

DACH1 Is a Cell Fate Determination Factor That Inhibits Cyclin D1 and Breast Tumor Growth†

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Obstacles to the expansion of cells with proliferative potential include the induction of cell death, telomere-based senescence, and the pRb and p53 tumor suppressors. Not infrequently, the molecular pathways regulating oncogenesis recapitulate aberrations of processes governing embryogenesis. The genetic network, consisting of the *dachshund* (*dac*), *eyes absent* (*eya*), *eyeless*, and *sine oculis* (*so*) genes, regulates cell fate determination in metazoans, with *dac* serving as a cointegrator through a So DNA-binding factor. Here, DACH1 inhibited oncogene-mediated breast oncogenesis, blocking breast cancer epithelial cell DNA synthesis, colony formation, growth in Matrigel, and tumor growth in mice. Genetic deletion studies demonstrated a requirement for *cyclin D1* in DACH1-mediated inhibition of DNA synthesis. DACH1 repressed cyclin D1 through a novel mechanism via a c-Jun DNA-binding partner, requiring the DACH1 α -helical DS domain which recruits corepressors to the local chromatin. Analysis of over 2,000 patients demonstrated increased nuclear DACH1 expression correlated inversely with cellular mitosis and predicted improved breast cancer patient survival. The cell fate determination factor, DACH1, arrests breast tumor proliferation and growth in vivo providing a new mechanistic and potential therapeutic insight into this common disease.

The *Drosophila dachshund* (*dac*) gene is the founding member of the DACH subfamily of nuclear proteins, which conduct an essential role in promoting differentiation of the *Drosophila* eye and limb (42). The *dac* gene forms part of a retinal determination (RD) signaling pathway in *Drosophila*. The RD pathway requires *eyeless* (*eya/Pax6*), *sine oculis* (*So, Six*), *eyes absent* (*eya/Eya*), and *dachshund* (*dac/Dach*), which function together as genetic components of this network (24, 25, 36). In *Drosophila*, *So* functions as a DNA-binding factor and *dac/eya* are transcription cofactors. Although *So/Six* binding sites have been identified in RD signaling target gene promoters, genome-wide analysis of DACH1-regulated genes identified a preponderance of AP-1-responsive genes. *Dachshund* is expressed prior to photoreceptor differentiation and is required for retinal morphogenesis. *Dachshund* itself is sufficient for inducing retinal fates since targeted misexpression results in ectopic eye formation from non-neuronal tissues (13, 54).

Although the RD gene network is best known for its role in eye specification, these genes, either individually or as a network, are expressed in postmitotic cells and contribute to diverse developmental processes in many cell types in all metazoans. The *So/Six* family governs proliferation of progenitor

populations prior to cell type specification. Misregulated expression of *Six* proteins occurs in human cancer (15, 21). *HSIX1* is the human homologue of the *Drosophila Six* gene that was originally isolated through its enrichment during the S phase of the cell cycle. The *Six* gene in *Drosophila* functions as a DNA-binding component of the transcription factor complex. *Six1* is overexpressed in breast cancer, and forced *Six1* expression attenuates a G₂ cell cycle check point (15, 21). *Six1* has been implicated as a dual-function regulator of metastasis, enhancing poorly metastatic rhabdomyosarcoma tumors (66). The molecular mechanism by which the RD pathway regulates human tumorigenesis is poorly understood.

Orderly cell cycle progression of nontransformed cells is orchestrated by coordinated induction of cyclin-dependent kinases that assemble in temporally and spatially defined complexes within the cell (43). Sequential phosphorylation of key substrates, including the retinoblastoma (pRb) protein, by cyclin D1 and cyclin E-cdk complexes promotes the timely induction of cellular DNA synthesis (27). Oncogenic disruption of the cell cycle machinery, through amplification or disruption of the cell cycle proteins themselves, is a common finding in human breast cancer. The *cyclin D1* gene encodes the regulatory subunit of a holoenzyme that contributes to the phosphorylation and inactivation of the pRb protein and is frequently overexpressed in human breast cancer epithelial cells. Specific oncogenic signals disrupt the cell cycle in a reproducible manner. Transformation by Ras requires the inactivation of the pRb and p53 pathways. Myc has activities compatible with the bypass of p21^{CIP1} (10, 14, 16, 28, 29, 51, 58) and/or activation

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of Arf and p53 (10), which impose a selection to the escape of cellular apoptosis (67). Genetic studies in mice have confirmed the fidelity of these molecular interactions in vivo. Thus, genetic deficiency for cyclin D1 provides resistance to Ras or ErbB2 but not c-Myc-induced tumorigenesis, whereas a distinct subset of genes antagonize Myc function including transforming growth factor β (TGF- β) and p21^{CIP1} (55).

Obstacles to the expansion of cells with proliferative potential include the induction of cell death, telomere-based senescence, and the pRb and p53 tumor suppressors. Not infrequently, the molecular pathways regulating oncogenesis, recapitulate aberrations of processes governing embryogenesis (1). The transcription factors encoded by the homeobox gene family play a vital role in growth and differentiation during normal development. This diverse group of proteins is divided into groups based on similarity among the homeodomain box and includes the MSX, Engrailed, PAX, and the SIX families. Deregulated homeobox gene expression is well known in cancer, with genes normally expressed in undifferentiated cells upregulated in cancer. Homeobox genes known to be deregulated in cancer include *HOX*, *MSX*, *HSIX1*, *GBX2*, the *PAX* genes, *CDX2*, *NKX3.1*, and *BARX2*. Recent microarray analysis studies demonstrated DACH1-repressed proliferative signaling in cultured cells (62). We examined the expression and function of DACH1 in normal and tumorous breast epithelium. We demonstrate DACH1 blocks cellular proliferation and contact-independent growth and identify a new mechanism by which DACH1 regulates cellular proliferation and growth in vivo.

MATERIALS AND METHODS

Plasmid construction. The expression plasmids for DACH1, DACH1 DS-domain alone (DS), or DACH1 DS-domain deleted (Δ DS) (which includes an N-terminal Flag peptide), the reporter plasmid 3-TP lux, 3XCRE luc the ponasterone-regulated expression system, and the full-length wild-type and mutant cyclin D1 promoter reporter constructions were previously described (5, 6, 62). The Flag-tagged DACH1 cDNA was subcloned into the MSCV-internal ribosome entry site (IRES)-green fluorescent protein (GFP). The SKI cDNAs was subcloned into the 3x Flag-CMV7.1 vector (Sigma, St. Louis, MO).

Cell sorting and DNA synthesis analysis. Magnetic activated cell sorting to enrich for transfected cells was conducted as previously described (7). Cell cycle parameters were determined by using laser scanning cytometry. Cells were processed by standard methods using propidium iodide staining of cell DNA. Each sample was analyzed by flow cytometry with a FACScan flow cytometer (Becton Dickinson Biosciences, Mansfield, MA) using a 488-nm laser. Histograms were analyzed for cell cycle compartments using ModFit version 2.0 (Verity Software House, Topsham, ME). A minimum of 20,000 events was collected to maximize statistical validity of the compartmental analysis.

Western blot analysis, tissue microarrays, immunohistochemistry, and purification of DACH1 antibody. Western blot analysis with antibodies to cyclin D1, cyclin E, cdk4, phosphorylated pRb (residue amino acid Ser⁷⁰⁸), Flag, p21^{CIP1/WAF1}, p27^{KIP1}, c-Jun, CREB, and the loading control guanine dissociation inhibitor (GDI) and immunohistochemistry for cyclin D1 and DACH1 were performed as previously described (35, 62). Affinity-purified polyclonal antibody to DACH1 was produced by Affinity BioReagents through immunizing rabbits with the peptide ERTIQDGRLYLKTVMY. Human breast tissue microarrays were from Imgenex (IMH-304) and NCI CBCTR 2001 TMA#2. The human breast cancer prognostic tissue microarray was previously described (2). This array consists of 2,197 formalin-fixed, paraffin-embedded breast cancer tissues with clinical-pathological characteristics, clinical follow-up information, and 283 normal/precancerous tissues. The level of DACH1 protein expression in tumors and normal human breast cancer in the TMAs was categorized by a semiquantitative score of the immunostaining intensity by light microscopy evaluation, using a standard methodology that determined a range of staining intensities from negative to strong with intermediate grades. The intensity of immunoperoxidase staining

was scored as 0 (negative), 1+ (a minimal to low level of positive staining), 2+ (moderate expression), or 3+ (strong staining). The evaluation and scoring of the cores of the tumors from the patients representing different breast tumor classifications and grades incorporated in the TMAs were conducted by a pathologist (R.G.R.). The results were viewed by two pathologists. Mitoses grade in tumor cells was determined according to the standard of Bloom, Richardson, and Elston.

