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# Cell Fate Determination Factor DACH1 Inhibits c-Jun–induced Contact-independent Growth

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The cell fate determination factor DACH1 plays a key role in cellular differentiation in metazoans. DACH1 is engaged in multiple context-dependent complexes that activate or repress transcription. DACH1 can be recruited to DNA via the Six1/Eya bipartite transcription (DNA binding/coactivator) complex. c-Jun is a critical component of the activator protein (AP)-1 transcription factor complex and can promote contact-independent growth. Herein, DACH1 inhibited c-Jun–induced DNA synthesis and cellular proliferation. Excision of c-Jun with Cre recombinase, in *c-jun<sup>fl/fl</sup>* 3T3 cells, abrogated DACH1-mediated inhibition of DNA synthesis. c-Jun expression rescued DACH1-mediated inhibition of cellular proliferation. DACH1 inhibited induction of c-Jun by physiological stimuli and repressed *c-jun* target genes (cyclin A,  $\beta$ -PAK, and stathmin). DACH1 bound c-Jun and inhibited AP-1 transcriptional activity. *c-jun* and *c-fos* were transcriptionally repressed by DACH1, requiring the conserved N-terminal (dac and ski/sno [DS]) domain. *c-fos* transcriptional repression by DACH1 requires the SRF site of the *c-fos* promoter. DACH1 inhibited c-Jun transactivation through the  $\delta$  domain of c-Jun. DACH1 coprecipitated the histone deacetylase proteins (HDAC1, HDAC2, and NCoR), providing a mechanism by which DACH1 represses c-Jun activity through the conserved  $\delta$  domain. An oncogenic v-Jun deleted of the  $\delta$  domain was resistant to DACH1 repression. Collectively, these studies demonstrate a novel mechanism by which DACH1 blocks c-Jun-mediated contact-independent growth through repressing the c-Jun  $\delta$  domain.

## INTRODUCTION

Homo- or heterodimeric transcription factors with basic region-leucine zipper structure, including Jun, Fos, ATF, and Maf subfamilies, regulate transcription of activator protein (AP)-1-responsive genes in a DNA sequence-specific manner. c-Jun encodes a critical component of the AP-1 transcription factor complex. Proliferative signals induce expression of both c-Fos and c-Jun (Karin *et al.*, 1997; Schreiber *et al.*, 1999). Mice genetically engineered to abolish either c-Jun or c-Jun NH<sub>2</sub>-terminal kinase (JNK) activity results in embryonic lethality (Jochum *et al.*, 2001). Of the four mitogen-activated protein kinase (MAPK) subfamilies, c-Jun activity is regulated primarily by Jun kinase (Morton *et al.*, 2003). The abundance of c-Jun is regulated by transcriptional induction and subsequent degradation through ubiquitination (Angel and Karin, 1991; Treier *et al.*, 1994; Fuchs *et al.*, 1996; Musti *et al.*, 1997).

The c-Jun protein structure consists of multiple functional domains, including an amino-terminal transactivation domain, a regulatory ( $\delta$  domain), a carboxy-terminal basic

DNA binding domain, and a leucine zipper protein dimerization domain. The  $\delta$  domain is critical for transcriptional activation by c-Jun. One of the first reported functions of the  $\delta$  domain was its engagement of cell type-specific inhibitors of c-Jun (Bohmann and Tjian, 1989; Baichwal and Tjian, 1990; Baichwal *et al.*, 1991). JNK docks to c-Jun on residues within the  $\delta$  domain (Kallunki *et al.*, 1995, 1996). Phosphorylation of c-Jun is thought to facilitate interaction of the c-Jun/AP-1 complex with coactivators. Transcriptional coactivators encoding histone acetyl transferase (HAT) activity, such as the CREB-binding protein (CBP) coactivator, bind to c-Jun facilitating the interaction between the AP-1 complex and the basal transcriptional machinery (Mayr and Montminy, 2001). JNK-mediated phosphorylation also accelerates c-Jun degradation by allowing recognition of the E3 ligase from the Fbw 7-containing Skp/Culin/F-box protein complex. In addition, JNK enhances activity of the E3 ligase, promoting degradation of c-Jun and JunB (Gao *et al.*, 2004; Nateri *et al.*, 2004).

