repeats that include the Notch ligand-binding domain and resulted in a classic *Notch1*$^{-/-}$ phenotype (refs. 31, 32; Supplementary Fig. S1).

At least 10 control embryos from each group (*Pofut1*$^{+/+}$ or *Pofut1*$^{+/-}$ and *Notch1*$^{+/+}$ or *Notch1*$^{+/-}$) were examined at E9.5 by *in situ* hybridization using cyclin D1 sense and antisense probes. There was no signal with the sense probe (Supplementary Fig. S2). In E9.5 control embryos, cyclin D1 transcript levels were high in the anterior forebrain and the posterior midbrain as observed previously (40, 41), in branchial arches, the otic vesicle, in an area immediately ventral to developing somites, and in the neural tube (Fig. 3*A-D*). *Pofut1*$^{-/-}$ mutant embryos at E9.5 ($n = 5$) showed a marked reduction in cyclin D1 gene expression in the brain, branchial arches, and otic vesicles (Fig. 3*A*). Interestingly, cyclin D1 expression increased slightly in the neural tube of *Pofut1*$^{-/-}$ embryos (Fig. 3*B*), an effect seen for other Notch target genes in *Pofut1*$^{-/-}$ embryos (28) and indicative of negative regulation of cyclin D1 expression by Notch receptor(s). *Notch1*$^{-/-}$ embryos at E9.5 ($n = 6$) also had markedly reduced expression of cyclin D1 in the brain, branchial arches, otic vesicle, and neural tube, although the tail retained expression (Fig. 3*C* and *D*). Despite the abnormal development of E9.5 *Pofut1*$^{-/-}$ and *Notch1*$^{-/-}$ embryos, the structures in which cyclin D1 expression was altered were clearly present.

At E8.5, mutant and control embryos are visually indistinguishable (28, 42). Control E8.5 embryos from *Notch1*$^{+/+}$ or *Notch1*$^{+/-}$ (n = 10) and *Pofut1*$^{+/+}$ or *Pofut1*$^{+/-}$ (n = 8) expressed cyclin D1 in two discrete regions, cranial and caudal, with a low level of expression in the neural tube (Fig. 3*G* and *H*). In the cranial region, transcripts were detected in rhombomeres r4, r6, and r7 as observed previously (41). *Pofut1*$^{-/-}$ embryos (n = 4) exhibited a loss of the organized rhombomere pattern of cyclin D1 expression (Fig. 3*G*). *Notch1*$^{-/-}$ embryos at E8.5 (n = 5) had reduced cyclin D1 expression in the hind brain and virtually no expression in distal rhombomeres (Fig. 3*H*). The diffuse cyclin D1 signal in the neural tube and tail of control E8.5 embryos was localized to the neural tube in *Notch1*$^{-/-}$ mutant embryos (Fig. 3*H*). The slight differences in cyclin D1 expression pattern between *Notch1*$^{-/-}$ and *Pofut1*$^{-/-}$ embryos suggests that cyclin D1 expression may be influenced by different Notch receptors depending on cellular context, although their overall similarity indicates that Notch1 is primarily responsible. Immunohistochemistry of *Pofut1*$^{+/+}$ or *Pofut1*$^{+/-}$ control embryos (n = 7; Fig. 3*E* and *F*) revealed cyclin D1 protein in the forebrain, midbrain, hind brain, and neural tube but no signal in branchial arches, suggesting a temporal difference between transcription and protein synthesis. Another notable difference was the detection of cyclin D1 protein but not transcripts in somites. In addition, the otic vesicle of control embryos expressed cyclin D1 protein uniformly. In E9.5 *Pofut1*$^{-/-}$ mutant embryos (n = 6), cyclin D1 protein was not detected in the forebrain, midbrain, or hind brain and was present in neural tube. However, similar levels of expression were seen in the otic vesicle of both *Pofut1*$^{-/-}$ and control embryos, in contrast to the reduction in cyclin D1 transcripts in mutant embryos (Fig. 3*A*).