Cell culture, plasmid transfection, luciferase reporter assays, and Matrigel culture. *Cyclin D1*^{-/-} cells derived from *cyclin D1*^{-/-} mice (64), p21^{CIP1}^{-/-} fibroblast cells (provided by L. Augenlicht) and p27^{KIP1}^{-/-} fibroblast cells (a gift from A. Koff) were cultured in Dulbecco modified Eagle medium with 10% fetal bovine serum in a humidified atmosphere with 5% CO₂ at 37°C. Other cells were cultured as recommended by the American Type Culture Collection and maintained in humidified atmosphere with 5% CO₂ at 37°C (59). Transfections were performed by using Superfect transfection reagent (QIAGEN, Valencia, CA) according to the manufacturer's protocol using 1.5 μ g of the reporter, 50 to 300 ng of expression plasmids, or equal molar amounts of control vector. The transfection efficiency was normalized by cotransfection with 0.2 μ g of pRL-CMV plasmid (Promega, Madison, WI) and measured with Promega's dual-luciferase reporter assay system according to the manufacturer's protocol. Control small interfering RNA (siRNA) was purchased from Santa Cruz. siRNAs for human DACH1 were designed and synthesized by Ambion, Inc. (gene accession number AF 356492). The target sequences were AAGGCCTCCTAAGAGGACTCA and AAGGACTTCGA GACCCTCTAC. DACH1-specific siRNAs or control siRNAs (100 or 200 nM) were transfected according to the Oligofectamine protocol (Invitrogen). Transfection efficiency was monitored by no-silencing fluorescein siRNA from QIAGEN. Statistical analyses were performed by using the Student *t* test. For three-dimensional culture, 1,000 cells mixed with 1:3 diluted growth factor reduced Matrigel matrix were seeded into BD biocoated cell culture inserts (24-well) with Matrigel basement membrane matrix from BD Bioscience (catalog no. 354447) and photographed after growth for 3 to 14 days. For confocal image analyses, MCF10A cells were mixed with 1:1-diluted matrigel and seeded into four-well chamber according to a previously described protocol (18).

Immunoprecipitation, immunoblotting, and Northern blot. 293T cells were used for the detection of protein-protein interaction in vivo, and immunoprecipitation-Western blotting was conducted as previously described using anti-Flag M2 antibody (Sigma) and immunoblotting with antibodies to CREB, c-Jun, or Flag (Santa Cruz Biotechnology). The guanine nucleotide dissociation inhibitor antibody (i.e., GDI) (35) was used as an internal control for protein abundance. For Northern blot analysis, total RNA was isolated by using TRIzol reagent. Cyclin D1 cDNA was randomly labeled using NEBlot Phototope kit, and the membrane was detected by Phototope-Star detection kit (New England Biolabs).

ChIP assay. Chromatin immunoprecipitation (ChIP) assays were performed as previously described (22). The human cyclin D1 promoter-specific primers used were as follows: AP-1 site, 5'-CTGCCTTCTACCTTGACCA-3' and 5'-TGAAGGGACGTCTACACCC-3'; and CRE site, 5'-GCCCCCTCCCGCT CCCATT-3' and 5'-TGGGGCTCTTCTGGGCAGC-3'. The sequences as a negative control in the cyclin D1 promoter were 5'-TTTCGGAAGCGTTT TCCC and 5'-AGCGGTTTCATTAGGAA. 293T cells were transiently co-transfected with expression plasmids encoding wild-type or mutant DACH1, with the cyclin D1 promoter constructions, -1745 or cyclin D1 -1745-AP-1/CRE mutant reporter, and then cultured for 36 h. The cells were cross-linked with formaldehyde buffer for 10 min at 37°C, and the procedure was performed as described earlier (22).

Nude mice study. Ponasterone A was purified from *taxus chinensis*, and 100 μ g of purified ponasterone A was introduced into 21-day slow release pellets produced by Innovative Research of American (Sarasota, FL) (5). Ponasterone A or placebo pellets were implanted subcutaneously into 4- to 6-week-old athymic female nude mice purchased from NCI. The following day, 2 \times 10⁶ MDA-MB-231 cells, stably expressing DACH1 or vector control, were injected subcutaneously, and the tumor growth was measured twice weekly by digital caliper. Ponasterone A or placebo pellets were implanted every 21 days.

Statistics. The Student *t* test and the Wilcoxon-Mann-Whitney test were used to analyze the significance of expression of DACH1. Kaplan-Meier survival curves were plotted and analyzed with the Cox proportional hazards regression. The statistical significance of difference between curves was tested by using the generalized Wilcoxon test.

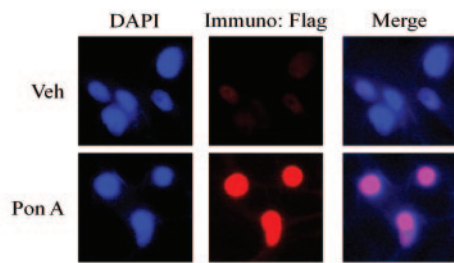
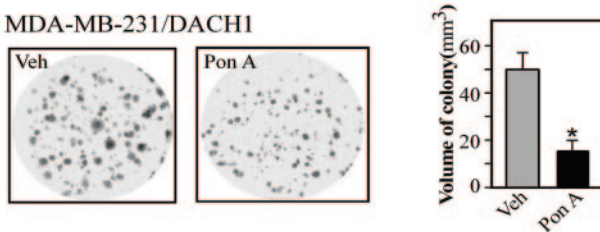
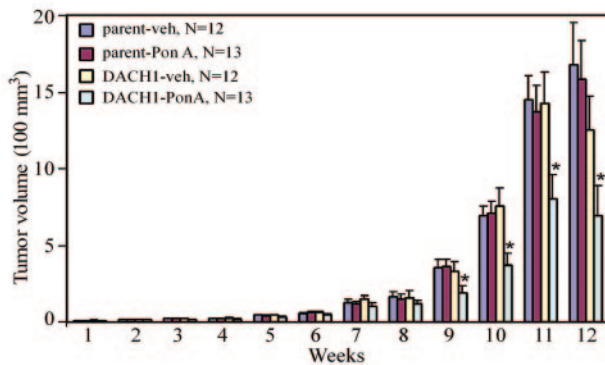
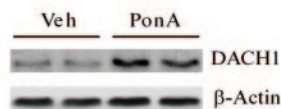
A. MDA-MB-231 DACH1 stable cells**B. MDA-MB-231/DACH1****C. MDA-MB-231 DACH1****D. Tumor**

FIG. 1. Inducible DACH1 expression inhibits colony formation and tumor growth in vivo. (A) Immunohistochemical stain for the Flag epitope of DACH1 in MDA-MB-231 stable ponasterone-inducible DACH1 cell lines showing nuclear colocalization with DAPI stain. (B) Ponasterone-inducible MDA-MB-231 stable cells were seeded onto six-well plates and stained with crystal violet after 14 days growth. Colony volumes (mean \pm the standard error of the mean [SEM], in mm^3) are shown on the right (*, $P < 0.01$). (C) Tumor growth of ponasterone-inducible MDA-MB-231 stable cells in athymic female nude mice ($n > 12$ separate animals in each group; *, $P < 0.05$). Mice were treated with the ponasterone A pellets or control pellets (vehicle), and the sizes of the tumors were assessed weekly. (D) Representative Western blot analysis of DACH1-inducible MDA-MB-231 cells from animals treated with vehicle or ponasterone A pellets.

RESULTS

DACH1 inhibition of colony formation and tumor growth in vivo. Recent studies examining the genetic signaling repressed by DACH1 identified clusters of genes promoting cellular proliferation, growth, and survival (62). To examine the function of DACH1 in regulating breast epithelial cell growth in vivo,

stable cell lines inducibly expressing DACH1 under the control of the ecdysone-regulated system were constructed in MDA-MB-231 cells. Ponasterone A induced expression of the Flag-tagged DACH1 expression vector as determined by immunohistochemistry and by Western blotting at 12 to 96 h after ponasterone A addition (Fig. 1A and see Fig. S1A in the supplemental material). The numbers and sizes of colonies were reduced $>70\%$ by DACH1 (Fig. 1B). To examine the effect of DACH1 on breast tumor growth in vivo, the ponasterone-inducible stable lines were implanted in nude mice, and a comparison was made between the sustained release pellets of ponasterone A and the control placebo pellets, with greater than 12 separate animals in each group. Ponasterone A pellets induced sustained expression of an ecdysone-regulated β -galactosidase reporter mouse (data not shown) (5). The induction of DACH1 expression reduced the growth of breast tumors in mice by 50% (Fig. 1C), with an inhibition of growth by DACH1 first detected at 9 weeks postimplantation ($329 \pm 66 \text{ mm}^3$ versus $186 \pm 27 \text{ mm}^3$, $P < 0.05$). Western blot analysis of tumors isolated from mice demonstrated the induction of DACH1 protein in mice treated with ponasterone A pellets compared to mice treated with placebo control pellets (Veh) (Fig. 1D).

DACH1 inhibition of breast epithelial cell DNA synthesis requires the DACH DS domain. To investigate the mechanism by which DACH1 blocked MDA-MB231 cellular growth, we examined the effect of DACH1 on DNA synthesis and cellular apoptosis. Although serum starvation does not arrest DNA synthesis in MDA-MB231 cells (47), the addition of serum produces an induction of the proportion of cells in the S phase of the cell cycle (Fig. 2A). Expression of DACH1 blocked serum-induced DNA synthesis (Fig. 2A) without affecting the fraction of apoptotic cells, inferred through analysis of the sub G_1 population and annexin V staining of the cells (Fig. 2B and C). MDA-MB-231 cells harbor mutation in the K-Ras gene. MCF-7 cells express amplification of c-Myc. To determine whether DACH1 was capable of inhibiting DNA synthesis in MCF-7 cells, cells were transfected with DACH1 expression vectors. To identify the domains of DACH1 involved in regulating DNA synthesis, a series of domain mutants were examined (Fig. 2D). MCF-7 cells cotransfected with DACH1 or its mutants were assessed by flow cytometry. DACH1 reduced the proportion of cells in the DNA synthetic (S) phase by 17% (Fig. 2E). One region of structural homology between DACH1 and the Ski/Sno proto-oncogene family (26) is predicted to form an α -helical structure (33) (DACH DS domain) that may convey general or specific DNA-binding activity (31). Deletion of the conserved DS domain abrogated inhibition of DNA synthesis. Expression of the related Ski protein did not inhibit DNA synthesis in MCF-7 cells. The inability of the Δ DS domain construct to inhibit DNA synthesis compared to the DS domain alone expression vector was not due to reduced expression of the Δ DS domain compared to the DS domain construct, since the Δ DS domain was expressed ~ 3 -fold better than the DS domain alone (see Fig. S1B in the supplemental material).