c-Jun contributes to contact-independent growth and is essential for the development of chemically induced tumors in mice (Eferl *et al.*, 2003). Both cellular Jun (c-Jun) and viral Jun (v-Jun) induce oncogenic transformation (Vogt, 2001). The retrovirally transduced allele of *c-Jun*, *v-Jun*, induces fibrosarcoma in chickens (for review, see Vogt, 2001). Oncogenic *v-jun* encodes a protein with complete deletion of the  $\delta$  domain and fails to bind JNK. The role of the  $\delta$  domain

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itself versus JNK phosphorylation sites within the  $\delta$  domain, in cell cycle control, cellular proliferation, and oncogenesis is complex. Although c-Jun promotes G<sub>1</sub>/S phase progression independently of its phosphorylation status (Wisdom *et al.*, 1999), c-Jun phosphorylation of serines 63 and 73 were required for Ha-Ras-induced cellular transformation in some (Smeal *et al.*, 1991), but not all studies (Kennedy *et al.*, 2003). Finally, although point mutation analysis demonstrated the oncogenic effects of the  $\delta$  domain deletion can be uncoupled from JNK signaling (Sprowles and Wisdom, 2003), mice and cells harboring a mutant allele of *c-jun* show reduced tumorigenesis by activated Ras signaling (Bannister *et al.*, 1991).

A variety of mechanisms attenuate c-Jun-mediated activity (Schutte *et al.*, 1989). Factors regulating c-jun stability inhibit c-Jun function through reducing its abundance. These factors include macrophage migration inhibitory factor (MIF) (Kleemann *et al.*, 2000), E3 ligase Fbw 7-containing Skp/Culin/F-box protein complex (SCF<sup>Fbw</sup>) (Gao *et al.*, 2004), and the E3 ligase Itch (Nateri *et al.*, 2004). Jab1 association with MIF inhibits Jab1-mediated AP-1 activity. Thus, the cytokine MIF serves to transduce cytokine signaling to nuclear c-Jun function. Components of the AP-1 complex itself can inhibit c-Jun activity. The AP-1 transcription factor family is composed of Jun, Fos, and ATF subunits, which interact through their leucine zipper motif and bind DNA through a basic region. Jun proteins form homo- and heterodimers. Several other basic proteins heterodimerize with c-Jun, including Maf (v-Maf, c-Maf, MafB, MafG, and MafK) proteins and the neural retina leucine zipper gene product 1 (Nr1), which may thereby regulate the activity of c-Jun (Angel and Karin, 1991). Finally, c-Jun-binding repressor proteins have been described. The c-Jun-interacting proteins JDP1 and JDP2 regulate UV-mediated apoptosis (Piu *et al.*, 2001). Recent genome-wide interrogation identified an inhibitor of AP-1-responsive target genes as the *DACH1* gene, which is known to be involved in compound eye development (Wu *et al.*, 2003).

Development of the compound eye in *Drosophila* is governed by a regulatory network of genes. The *eyeless*, *sine oculis*, *eyes absent*, and *dachshund* genes are required for normal eye development, and ectopic expression of *eyeless*, *eyes absent*, and *dachshund* induce ectopic eye formation. This regulatory pathway, conserved among metazoans and the vertebrate homologues of *eyeless* (Pax6), *sine oculis* (Six1-6), *eyes absent* (*Eya1-4*), and *dachshund* (*DACH1-2*), contributes to organism development (Kawakami *et al.*, 2000). The six genes encode a conserved Six and Homeo domain sequence-specific DNA binding family of transcription factors. Eya functions as a coactivator for Six proteins, and functions in a phosphatase-dependent manner (Lee *et al.*, 2000a). The *dachshund* genes encode cointegrator proteins recruited to Six binding sites at the promoters of target genes that promote cellular differentiation and cell cycle exit. A conserved domain (dac and ski/sno domain 1), which has significant identity with Ski/Sno ([DS] domain), is conserved from *Drosophila* to the human (Chen *et al.*, 1997; Davis *et al.*, 1999; Kawakami *et al.*, 2000). The *DACH1* protein binds the CBP coactivator or interacts directly with corepressors to regulate transcriptional activity (Ikeda *et al.*, 2002; Li *et al.*, 2003). The recruitment of *DACH1* to Six binding sites is regulated through mechanisms that have as yet to be defined.