Cyclin D1 up-regulation is necessary for Notch1 induction of cell cycle progression and transformation in RKE-ER-N^{ic} cells. The fact that the cyclin D1 gene is a target of Notch signaling

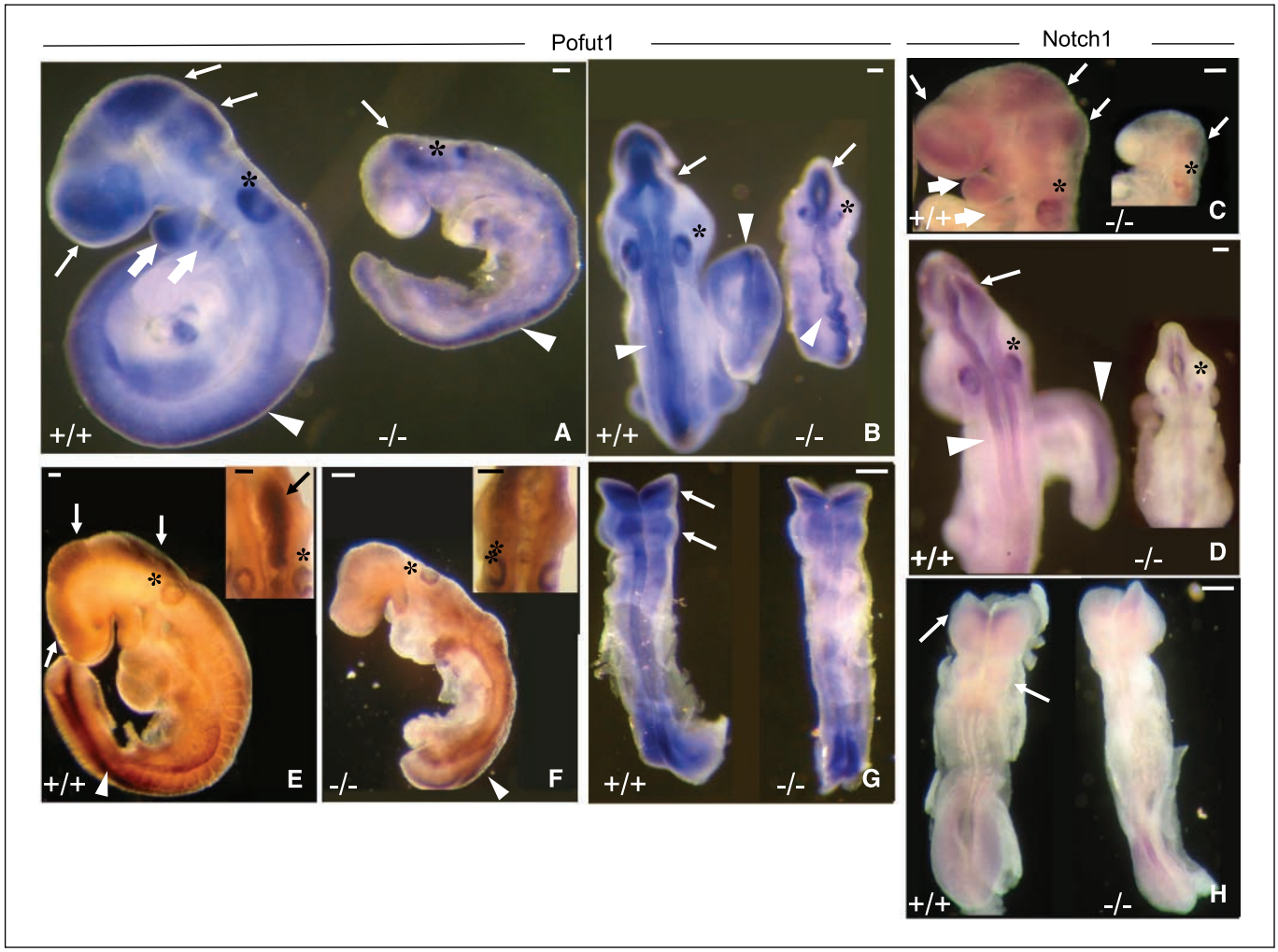

Figure 3. Embryos defective in global Notch signaling or Notch1 have reduced cyclin D1 expression. *A,* lateral view of control (*left*) and *Pofut1*$^{-/-}$ (*right*) embryos. *Thin arrows,* forebrain, midbrain, and hind brain; *thick arrows,* branchial arches; *asterisks,* otic vesicle; *arrowheads,* neural tube. *B,* dorsal view of E9.5 control (*left*) and *Pofut1*$^{-/-}$ embryo. *Thin arrows,* hind brain; *asterisks,* otic vesicle; *arrowheads,* neural tube with maximum expression close to the hind brain and in the tail. *C,* E9.5 control and *Notch1*$^{-/-}$ embryo heads. *Thin arrows,* forebrain, midbrain, and hind brain; *thick arrows,* branchial arches; *asterisks,* otic vesicle. *D,* dorsal view of E9.5 control (*left*) and *Notch1*$^{-/-}$ embryo. *Thin arrow,* hind brain; *asterisks,* otic vesicle; *arrowheads,* neural tube with maximum expression close to the hind brain and in the tail. *E,* whole mount immunohistochemistry of E9.5 *Pofut1*$^{-/-}$ control embryos. *Thin arrows,* midbrain and hind brain; *asterisk,* otic vesicle; *arrowhead,* neural tube. *F,* whole mount immunohistochemistry of an E9.5 *Pofut1*$^{-/-}$ embryo. Cyclin D1 protein is detected in the otic vesicle (*asterisk*) and tail (*arrowhead*) but not in the brain. *G,* dorsal view of an E8.5 control (*left*) and *Pofut1*$^{-/-}$ embryo (*right*; rostral region up). *Thin arrows,* individual rhombomeres. Tail expression is diffuse in control; expression reduced in mutant. *H,* dorsal view of an E8.5 control (*left*) and *Notch1*$^{-/-}$ embryo (*right*; rostral region up). *Thin arrows,* individual rhombomeres. Tail expression is diffuse in control; expression reduced in mutant, except in neural tube. Bar, 200 μm.