Phosphorylation and inactivation of the pRb protein and the *cyclin D1* gene product play a key role in DNA synthesis of MDA-MB231 cells. To investigate the candidate target cell cycle proteins regulated by DACH1, responsible for the inhi-

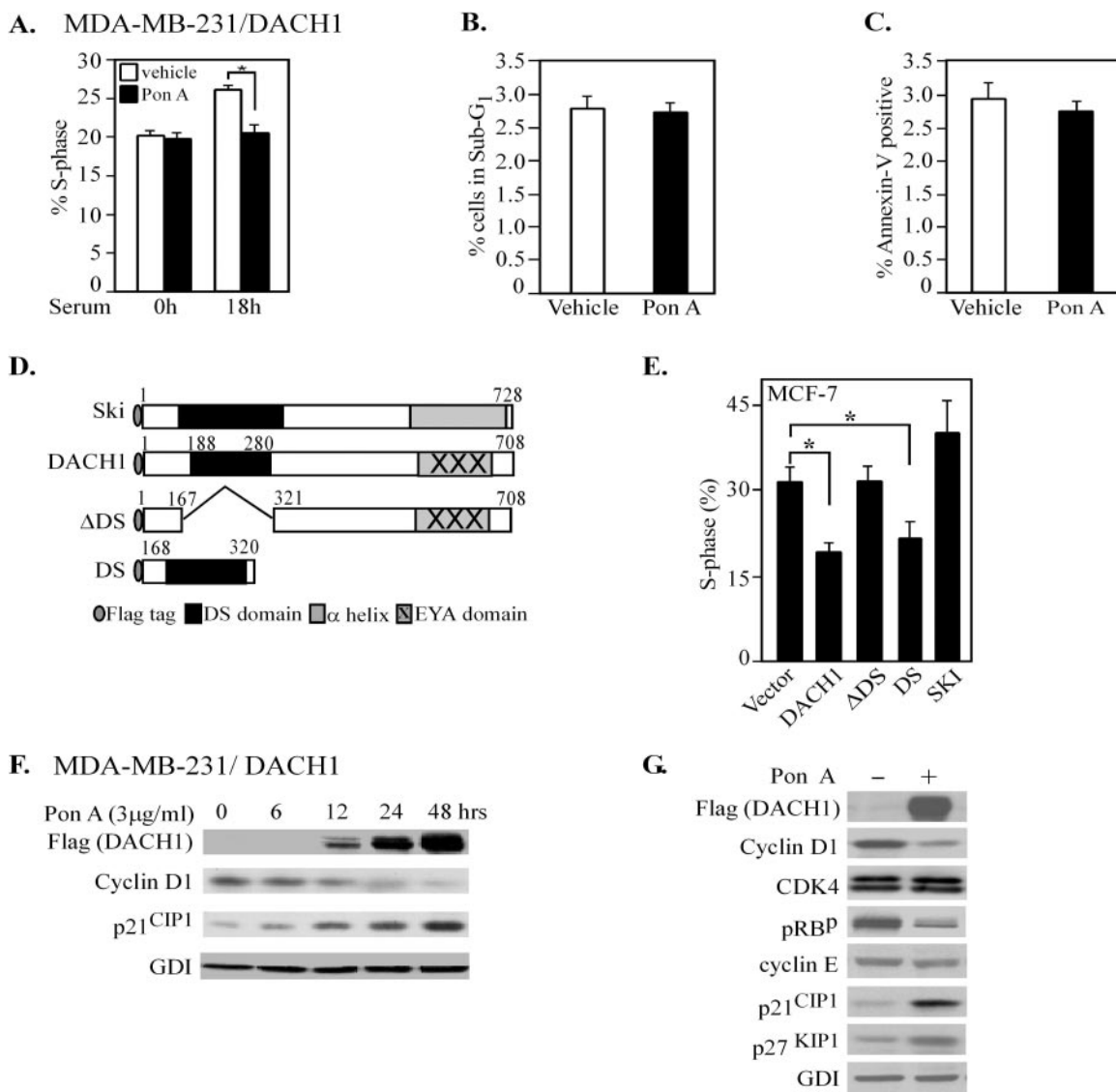


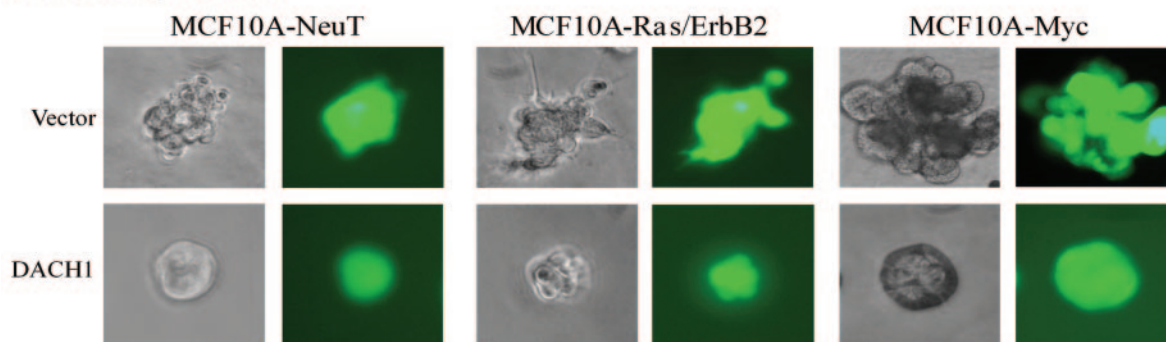
FIG. 2. *DACH1* inhibits DNA synthesis through the DS domain. (A) MDA-MB-231 cells expressing inducible *DACH1* were characterized for the effects of *DACH1* expression on serum-induced DNA synthesis. The effect of *DACH1* expression on S phase or apoptosis assessed by Sub-G₁ (B) and annexin V stain (C) is shown after 18 h of serum addition. The data are means ± the SEM for >5 separate experiments. (D) Schematic representation of *DACH1* expression vectors. (E) Cell cycle analysis of MCF-7 cells transfected with *DACH1* expression vectors. The percent MCF-7 cells in S phase is shown compared to the vector control. The data are mean change in S phase ± the SEM of *DACH1*-transfected MCF-7 cells (*n* = 4). (F) Dose-dependent induction of *DACH1* by ponasterone A and the corresponding cyclin D1 and p21^{CIP1} expression in MDA-MB-231 cells. (G) Cell-cycle-related protein abundance as determined by Western blotting in MDA-MB-231 cells after 48 h of ponasterone A treatment.

bition of DNA synthesis, Western blot analysis was conducted. Cells were treated with ponasterone A to induce expression of *DACH1*, and serial time points were analyzed. *DACH1* induction was observed at 12 h, contemporaneous with a reduction in cyclin D1 and an induction of p21^{CIP1}. The abundance of the protein loading control GDI was unchanged (Fig. 2F). To determine target genes involved in blocking serum-induced DNA synthesis, cells were serum starved and, after induction of *DACH1* with ponasterone A, the cells were treated with serum for 18 h. After 24 h of *DACH1* expression, the cyclin D1 levels were reduced 60% while increasing the abundance of p21^{CIP1} and p27^{KIP1} (Fig. 2G). A phospho-specific antibody

directed to the Ser⁷⁸⁰ site phosphorylation of the pRb protein showed a 60% reduction in pRb phosphorylation. Cyclin E and cdk4 levels were unchanged (Fig. 2G).

***DACH1* inhibits DNA synthesis and oncogene-induced invasive phenotype induced by Ras or Myc through distinct pathways.** The creation of genetically defined human cancer cells has defined with greater precision the regulatory pathways contributing to the tumorigenic phenotype (19, 23). MCF10A cells are a spontaneously immortalized human luminal epithelial cell line (56). When grown on basement membrane, the cultures of MCF10A cells form growth-arrested three-dimensional structures, termed acini. These structures, comprised of