In view of the finding that c-Jun plays a key role in oncogene-induced transformation and that *DACH1* functions as an inhibitor of AP-1-responsive gene expression, we investigated the potential role for *DACH1* in c-Jun-mediated cellular growth. *DACH1* inhibited c-Jun-mediated DNA synthesis and contact-independent growth. AP-1 activity was

repressed by *DACH1* requiring a conserved ([DS]) domain. Repression of c-Jun transcription and transactivation by *DACH1* required the *DACH1* DS domain. *DACH1* bound a corepressor complex, including HDAC1/HDAC3 and repressed c-Jun transactivation through the c-Jun  $\delta$  domain. c-Jun transactivation and contact-independent growth is controlled by the cell fate determination factor *DACH1*.

## MATERIALS AND METHODS

### Plasmid Constructions

The expression plasmids that include an N-terminal FLAG peptide for *DACH1*, *DACH1* DS-domain alone (DS), or *DACH1* DS-domain deleted ( $\Delta$ DS) were described previously (Wu *et al.*, 2003). The FLAG-tagged *DACH1* c-DNA was subcloned into the MSCV-IRES-GFP and pLRT vector. The full-length *DACH1* cDNA was subcloned in frame with the VP16 activation domain in the pVP16 vector to form a *DACH1*-VP16 fusion protein. *DACH1* short hairpin RNA (shRNA) in the pSM2 expression vector was purchased from Open Biosystems (Huntsville, AL) and subcloned into the LMP retroviral vector. The Ski cDNAs was subcloned into the 3x Flag-CMV7.1 vector (Sigma-Aldrich, St. Louis, MO). The expression vector encoding adenovirus-induced Cre expression or control viruses were described previously (Wang *et al.*, 1995). The cyclin A promoter (Katabami *et al.*, 2005) and stathmin promoter (Kinoshita *et al.*, 2003) reporter genes were described previously. The wt *c-fos*-LUC, serum response element (SRE), or ternary complex factor (TCF) mutants (PM12 and PM18) were described previously (Wang *et al.*, 1998). AP-1 Luc (3TPlux), *c-Jun* Luc and *JunB* Luc were described previously (Watanabe *et al.*, 1996). The expression vectors for c-Jun linked to Gal4 or E2 and truncated c-Jun were described previously (Baichwal and Tjian, 1990; Baichwal *et al.*, 1991). The Ha-Ras expression vector was described previously (Albanese *et al.*, 1995).

### Cell Culture, DNA Transfection, and Luciferase Assays

Cell culture, DNA transfection, and luciferase assays were performed as described previously (Fu *et al.*, 2000, 2002, 2003). The NIH3T3, HEK293T, and MCF-7 cell line were cultured in DMEM supplemented with 10% fetal calf serum, 1% penicillin, and 1% streptomycin. *c-jun*<sup>fl/fl</sup> 3T3 cells were derived from mouse embryo fibroblast from *c-jun*<sup>fl/fl</sup> transgenic mouse (Zenz *et al.*, 2003) by using the standard 3T3 protocol. Rat1a-J4 cells express c-Jun in a doxycycline-controlled manner (Katabami *et al.*, 2005). Cells were plated at a density of  $1 \times 10^5$  cells in a 24-well plate on the day before transfection with Superfect according to the manufacturer's protocol (QIAGEN, Valencia, CA). At least two different plasmid preparations of each construct were used. In cotransfection experiments, a dose response was determined in each experiment with 50 and 200 ng of expression vector and the promoter reporter plasmids (1  $\mu$ g). Luciferase activity was normalized for transfection by using  $\beta$ -galactosidase reporters as an internal control. Luciferase assays were performed at room temperature by using an Autolumat LB 953 (Berthold Technologies, Bad Wildbad, Germany). The -fold effect was determined for 50–200 ng of expression vector with comparison made to the effect of the empty expression vector cassette and statistical analyses was performed using the Mann-Whitney *U*-test.