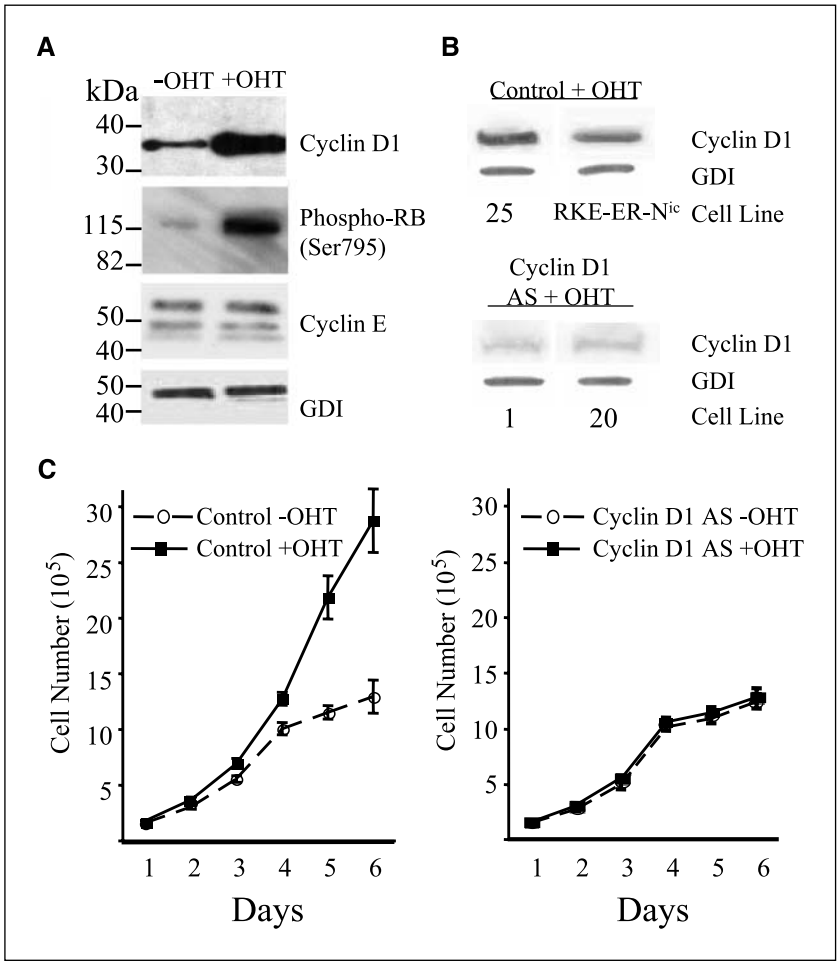

Figure 4. Cell proliferation induced by Notch1 ICD is dependent on increased cyclin D1 protein production in RKE-ER-N^{ic} cells. *A,* RKE-ER-N^{ic} cells were treated with 1 μmol/L OHT or ethanol vehicle for 12 hours. Whole-cell lysates were prepared, and western blot analysis was done with anti–cyclin D1 followed after stripping by anti–cyclin E, anti-phospho-Rb, and anti-GDI antibodies on the same blot. *B,* RKE-ER-N^{ic} cells expressing a cyclin D1 antisense cDNA (lines 1 and 20), control cells that survived antibiotic selection but had not down-regulated cyclin D1 (line 25), and parental RKE-ER-N^{ic} cells were treated with 1 μmol/L OHT for 6 hours. Whole-cell lysates were subjected to western analysis for cyclin D1 followed after stripping by anti-GDI antibodies. The lysates were analyzed on a single gel but in noncontiguous lanes. *C,* proliferation of RKE-ER-N^{ic} cells stably expressing cyclin D1 antisense cDNA and control cells in the presence and absence of 2 μmol/L OHT. Cells were seeded at 10^5 per well in triplicate, and the cell number was counted daily. Data from two experiments done in triplicate (n = 6) on cell line 25 (control; *left*) and line 1 (cyclin D1 antisense; *right*). RKE-ER-N^{ic} cells and line 20 (cyclin D1 antisense) gave similar results.

in normal and transformed cells begged the question of whether cyclin D1 was playing a primary or secondary role in Notch-induced cell transformation. RKE-ER-N^{ic} cells stably expressing Notch1^{ic}-ER chimeric protein are transformed in a hormone-dependent manner and specifically increase cyclin D1 transcripts, with no change in cyclin E, E2F-1, or Cdc25A mRNA levels (12, 13). We determined that the protein level of cyclin D1 is also increased after OHT treatment of RKE-ER-N^{ic} cells (Fig. 4). Following culture in 0.1% serum for 48 hours, cells were treated for 12 hours with or without 1 μmol/L OHT and analyzed by western blot. Hormone activation of RKE-ER-N^{ic} cells markedly increased the amount of cyclin D1 protein (Fig. 4*A*). The level of phospho-Rb was also increased over control cells (Fig. 4*A*), and an increase from ~30% to ~50% in the percentage of cells in G_2-M was observed in OHT-treated cells by propidium iodide staining and fluorescence cytometry (data not shown). No increase in the level of cyclin E was observed on Notch1 ICD overexpression (Fig. 4*A*).