A. 3D matrigel culture



B. Confocal

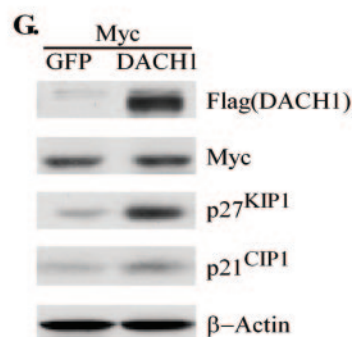
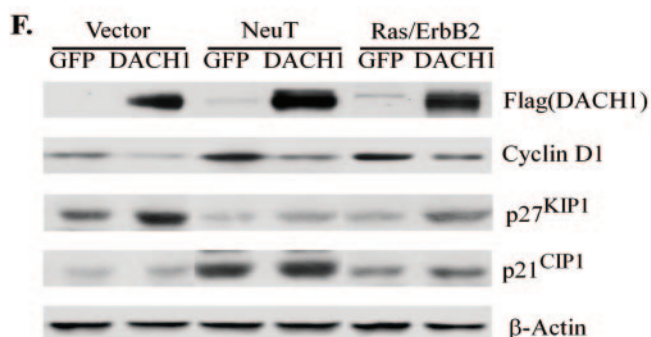
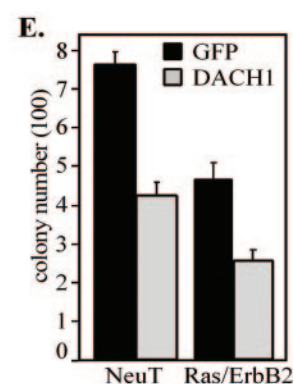
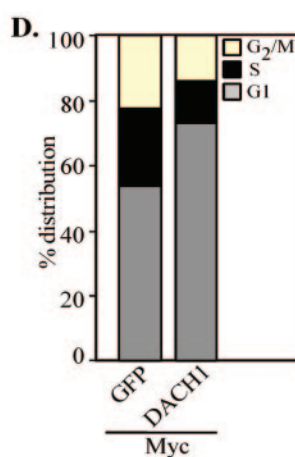
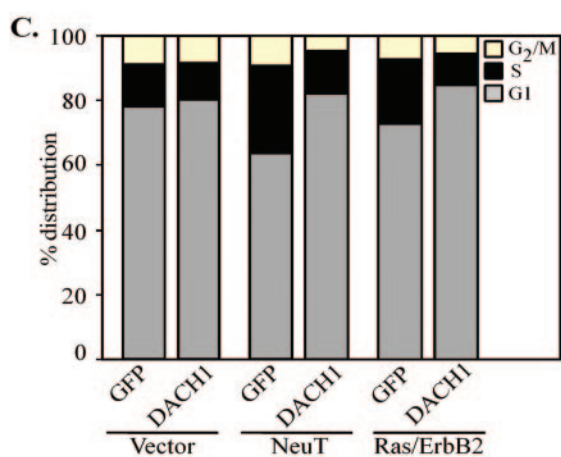
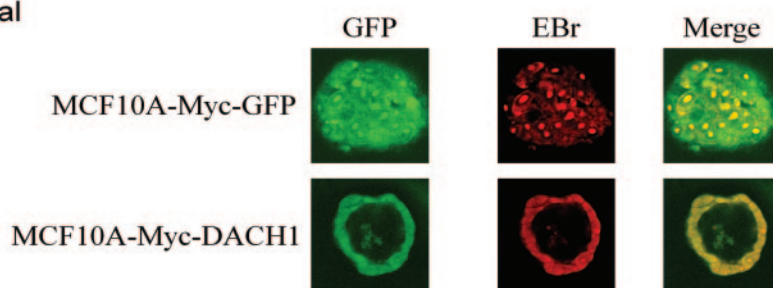


FIG. 3. DACH1 inhibits DNA synthesis induced by NeuT, Ras/ErbB2 or Myc. (A) The immortalized MCF10A cells were transfected with NeuT, Ras/ErbB2, or c-Myc. Cells were transfected with MSCV-IRES-GFP or MSCV-DACH1-IRES-GFP and selected through GFP FACS for analysis. Morphology of three-dimensional growth in Matrigel at 10 days was depicted by phase-contrast and fluorescence microscopy of acinar structures. (B) Confocal image of GFP and ethidium bromide staining after fixation. Transformed MCF10A cells grow as a solid ball in which the central acinar structure is filled with proliferating cells. The expression of DACH1 reverts the oncogenic morphology with a return to the nontransformed MCF10A phenotype with polarized epithelium and an acinar-like lumen structure. (C and D) Cell cycle distribution. (E to G) Colony formation assay results (E) and cell cycle control proteins as determined by Western blotting of transformed MCF10A cells with the indicated antibodies (F and G).

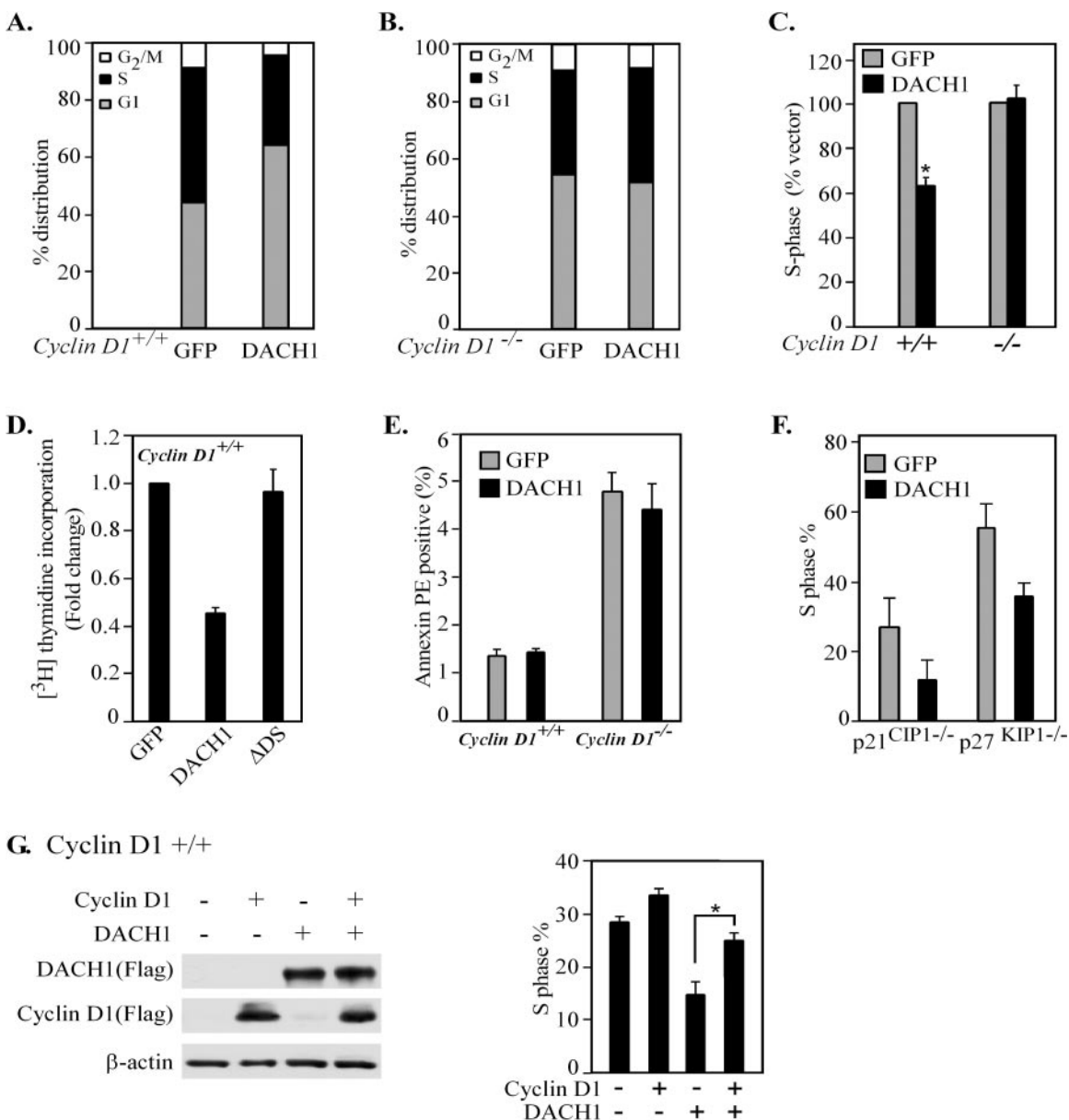


FIG. 4. DACH1 inhibition of DNA synthesis requires cyclin D1. (A to F) DACH1 regulation of cell cycle distribution by FACS analysis (A to C and F) or DNA synthesis using [³H]thymidine uptake (D) and apoptosis using annexin V staining in 3T3 cells derived from the MEFs of mice with either the *cyclin D1* (A to E) or the *p21*^{CIP1} or *p27*^{KIP1} (F) gene deleted compared to sibling controls of the same strain background. Cell cycle distribution was assessed using cells transduced with MSCV-IRES-GFP or MSCV-DACH1-IRES-GFP through GFP FACS sorting. Western blotting confirmed the expression of DACH1 with GDI as the loading control (see Fig. S2 in the supplemental material). (G) *cyclin D1*^{+/+} cells were transduced with expression vectors encoding Flag epitope-tagged cyclin D1 or DACH1. Western blot analysis demonstrated the expression of DACH1 and cyclin D1. FACS analyses demonstrated that cyclin D1 rescues the inhibition of DNA synthesis by DACH1.

a single layer of polarized epithelial cells surrounding a hollow lumen, resemble mammary acini and undergo morphological changes in response to transforming oncogenes (50, 56). The morphology of MCF10A cellular colony growth in three-dimensional Matrigel culture is characteristic for specific oncogenic pathways (9, 52). To define which oncogenic signaling pathways are inhibited by DACH1, MCF10A cells were transduced with expression vectors encoding oncogenic ErbB2 (NeuT), Ras, and ErbB2 (Ras/ErbB2) or c-Myc. These transformed cells were then transduced with viral expression vectors

encoding DACH1 (MSCV-IRES-GFP or MSCV-DACH1-IRES-GFP) and selected through fluorescence-activated cell sorting (FACS) of GFP-expressing cells. Analyses were conducted of morphology in Matrigel (Fig. 3A and B), cell cycle distribution (Fig. 3C and D), colony formation ability (Fig. 3E), and cell cycle control proteins abundance determined by Western blotting (Fig. 3F and G). MCF10A cells form acinus-like spheroids. MCF10A-NeuT cells grown in Matrigel formed complex acinus-like structures (39). The activation of ErbB2 in MCF10A cells is known to induce this multiacinar phe-