### Small-interfering RNA (siRNA) Transfection, Western Blot, and Immunoprecipitation Assays

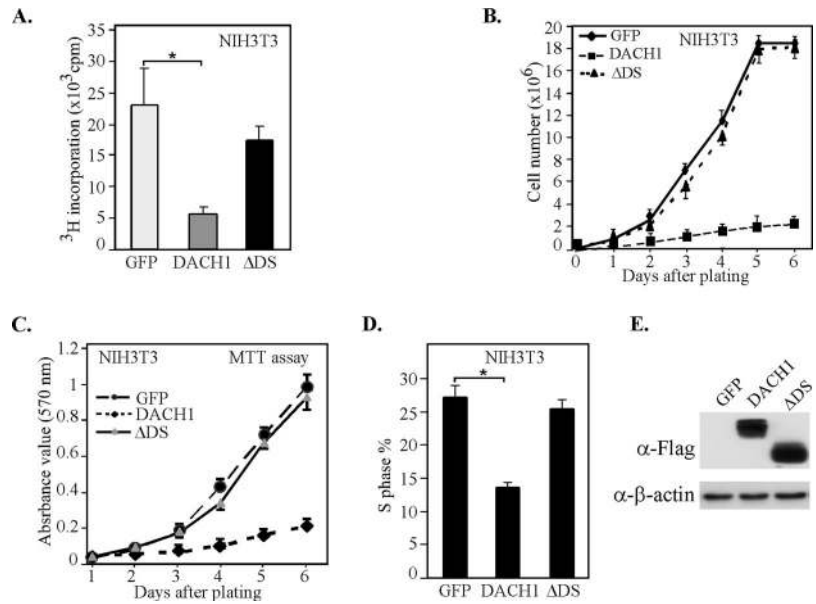
The *Dach1* siRNA target sequence is 5'-AAAGTGGCTTCCTTACGGTG. Control siRNA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). *Dach1*-specific siRNAs or control siRNAs (100 or 200 nM) were transfected following the Oligofectamine protocol (Invitrogen, Carlsbad, CA). Transfection efficiency was monitored by no-silencing fluorescein siRNA from QIAGEN. 3T3 cells were infected with *DACH1* shRNA or control retrovirus and selected by puromycin. Western blot analysis using antibodies to c-Jun (Santa Cruz Biotechnology), cyclin A (Santa Cruz Biotechnology),  $\beta$ -PAK (Santa Cruz Biotechnology), E2 tag (Abcam, Cambridge, MA), FLAG tag (Sigma-Aldrich), and the loading control guanine dissociation inhibitor (GDI) was conducted as described previously (Lee *et al.*, 2000b; Wu *et al.*, 2003). HEK293T cells were used for the detection of protein-protein interaction in vivo and immunoprecipitation was conducted as described previously using an anti-hemagglutinin (HA) antibody and immunoblotting with antibodies to HDAC1 (SC-7872), HDAC3 (SC-17795), NCoR (SC-8994), p300 (SC-585), and c-Jun (SC-44 and SC-1694).