To determine if the transforming ability of Notch1 ICD is dependent on increased levels of cyclin D1 protein, RKE-ER-N^{ic} cell lines stably expressing a human cyclin D1 antisense construct were generated. This cDNA was shown previously to strongly reduce cyclin D1 levels and inhibit ErbB2-induced transformation of Rat-1 cells (15, 35, 43). Transfectant colonies identified by western analysis to have the least cyclin D1 protein after OHT treatment (Fig. 4*B*) were compared with control cells in a cell proliferation assay. The results show that OHT strongly enhanced the proliferation of control RKE-ER-N^{ic} cells (Fig. 4*C, left*). In the absence of OHT, control RKE-ER-N^{ic} cells stopped proliferating on contact inhibition. By contrast, hormone induction did not promote proliferation in RKE-ER-N^{ic} cells expressing the cyclin D1 antisense cDNA. Rather, these cells stopped proliferating on contact in the presence or absence of hormone (Fig. 4*C, right*). Thus, cyclin D1 protein up-regulation is essential for Notch1 ICD to induce proliferation of RKE-ER-N^{ic} cells.

The cell lines expressing cyclin D1 antisense were also tested for their ability to grow in a semisolid medium in the presence and absence of OHT. RKE-ER-N^{ic} cells readily formed colonies in the presence of OHT as expected (13), whereas few colonies formed in the absence of hormone treatment (Fig. 5*A* and *B*). RKE lines expressing both ER-N^{ic} and the cyclin D1 antisense construct, however, formed ~11-fold fewer colonies (Fig. 5*C*) of ~1/9th the surface area of those with vector alone (Fig. 5*D*). Taken together, these results show that overexpression of Notch1 ICD that does not lead to increased cyclin D1 protein levels fails to transform RKE-1 cells. Thus, cyclin D1 up-regulation is a necessary component of the transformation of RKE-1 cells by Notch1 ICD.

Discussion

The cyclin D1 promoter reporter constructs tested herein have conclusively identified the CSL binding site revealed previously by

in vitro EMSA (12) as a functional Notch signaling response element *in vivo* and as the only site that responds to activated Notch1 in the full-length cyclin D1 promoter. The generality of the effect of Notch signaling on cyclin D1 promoter activation in RKE-1, CHO, and mammary tumor cells expressing Notch1 or Notch4 ICD suggested that cyclin D1 up-regulation may be a means by which activation of the Notch pathway induces cell proliferation and tumor formation.