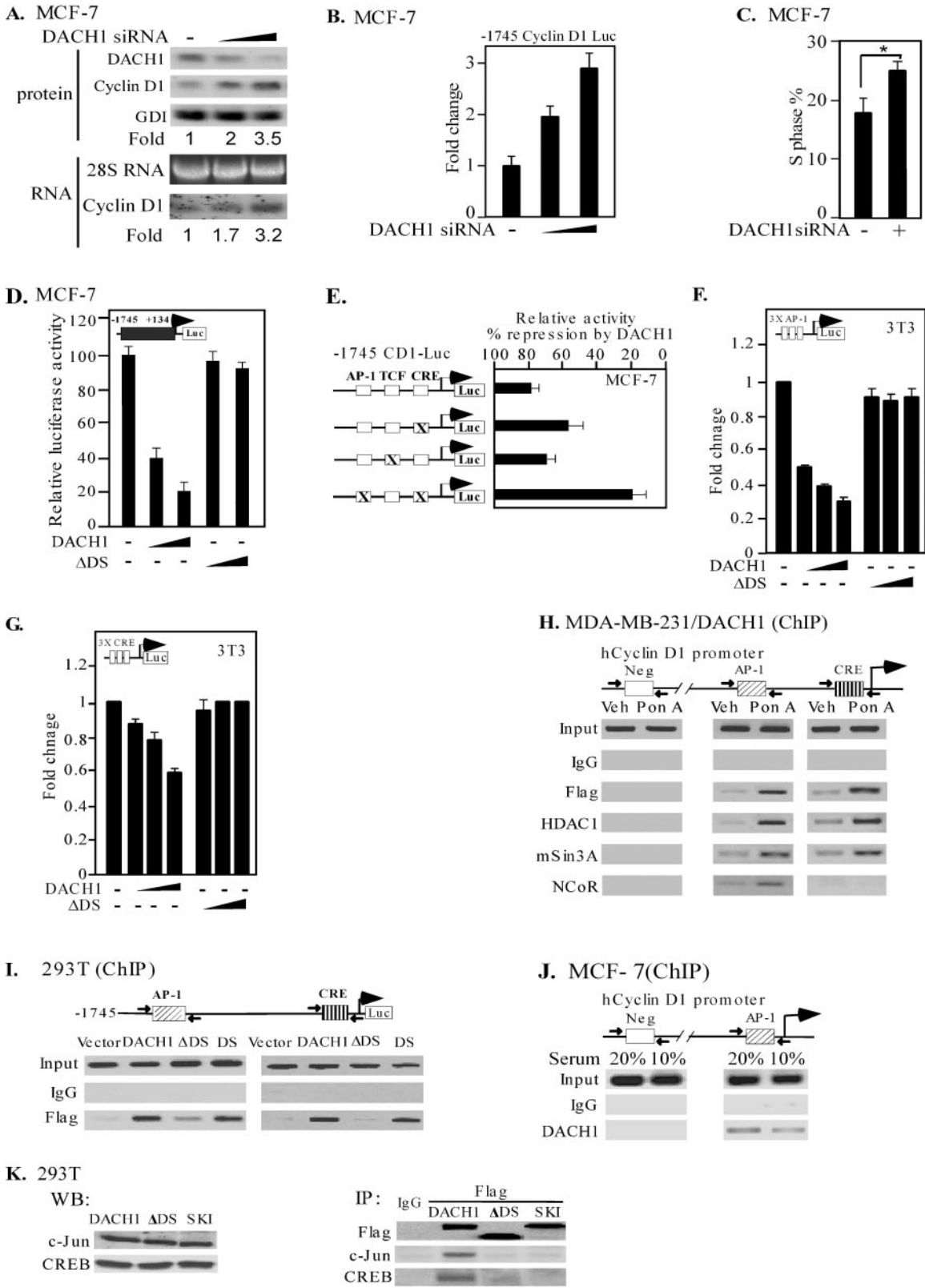


FIG. 5. DACH1 recruits HDAC to the AP-1/CRE site of the cyclin D1 promoter in the context of local chromatin. (A) MCF-7 cells treated with DACH1 or control siRNA. Endogenous cyclin D1 protein abundance and cyclin D1 mRNA level were detected; GDI was used as a loading control for Western blot analyses, and 18S RNA was used as a loading control for Northern blot analyses. (B) Cyclin D1 promoter activity assessed in MCF-7 cells transfected with DACH1 siRNA. Promoter activity is shown as the means \pm the SEM for six separate transfections. (C) Mean S-phase analyses of DACH1 siRNA transfected MCF-7 cells. (D and E) Cyclin D1 promoter activity in cells transfected with increasing amounts

notype through excessive cellular proliferation and changes in apicobasal polarization (17, 44). *DACH1* transduction of oncogenic NeuT transformed MCF10A cells abolished the formation of these multicellular structures, reversing the phenotype to the spherical morphology of parental MCF10A (Fig. 3A). The MCF10A-Ras/ErbB2 colonies consisted of multicellular spheroids in which individual cells protruded long spikes. *DACH1* transduction of these Ras/ErbB2 transformed MCF10A cells reversed the phenotype and abolished the formation of these spike structures (Fig. 3A). MCF10A-c-Myc cells grew as multicellular acinus-like structures, and *DACH1* transduction resulted in the formation of well-polarized spheroids with lumen formation (Fig. 3A and B). Collectively, these studies demonstrate that *DACH1* inhibits oncogene-induced morphological abnormalities in MCF10A cells.

DACH1 inhibited NeuT, Ras/ErbB2, and c-Myc-induced DNA synthesis as assessed by a reduction in the proportion of cells distributed within the S phase of the cell cycle (Fig. 3C and D). *DACH1* reduced primarily the S phase but also the G₂/M fraction of c-Myc-expressing MCF10A cells. MCF10A cells transformed by oncogenic NeuT or Ras/ErbB2 gain the capacity to sustain contact-independent growth and form colonies in soft agar. *DACH1* blocked colony formation induced by either NeuT or Ras/ErbB2 (Fig. 3E). To determine the components of the cell cycle regulated by *DACH1* that may contribute to the inhibition of cellular growth, Western blot analyses were conducted of the oncogene-transduced MCF10A cells. We compared the effect of transducing the MCF10A cells to the effect of control vector. In randomly cycling cells, the abundance of cyclin D1 encodes a rate-limiting step in DNA synthesis (46, 68). In parental MCF10A, cyclin D1 abundance was reduced by *DACH1*, and p27^{KIP1} was induced (Fig. 3F) without a significant change in S-phase distribution. In NeuT or Ras/ErbB2-transformed MCF10A cells, cyclin D1 and p21^{CIP1} abundance was induced, a finding consistent with previous studies in which Raf induced cyclin D1 in MCF10A cells (50). DNA synthesis was inhibited by *DACH1*, associated with a reduction in cyclin D1 and induction of p27^{KIP1}. c-Myc repressed p21^{CIP1} and p27^{KIP1}, and *DACH1* partially derepressed p21^{CIP1} and p27^{KIP1} abundance but did not affect cyclin D1 expression (Fig. 3G). The analysis of *DACH1* in oncogene-transduced MCF10A cells indicates that *DACH1* consistently reduces oncogene-induced expression of cyclin D1. Collectively, these studies show that *DACH1* inhibits oncogene-induced DNA synthesis, malignant morphological changes in Matrigel, and contact-independent growth as assessed by colony formation.

In view of the finding that *DACH1* inhibited Ras and oncogenic ErbB2 induced cyclin D1 expression, we investigated the requirement for cyclin D1 in *DACH1*-mediated inhibition of DNA synthesis. To examine further the role of cyclin D1, p21^{CIP1} and p27^{KIP1} in *DACH1*-mediated cell cycle arrest, 3T3 cells were derived from mouse embryonic fibroblasts (MEFs) of mice with deletions of either the *cyclin D1*, the p21^{CIP1}, or the p27^{KIP1} gene, and a comparison made with MEFs from sibling controls of the same strain background for each line. The cell cycle distribution was assessed by using cells transduced with MSCV-IRES-GFP or MSCV-*DACH1*-IRES-GFP through GFP FACS sorting. Western blot analysis confirmed the expression of *DACH1*. GDI was used as a protein loading control (see Fig. S2 in the supplemental material). *DACH1* inhibited DNA synthesis in *cyclin D1*^{+/+} but not *cyclin D1*^{-/-} cells assessed by either FACS analysis or tritiated thymidine uptake, and the inhibition of DNA synthesis by *DACH1* required the *DACH1* DS domain (Fig. 4A to D). Apoptosis rates were unaffected by *DACH1* in either *cyclin D1*^{+/+} or *cyclin D1*^{-/-} cells (Fig. 4E). Deletion of p21^{CIP1} or p27^{KIP1} altered the relative distribution of cells within the cell cycle. The proportion of cells in S phase inhibited by *DACH1* in 3T3 cells with either the p21^{CIP1} or p27^{KIP1} gene deleted (Fig. 4F) was similar to that of the parental control cells. These studies indicate that cyclin D1, but not p21^{CIP1} or p27^{KIP1}, are required for *DACH1*-mediated inhibition of DNA synthesis in 3T3 fibroblasts. To further test the role of cyclin D1 in *DACH1*-induced growth arrest, *cyclin D1*^{+/+} or *cyclin D1*^{-/-} cells were infected with Flag-tagged cyclin D1, *DACH1*, or both cyclin D1 and *DACH1*, and Western blotting confirmed the expression of exogenous cyclin D1 and *DACH1*. Cell cycle analyses demonstrated the rescue of *DACH1*-mediated growth inhibition upon the expression of cyclin D1 (Fig. 4G).