### Cell Cycle and DNA Synthesis Analysis

Cell cycle parameters were determined using laser scanning cytometry. Cells were processed by standard methods by using propidium iodide staining of cell DNA. Each sample was analyzed by flow cytometry with a FACScan flow cytometer (BD Biosciences, Mansfield, MA) by using a 488-nm laser. Histograms were analyzed for cell cycle compartments using ModFit version 2.0



**Figure 1.** DACH1 inhibits cellular proliferation and DNA synthesis of NIH3T3 cells. (A) NIH3T3 cells were transfected with expression vectors encoding either DACH1, or a mutant of DACH1 deleting the DS domain ( $\Delta$ DS) or a control vector (GFP). Forty-eight hours after transfection, tritiated thymidine incorporation was determined ( $*p < 0.01$ ). The cell proliferation rate was assessed for NIH3T3 cells transduced with retroviral expression vectors encoding the GFP, DACH1, or  $\Delta$ DS. Proliferation analyses were conducted by either counting cellular number (B) or MTT assay (C). (D) FACS analysis of NIH3T3 cells transduced with retroviral expression vectors. The proportion of cells in S phase is shown. Data are mean  $\pm$  SEM of  $n = 6$  separate experiments ( $*p < 0.01$ ). (E) Western blot analysis to the FLAG-tag at the N terminus of DACH1 or DACH1 $\Delta$ DS.  $\beta$ -Actin is used as a loading control showing similar level of expression of the wt and mutant DACH1 proteins.



(Verity Software House, Topsham, ME). A minimum of 20,000 events was collected to maximize statistical validity of the compartmental analysis. DNA synthesis was analyzed by [ $^3$ H]thymidine (TdR) incorporation. Cells ( $10^5$ ) were plated into 24-well plate and cultured for 36 h. Then, 1  $\mu$ Ci of [ $^3$ H]TdR was added to each well, and culture was continued for 2 h. Cells were washed and fixed before measuring incorporated [ $^3$ H]TdR by liquid scintillation.

#### Cell Proliferation Assays

For the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay, NIH3T3 cells infected with MSCV-IRES-GFP, MSCV-DACH1-IRES-GFP, or MSCV-DACH1 $\Delta$ DS-IRES-GFP were seeded into 96-well plates in normal growth medium, and cell growth was measured every day by MTT bromide assays. c-Jun-expressing Rat1a-J4 cells were plated in growth medium in either the presence or absence of 2  $\mu$ g/ml doxycycline. To measure growth curve, cells were seeded into 12-well plates and serially counted for 5–6 d.

#### Colony Formation Assay

Rat1a-J4 cells ( $4.0 \times 10^3$ ) were plated in triplicate in 3 ml of 0.3% agarose (sea plaque) in complete growth medium in the presence or absence of 2  $\mu$ g/ml doxycycline overlaid on a 0.5% agarose base, also in complete growth medium. Two weeks after incubation, colonies  $>50 \mu$ m in diameter were counted using an Omnicon 3600 image analysis system. The colonies were visualized after staining with 0.04% crystal violet in methanol for 1–2 h.

#### Chromatin Immunoprecipitation (ChIP) Assay

ChIP assays were performed as described previously (Fu *et al.*, 2004). Polymerase chain reaction (PCR) primers were as follows: for murine cyclin A: forward, 5'-CCTCAGGCTCCCGCCCTGTAAGATCC and reverse, 5'-TCAAGTAGCCCGCGACTATTGAATAT; and for murine cyclin D1 AP-1 site: forward, 5'-CCGGTGTCTGGTTCCTGGA and reverse, 5'-CCCCGAAA-ATCCAGCAACA. The cells were cross-linked with formaldehyde buffer for 10 min at 37°C, and the procedure was followed as described in Fu *et al.* (2004). Double ChIP assay was performed first using polyclonal c-Jun antibody, and then elution was diluted with 10X volume of immunoprecipitation (IP) buffer and then immune-precipitated with anti-FLAG M2 antibody following standard protocol.