Stimulation of Notch receptors by the Notch ligand Jagged1 also resulted in activation of the cyclin D1 promoter through the CSL site. This activation was modulated by Lfng, consistent with cyclin D1 being a physiologic Notch target gene. *In vivo* cyclin D1 transcripts in specific brain regions and elsewhere were greatly reduced in mouse embryos deficient in Notch signaling through all four Notch receptors (*Pofut1*$^{-/-}$) or with inactivated Notch1 (*Notch1*$^{-/-}$). However, cyclin D1 cannot be an essential target of Notch signaling in development because cyclin D1 null mice are viable (17, 18) and deletion of all three D-type cyclins does not result in a Notch phenotype (44). However, it is possible that the neurologic abnormalities observed in cyclin D1 mutant mice may reflect a reduction in Notch1 signaling because Notch1 is a key regulator of neuronal differentiation (45). Cyclin D1 RNA expression was described previously in the neural groove of the developing chick (46) and in mouse brain at ~E8.0 and E9.0 (40, 41). We obtained identical results in control mouse embryos and we revealed the pattern of cyclin D1 protein expression at these stages by immunohistochemistry. In the previous studies, sonic hedgehog signaling was shown to be responsible for the majority of cyclin D1 expression in the neural groove of the chick (46) and in the mouse brain (40, 41), whereas the role of Notch was unexplored. Interestingly, Notch signaling has recently been shown to be an important factor in the growth of medulloblastomas induced by sonic hedgehog signaling in transgenic mice (47), suggesting cyclin D1 as a point of intersection between the Notch and sonic hedgehog signaling pathways. The finding that cyclin D1 is a physiologic target of Notch signaling is of value in understanding the role of the Notch pathway in cell proliferation, differentiation, and tumorigenesis.

The ability of Notch1 ICD to function as an oncogene when overexpressed has been known because it was cloned as TAN-1 in human acute T-cell lymphoblastic leukemia (48). However, few mechanisms by which Notch1 transforms cells have been identified. Here, we identify a new mechanism for transformation by activated Notch1. By inhibiting the increase in cyclin D1 level