***DACH1* repression of the cyclin D1 promoter requires the AP-1/CRE DNA-binding site.** *DACH1* inhibited DNA synthesis in the breast cancer epithelial cell lines MCF-7 and MDA-MB-231 and in NIH 3T3 cells. We sought to determine whether physiological levels of *DACH1* regulated cyclin D1 abundance. The administration of siRNA directed to endogenous *DACH1* in MCF-7 cells reduced *DACH1* abundance and induced cyclin D1 protein and mRNA abundance (~3-fold), indicating physiological levels of *DACH1* repress cyclin D1 (Fig. 5A). Since *DACH1* expression inhibited MCF-7 cell DNA synthesis (Fig. 2) and the abundance of cyclin D1 is rate limiting in DNA synthesis of MCF-7 cells (68), the role of *DACH1* in regulating cyclin D1 was next assessed in these cells. To examine the role of endogenous *DACH1* in regulating

of expression vector encoding *DACH1* or a *DACH1* mutant with the DS domain deleted (Δ DS) in MCF-7 cells. The vector control value was set as 100. Cyclin D1 promoter point mutants were compared for repression by *DACH1*. The data (means \pm the SEM) are shown as the percent repression compared to the vector for eight separate experiments. (F and G) Heterologous reporters encoding the cyclin D1 AP-1 and CRE sites linked to the luciferase reporter gene were assessed for regulation by *DACH1* in 3T3 cells. (H) ChIP assay using oligonucleotides directed to DNA sequences of the endogenous human cyclin D1 promoter AP-1 or CRE sites or a control DNA sequence at -3060. MDA-MB-231 cells expressing inducible Flag-tagged *DACH1* are shown treated with PonA or vehicle. (I) 293T cells cotransfected with -1745 cyclin D1 promoter and Flag-tagged wild-type or mutant *DACH1* expression vectors. ChIP assays were conducted using oligonucleotides directed to DNA sequences of the human cyclin D1 AP-1 or CRE sites. (J) ChIP analyses of endogenous *DACH1* binding to the human cyclin D1 promoter comparing the -3060 region (negative) and the cyclin D1 promoter AP-1 site. (K) 293T cells were transfected with control or *DACH1* or Ski expression vector and analyzed by Western blotting to detect c-Jun and CREB. Immunoprecipitation was conducted with antibodies directed to the Flag epitope of the *DACH1*, Δ DS, and Ski expression vectors and immunoprecipitation analyzed for c-Jun or CREB.

the cyclin D1 promoter, DACH1 siRNA was compared to control siRNA for its effect in regulating the cyclin D1 promoter. DACH1 siRNA induced the full-length cyclin D1 promoter threefold (Fig. 5B). Consistent with previous studies demonstrating that cyclin D1 abundance is rate limiting in MCF-7 cell DNA synthesis (40), DACH1 siRNA induced the S phase in MCF-7 cells (Fig. 5C). The cyclin D1 promoter was repressed in a dose-dependent manner by the expression of DACH1, and deletion of the DACH1 DS domain abrogated repression of the cyclin D1 promoter (Fig. 5D). In contrast to DACH1, the related Ski protein did not inhibit cyclin D1 promoter activity (not shown). Mutation of the AP-1 and CRE site of the cyclin D1 promoter reduced DACH1 repression by 80%, suggesting that these sequences together played a key role in repression by DACH1 (Fig. 5E). DACH1 repressed cyclin D1 promoter activity in 293T and NIH 3T3 cells and again repression required both the AP-1 and the CRE sites (data not shown). The cyclin D1 promoter AP-1 and CRE sites serve as both basal level and inducible enhancer elements; therefore, experiments were conducted to determine whether these elements were sufficient for repression by DACH1. The CRE and AP-1 sites were sufficient for repression by DACH1, and repression required the DACH1 DS domain (Fig. 5F and G).

To determine the mechanisms by which DACH1 repressed the cyclin D1 promoter, ChIP assays were conducted to examine the occupancy of DACH1 at the endogenous human cyclin D1 promoter in human breast cancer cells. Comparison was made between the inducible DACH1 stable cell line and the vehicle control. Using oligonucleotides directed to the endogenous human cyclin D1 AP-1 and CRE sites, ChIP assays conducted with the anti-Flag antibody demonstrated the presence of DACH1 in the context of the local chromatin structure of the endogenous human cyclin D1 promoter upon the induction of DACH1 expression (Fig. 5H). HDAC1, mSin3A, and NCoR were recruited by DACH1 to form a multimeric repressor complex at the AP-1 site of the endogenous human cyclin D1 promoter. Amplification was not observed using oligonucleotides directed toward sequences within the -3060 region of the cyclin D1 promoter. No amplification was observed using either the vehicle control cells or immunoglobulin G in the ponasterone-inducible DACH1 stable cell line (Fig. 5H). To determine which region of the DACH1 protein was necessary for the formation of a DNA-associated complex at the cyclin D1 promoter in the context of its local chromatin structure, 293T cells were transfected with the human cyclin D1 luciferase promoter and wild-type or mutant DACH1 expression vectors. ChIP assay using the Flag antibody demonstrated that both wild-type DACH1 and the DS domain alone were recruited to the AP-1 and the CRE site, whereas the DS domain deleted mutant was defective in recruitment to the cyclin D1 promoter (Fig. 5I). Since DACH1 regulated DNA synthesis and cyclin D1 expression and promoter activity, we sought to determine whether endogenous DACH1 bound the cyclin D1 promoter in the context of local chromatin. Endogenous DACH1 was associated with the cyclin D1 promoter AP-1 site but was not identified at the -3060 region. Serum addition did not significantly alter DACH1 occupancy in ChIP assays (Fig. 5J).

Since DACH1 was recruited in a DNA sequence-specific

manner to the cyclin D1 AP-1/CRE site and is not thought to bind a defined DNA sequence directly, we sought to determine whether DACH1 formed a complex with AP-1 proteins in cultured cells. Immunoprecipitation was conducted with the Flag antibody using cell extracts derived from 293T cells transfected with DACH1 or Ski, normalized for equal amounts of c-Jun and CREB by Western blotting (Fig. 5K). Equal amounts of DACH1 and c-Jun were coprecipitated with the Flag antibody; however, only DACH1 coprecipitated c-Jun and CREB. Deletion of the DACH1 DS domain abolished binding to c-Jun and CREB (Fig. 5K). These findings are consistent with a model in which DACH1 binds c-Jun and is recruited to an AP-1 site in the context of its local chromatin structure, requiring the DS domain of DACH1 to recruit the corepressors HDAC1, NCoR, and mSin3A to repress cyclin D1 gene expression.

DACH1 expression is regulated during mammary gland development and reduced in metastatic breast cancer. In view of the finding that endogenous DACH1 inhibited cyclin D1 expression and that DACH1 expression inhibited DNA synthesis in breast cancer cell lines, we investigated the distribution and physiological regulation of DACH1 in human and murine breast epithelium. DACH1 expression was examined in breast epithelium and breast cancer cell lines by immunohistochemical staining and Western blotting with a DACH1-specific antibody. The specificity of the antibody was confirmed through Western blotting of 293T cells transfected with expression vectors encoding the DACH1 wild-type or the DACH1 Δ DS domain (Fig. 6A). Immunoreactive DACH1 was detected in MCF-7, MCF-10A, and MDA-MB-231 cells and several other breast cancer cell lines (Fig. 6B). As a form of positive control for immunohistochemical staining, DACH1 was identified in the embryonic eye as previously described (8) (Fig. 6C). To determine whether DACH1 expression is regulated during normal physiological changes in the breast, DACH1 immunohistochemistry was assessed during murine mammary gland development and lactation. DACH1 expression was detectable in normal virgin mammary gland, increased at 10 days pregnancy, and decreased in lactation (day 10) and involution (day 10) (Fig. 6D). The homeodomain protein Six1, which functions in developmental networks including eye development in *Drosophila*, is expressed in mammary carcinoma but is absent or detected at low levels only in normal mammary tissues (15, 21). Six1 expression, although detectable in breast cancers, was weakly expressed in the normal murine mammary epithelium (see Fig. S3 in the supplemental material), as recently shown (15). The expression of Six1 was dramatically increased during pregnancy, coinciding with high expression of cyclin D1. We tested whether Six1 was capable of inducing cyclin D1. HEK293T cells were transiently transfected with the cyclin D1 promoter luciferase reporter and expression vectors encoding either Six1 or DACH1. A dose-dependent activation of the cyclin D1 promoter by Six1 and repression by DACH1 were observed (see Fig. S3B in the supplemental material), a finding consistent with recent findings that Six1 induces cyclin D1 promoter in a rhabdomyosarcoma cell line (65). Collectively, these findings are consistent with a model in which the relative abundance of the Eya/Six complex are under physiological control and may together contribute to the relative abundance of cyclin D1 in a given cell type.