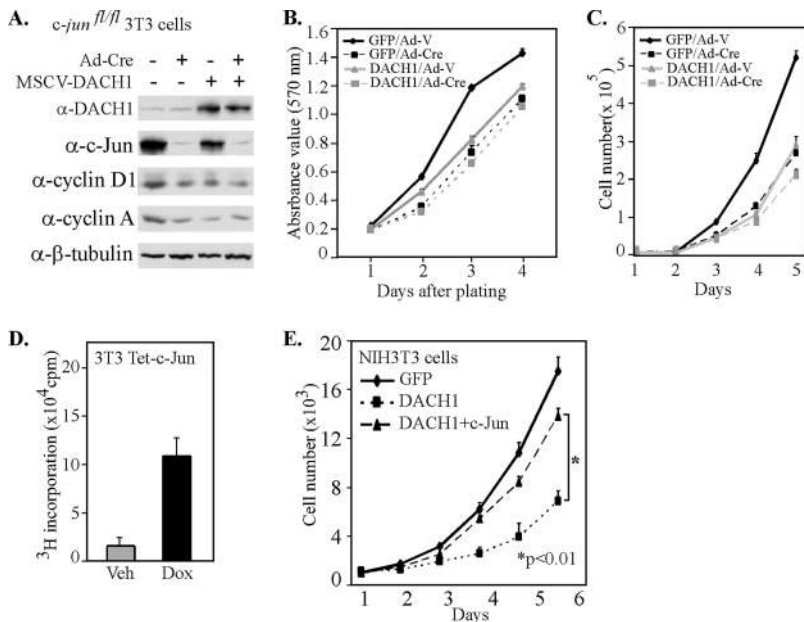
## RESULTS

### c-Jun Is Required for DACH1-mediated Inhibition of Cellular Proliferation

To determine the role of Dachshund1 in fibroblast cellular proliferation and growth, NIH3T3 cells were transduced with a retroviral expression plasmid encoding DACH1 linked to green fluorescent protein (GFP) through an internal ribosomal entry site. Homogeneously transduced populations of NIH3T3 cells were compared with cells trans-

duced with an empty expression vector (MSCV-IRES-GFP). DACH1 expression inhibited tritiated thymidine uptake by 75% (Figure 1A). To determine the domain of DACH1 involved in cellular proliferation, a mutation of the conserved DS domain was assessed. NIH3T3 cells transduced with the mutant DACH1 protein, which was expressed in equal amounts by Western blotting, failed to inhibit tritiated thymidine uptake (Figure 1A). Cellular proliferation assays were conducted by serial counting of cell number (Figure 1B) and the MTT stain (Figure 1C). Cells transduced with DACH1 failed to proliferate, whereas cells transduced with either a mutant DACH1 deleted of the DS domain or the GFP vector alone proliferated with similar efficiency. Fluorescence-activated cell sorting (FACS) analysis of DACH1-transduced NIH3T3 cells demonstrated an inhibition of DNA synthesis that was dependent upon the DS domain (Figure 1D). Expression of the DACH1 mutant, DACH1 $\Delta$ DS was similar to the DACH1 wild type as assessed by Western blot analysis of the FLAG epitope at the N terminus of each protein (Figure 1E).

Previous studies had shown AP-1 reporter activity was inhibited by DACH1 (Wu *et al.*, 2003). Endogenous AP-1 activity contributes to 3T3 cell proliferation. To examine the importance of c-Jun as an endogenous target of DACH1-mediated inhibition of DNA synthesis, we used c-Jun-deficient 3T3 cells. c-Jun $^{-/-}$  mice die in gestation from cardiovascular and hepatic defects (Eferl *et al.*, 1999). Significant functional differences have been observed in mouse embryonic fibroblasts derived from embryonic stem cell knockout, compared with cells derived by acute target gene excision by using floxed alleles and Cre recombinase (Sage *et al.*, 2003). We therefore derived c-Jun $^{fl/fl}$  3T3 cells from c-Jun $^{fl/fl}$  mice (Zenz *et al.*, 2003) by using a standard protocol (Todaro and Green, 1963). The c-Jun $^{fl/fl}$  3T3 cells were transduced with Cre recombinase (Sage *et al.*, 2003), and the deletion of c-Jun was confirmed by Western blot analysis (Figure 2A). These cells were transduced with either a DACH1 retroviral expression vector or equal amount of control viral vector, and Western blot analysis and cellular proliferation assays were conducted. c-Jun deletion was associated with a reduction in cyclin D1 and cyclin A expression consistent with prior