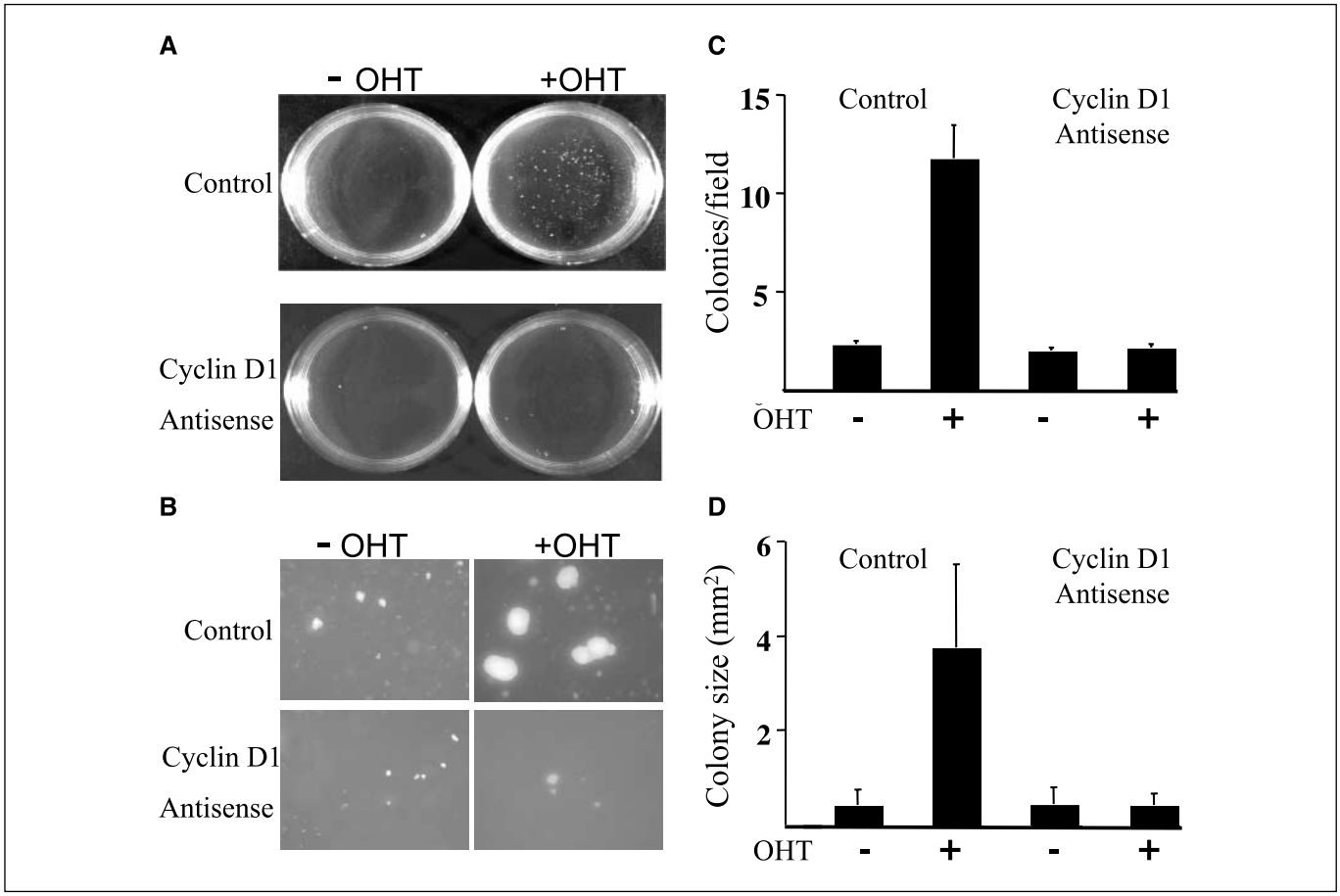

Figure 5. Notch1 ICD-induced proliferation of RKE-ER-N^{ic} cells in soft agar is dependent on increased cyclin D1 protein expression. *A,* RKE-ER-N^{ic} cells stably expressing cyclin D1 antisense cDNA (line 1) or control (line 25) were seeded into soft agar and maintained in culture in the presence (+*OHT*) or absence (−*OHT*) of 1 μmol/L OHT for ~26 days before plates were photographed from above. Similar results were obtained with cyclin D1 antisense cDNA (line 20) and RKE-ER-N^{ic} cells. *B,* photomicrographs of representative ×40 fields of the colonies shown in (*A*). *C,* quantitation of average colony number per field. All of the colonies in six random ×10 field images per plate were photographed and counted using NIH Image software. *D,* quantitation of average colony size. The surface area of 20 colonies in random ×40 field images was measured using NIH Image software.

that follows overexpression of activated Notch1, proliferation and transformation of RKE-ER-N^{ic} cells were prevented. It will be important to determine if this mechanism is responsible for Notch-induced tumorigenesis *in vivo*. For example, expression of activated Notch1 in mammary gland causes mammary tumors that exhibit increased expression of cyclin D1 (5). This may represent a functional correlation analogous to the dependence of RKE-1 cells on cyclin D1 for transformation by Notch1 ICD described here and could be tested by generating mice expressing a MMTV-activated Notch1 transgene in a conditional cyclin D1 null mammary epithelial cell background. Mechanisms of Notch1 transformation are clearly cell type specific because cyclin D3, and not cyclin D1, mediates transformation by activated Notch1 in T-cell leukemia (49), and β-catenin rather than cyclin D1 is necessary for Notch1 to cause primary melanoma cells to proliferate and progress (50). Furthermore, transformation of breast myoepithelial cells by Wnt signaling requires the expression of Notch ligands and correlates with up-regulation of cyclin D2 but not Notch1 or cyclin D1 (34). Thus, although the requirement for cyclin D1 in cellular transformation of RKE-1 cells by Notch1 described here is an important new mechanism of Notch-induced cellular transformation, it is expected to occur in only some cell types that are difficult to predict.

In summary, this study shows that cyclin D1 is a direct target of ligand-induced Notch signaling *in vitro* and is also a target *in vivo* and that up-regulation of cyclin D1 levels is required for transformation of RKE-1 cells by Notch1. This is a new means by which Notch1 signaling may regulate proliferation and differentiation under physiologic conditions and a previously undescribed mechanism by which Notch1 may transform cells.

Addendum

While this article was under review, cyclin D1 gene expression was shown to be a target of Notch1 in the retina (51).

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