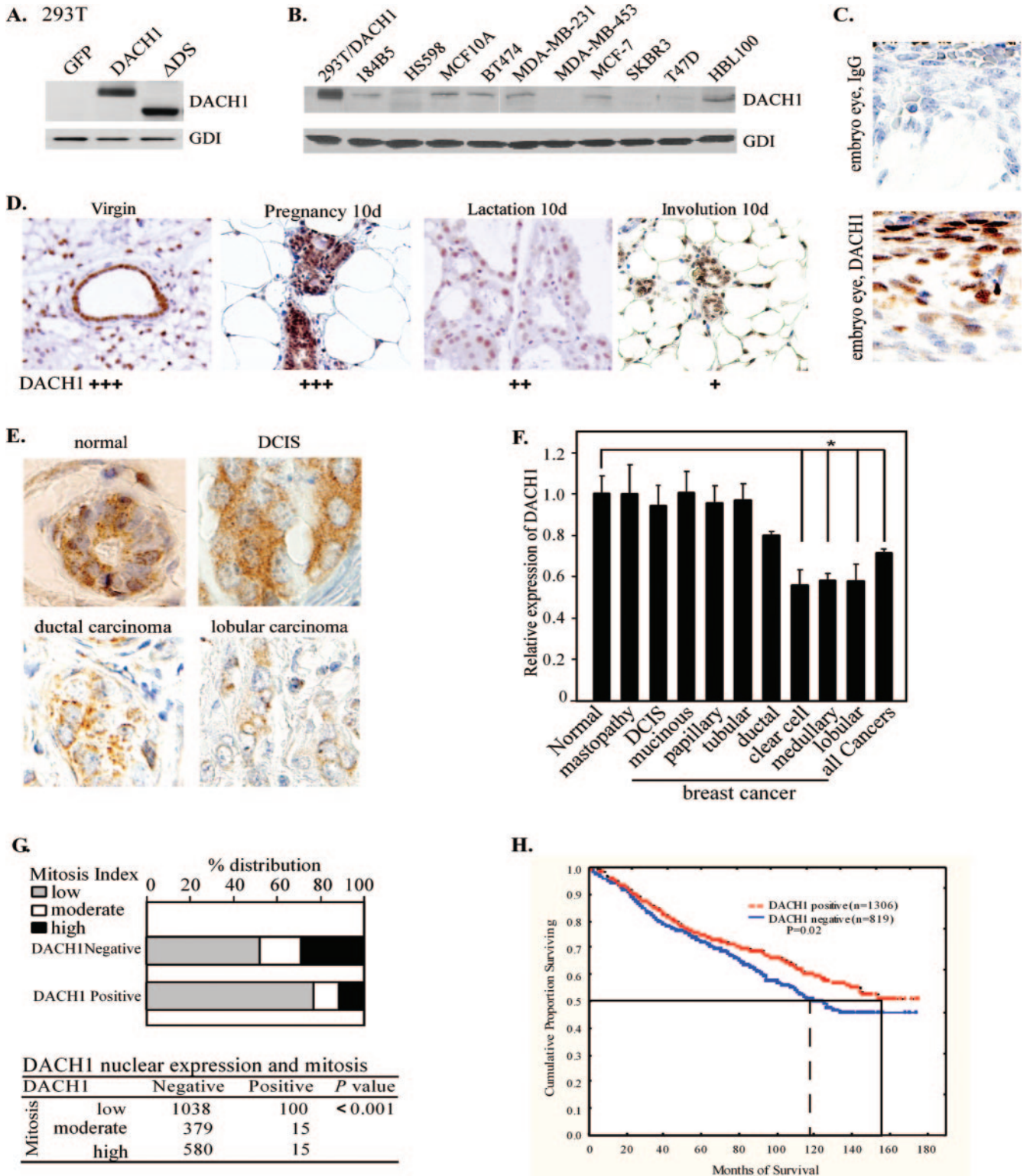


FIG. 6. DACH1 expression in human breast cancer cell lines, mouse mammary gland, and breast carcinoma tissues. (A) 293T cells were transiently transfected with control or DACH1 expression vector and analyzed by Western blotting with a DACH1 specific antibody. (B) Western blot for DACH1 abundance of breast epithelial cell lines, breast cancer cell lines, and control extracts of 293T cells transfected with DACH1 (lane 1). GDI was used as a loading control. (C) DACH1 expression in mouse embryonic eye was used as a positive control. (D) DACH1 immunohistochemistry during the murine mammary gland development of the virgin, at pregnancy (day 10), at lactation (day 10), and at involution (day 10). (E) Representative DACH1 expression in normal breast epithelium, ductal carcinoma in situ, or invasive human breast cancer samples using DACH1 specific antibodies. (F) Analysis of DACH1 expression in subcategory of human breast tissue microarray (*, $P < 0.05$). (G) Relationship of DACH1 expression to mitosis index. (H) DACH1 expression and prognosis of human breast carcinoma ($n = 2,125$ patients).

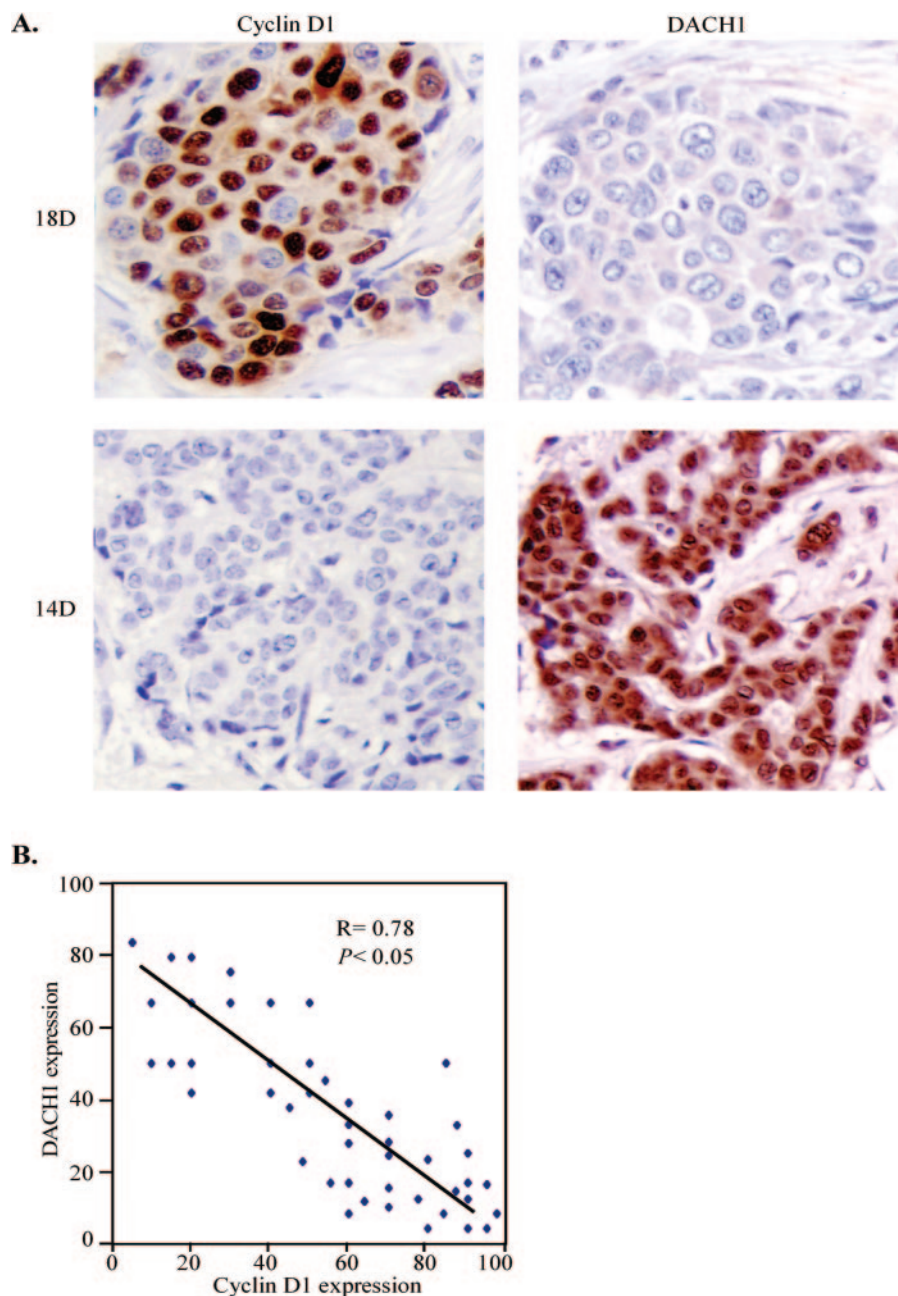


FIG. 7. Relative expression of DACH1 and cyclin D1. (A) Representative immunostaining of cyclin D1 and DACH1 in paired breast cancer tissues. (B) Inverse correlation of cyclin D1 and DACH1 in breast cancers ($n = 46$).

In order to clarify the DACH1 expression profile during the development of breast cancer and its prognostic significance, we analyzed DACH1 expression in a breast tissue microarray containing normal mammary epithelial cells, mastopathy, carcinoma in situ, and over 2,100 breast cancer samples for which detailed clinical-pathological characteristics and follow-up information were available. The results indicated that DACH1 was expressed in normal mammary epithelial ($n = 81$) but was significantly reduced in breast cancer ($n = 2117$). Similar DACH1 immunostaining was seen in mastopathy ($n = 17$) and ductal carcinoma and lobular carcinoma in situ ($n = 63$) com-

pared to normal breast tissue (Fig. 6E; see also Fig. S4 in the supplemental material). The separate analysis of different tumor subtypes revealed significantly reduced expression in clear cell carcinoma or cribriform carcinoma ($n = 75$), lobular carcinoma ($n = 300$), and medullary carcinoma ($n = 58$) (Fig. 6F). DACH1 expression was inversely related with tumor diameter ($P < 0.001$), local tumor stage ($P < 0.0001$), and nodal metastasis ($P = 0.04$). Importantly, nuclear expression of DACH1 correlated inversely with mitosis ($P < 0.001$). The finding that DACH1 expression was reduced in invasive breast cancer compared to ductal carcinoma in situ prompted us to examine

additional breast cancer tissue arrays that allowed direct comparison of invasive versus noninvasive breast cancer. Reduced *DACH1* expression in invasive human breast cancer was confirmed with two additional distinct *DACH1* antibodies on two additional distinct arrays (see Fig. S5 and S6 in the supplemental material). Semiquantitative immunostaining for *DACH1* of invasive breast cancers ($n = 124$) demonstrated less intense and predominantly cytoplasmic, rather than nuclear, staining compared to either the normal breast epithelium ($n = 23$) or ductal carcinoma in situ ($n = 19$) (see Fig. S6 in the supplemental material). Within the invasive breast cancer group, immunostaining was less intense among patients with lymph node involvement (see Fig. S6 in the supplemental material). Analysis of the prognostic significance of *DACH1* expression in breast cancers demonstrated that *DACH1* expression is associated with favorable survival time in all cancer samples (Fig. 6G). Kaplan-Meier survival curves showed significantly better survival for *DACH1*-positive versus *DACH1*-negative patients ($P = 0.02$). This finding was confirmed by Cox proportional hazards regression, with only *DACH1* as a predictor of survival. In univariable modes, *DACH1*-positive patients were estimated to have an $\sim 20\%$ lower mortality than *DACH1*-negative patients (hazard ratio = 0.81, $P = 0.005$). Thus, *DACH1* levels are regulated during normal mammary gland development in the epithelial cells, with reduced expression in invasive human breast cancer. When examined collectively across all breast cancer types, *DACH1* abundance is significantly inversely correlated with patient survival.

Since *DACH1* repressed cyclin D1 expression in cultured cells, we examined the relationship between *DACH1* and cyclin D1 expression by costaining for both proteins in human breast cancer samples. The expression of *DACH1* and cyclin D1 was detected by immunohistochemistry. The semiquantitative analyses of 46 breast cancer tissues revealed that nuclear staining of *DACH1* was inversely correlated with the expression of cyclin D1 (Fig. 7).

DISCUSSION

Studies in *Drosophila* have identified a genetic network consisting of the *eyeless*, *sine oculis* (*so*), DNA-binding homeodomain factor, and the *eyes absent* (*eya*) and *dachshund* (*dac*) cofactors. Remarkably, each of these genes is sufficient for eye determination and function together in a molecular network during development. The mechanism by which *Dachshund* regulates cellular proliferation and differentiation was previously unknown. In the present studies, *Dach1* expression was regulated during normal murine mammary gland development, and *DACH1* was expressed in human breast cancer with reduced expression in metastatic breast cancer. *DACH1* inhibited both ErbB2 and c-Myc-induced oncogenic morphogenesis in three-dimensional basement membrane cultures. *DACH1* blocked contact-independent growth of human breast cancer cells in vivo. *DACH1* inhibited ErbB2- and c-Myc-induced DNA synthesis without affecting cellular apoptosis. *DACH1*-dependent inhibition of DNA synthesis involved cyclin D1 and p21^{CIP1}. Together, these studies demonstrate that the *Drosophila* cell fate determination network directly intersects the genetic determinants of cell cycle control through cyclin D1/p21^{CIP1}.

Cyclin D1 is sufficient for mammary tumorigenesis in trans-

genic mice, collaborates in oncogenesis through pRb inactivation, and functions as a common downstream target induced by oncogenic signals (48, 64). In the present study, cyclin D1 abundance was repressed by *DACH1*. Reduction in *DACH1* by siRNA induced cyclin D1 abundance and S-phase progression, suggesting that endogenous *DACH1* is a physiological regulator of cyclin D1 in human breast cancer epithelial cells. Inhibition of DNA synthesis by *DACH1* was abrogated by genetic deletion of *cyclin D1*, demonstrating the *cyclin D1* gene is a functional genetic target of *DACH1* function in mammalian cells. *DACH1* reduced the proportion of MCF-7 cells in S phase. *DACH1* inhibition of DNA synthesis, cyclin D1 abundance, and promoter activity required the conserved DS domain. Cyclin D1 abundance is limiting in the induction of MCF-7 cell S-phase progression, DNA synthesis, and cellular proliferation (46, 68). The cyclin D1 gene is known to encode a labile, growth factor- and oncogene-inducible protein required for mammary tumorigenesis induced by Ras and ErbB2. The present study extends these findings, demonstrating that cyclin D1 functions at a genetic interface with the pathways regulating cell fate determination.

The present study identified a novel mechanism by which *DACH1* regulates gene expression. Although a direct DNA-binding capacity for *DACH1* has not been identified, *DACH1* is known to form part of a DNA-binding complex, with the So/Six homeodomain proteins providing the DNA specific binding capacity (37). We now show that *DACH1* was recruited within chromatin complexes, with DNA sequence-specific binding capabilities provided by c-Jun or CREB. These findings raise the possibility that *DACH1* may function as one of the previously hypothesized c-Jun repressor binding proteins (60). The DS domain of *DACH1*, which is predicted to form a helix-turn-helix structure, was required for recruitment to AP-1/CRE sites. Proteins conveying intrinsic or associated histone deacetylase (HDAC) activity (NCoR, Sin3A, and HDAC1) were also recruited with *DACH1* to the sites of *DACH1*-dependent repression. The present study extends the mechanisms by which the cell fate determination factor complex regulates gene transcription by demonstrating the ability of *DACH1* to use AP-1/CRE binding proteins as a molecular scaffold to recruit HDAC-containing repressor complexes to these sites. These findings are important in identifying the molecular mechanisms by which *DACH1* may regulate the molecular genetic targets previously identified by high-density microarray analysis (62).

The three-dimensional culture of MCF10A on a reconstituted basement membrane results in polarized growth-arrested acinar-like spheroids, which are thought to recapitulate aspects of glandular architecture in vivo (18, 30). Oncogenic disruption of this morphogenetic process has identified distinct changes induced by different oncogenes. *DACH1* reversed the ErbB2- and c-Myc-induced morphological changes of MCF10A cells in Matrigel. Furthermore, *DACH1* inhibited DNA synthesis in cell lines harboring distinct activating mechanisms, through mutation in K-Ras (MDA-MB-231) or amplification of c-Myc (MCF-7 cells) (32). *DACH1* inhibition of MCF10A-c-Myc-induced DNA synthesis correlated with the induction of p21^{CIP1}, consistent with previous findings that c-Myc promotes growth in part through p21^{CIP1} (14) and Smad-dependent mechanisms (20).

c-Jun contributes to cellular proliferation and tumorigenesis (53). *c-Jun* promotes G₁ phase cell cycle progression, and immunoneutralizing antibodies to c-Jun inhibit DNA synthesis (11). MEFs deficient in c-Jun exhibit a severe defect in proliferation due to extension of the G₁ transition time (49). DACH1 repressed AP-1 activity, associating with c-Jun in the context of local promoter chromatin. DACH1 purified whole-cell extracts contained AP-1 binding activity, and DACH1 coprecipitated c-Jun through the DS domain. DACH1 repression of cyclin D1 expression, DNA synthesis, and cell survival are consistent with previous findings that AP-1 induces cyclin D1 transcription (4, 12, 61). Cyclin D1 contributes to cell survival since *cyclin D1*^{-/-} MEF cells display both reduced rates of G₁ phase progression and increased cellular apoptosis (3). DACH1, but not the related Ski, blocked serum-induced DNA synthesis in both mammary epithelial cells and in fibroblasts, demonstrating dissociable functions of these homologous proteins. The current model suggests DACH1, like Sno/Ski and NCoR (41, 57, 63), is recruited with DNA-binding proteins. DACH1, but not Ski, repressed the transcription of *c-Jun*. Recruitment to distinct DNA-binding proteins through the DS domain may contribute to the transcriptional specificity of the DACH1 versus Ski complexes (41).

Several recent findings suggest the RD pathway may regulate aberrant cellular growth and metastasis. Six1 expression is enhanced in metastatic rhabdomyosarcoma and contributes to cellular migration (66). Six6 functions as a tissue-specific repressor in association with Dach corepressors through Six6 binding sites (37), and the EWS/NOR1 translocation, implicated in extraskeletal myxoid chondrosarcoma, is repressed by Six3 (34). It has been hypothesized that Six1, which regulates DNA-damage-induced G₂-phase arrest, may regulate migration through *Lbx1h* (66). In view of the reduction in DACH1 in metastatic human breast cancer and findings that cyclin D1 promotes cellular migration, through altering the adhesion and phosphorylation of tyrosine-phosphorylated paxillin (38, 45), it will be of interest to determine whether DACH1 contributes to cellular migration. The accumulating evidence that the RD pathway contributes to tumorigenesis through intersecting cell cycle control raises the possibility that this pathway may be a useful new therapeutic prospect.

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