**Figure 2.** DACH1 inhibition of cellular proliferation requires *c-jun*. (A) Western blot analysis of *c-jun*<sup>fl/fl</sup> 3T3 cells transduced with adenovirus expressing Cre recombinase. Cells were transduced with the MSCV-DACH1 vector as indicated. Western blot analysis shows Ad-Cre excision of *c-jun* abrogated *c-jun* expression. Antibodies are to cyclin D1, cyclin A, and  $\beta$ -tubulin. Cellular proliferation assessed by MTT assay (B) and by cell counting of DACH1-transduced cells (C) shown in A. (D) Proliferation assay of 3T3 Tet-*c-Jun* cells, in which 2  $\mu$ g/ml doxycycline for 48 h was used to induce *c-Jun* protein level. (E) NIH3T3 cells transduced with pLRT-GFP, pLRT-DACH1, or pLRT-DACH1 and pLRT-*c-Jun* expressing vector. Cellular number was determined daily for 6 d with 2  $\mu$ g/ml doxycycline. *c-Jun* expression reversed DACH1-mediated inhibition of cellular proliferation ( $p < 0.01$ ).

findings that *c-jun* induces cyclin D1 and cyclin A (Albanese *et al.*, 1995). DACH1 expression in *c-jun*<sup>fl/fl</sup> cells reduced cyclin D1 abundance 30%, and cyclin A expression 50% (Figure 2A). DACH1 expression in parental *c-jun*<sup>fl/fl</sup> 3T3 cells reduced proliferation rates >35%. In cells deleted of *c-jun* (*c-jun*<sup>fl/fl</sup> + Cre), expression of DACH1 failed to inhibit cellular proliferation (Figure 2B). A detailed time course was conducted to determine the role of *c-jun* in DACH1-mediated inhibition of cellular proliferation through daily counting of cells (Figure 2C). Cellular proliferation of wild-type cells was inhibited by DACH1 expression; however, the proliferation rate of cells deleted of *c-jun* was not affected by DACH1 expression (Figure 2C). To determine whether expression of *c-Jun* could overcome DACH1-mediated repression of cellular proliferation, a cell line encoding a doxycycline-inducible *c-jun* cDNA was used (3T3 Tet-*c-Jun*). The induction of *c-Jun* expression by the addition of doxycycline stimulated cellular proliferation (Figure 2D). Transduction of the NIH3T3 cells with DACH1 reduced cellular proliferation >60% at 6 d. Induction of *c-Jun* abundance, through the addition of doxycycline, restored cellular proliferation by >85% (Figure 2E). Together, these studies demonstrate DACH1 inhibition of cellular proliferation requires *c-jun* and can be overcome by *c-Jun* expression.

#### DACH1 Inhibits Colony Formation Requiring the Conserved DS Domain

The expression of *c-Jun* is sufficient to induce contact-independent growth of Rat1a cells (Schutte *et al.*, 1989). The stable cell line expressing doxycycline-inducible *c-Jun* was infected with an MSCV virus encoding either GFP, DACH1 wt or DACH1 mutant. Western blot analysis demonstrated the induction of *c-Jun* by doxycycline and transfected DACH1 protein was detected by an antibody directed to the N-terminal FLAG epitope (Figure 3A). DACH1 also inhibited basal proliferation of Rat1a-J4 cells (Figure 3B). Previous studies have demonstrated the importance of *c-Jun* in promoting cell cycle transition (Ryseck *et al.*, 1988; Kovary and Bravo, 1991). The *c-Jun*-induced thymidine uptake, reflecting DNA synthesis, was attenuated by DACH1 expression (Figure 3C). Colony formation was induced by *c-Jun* expres-

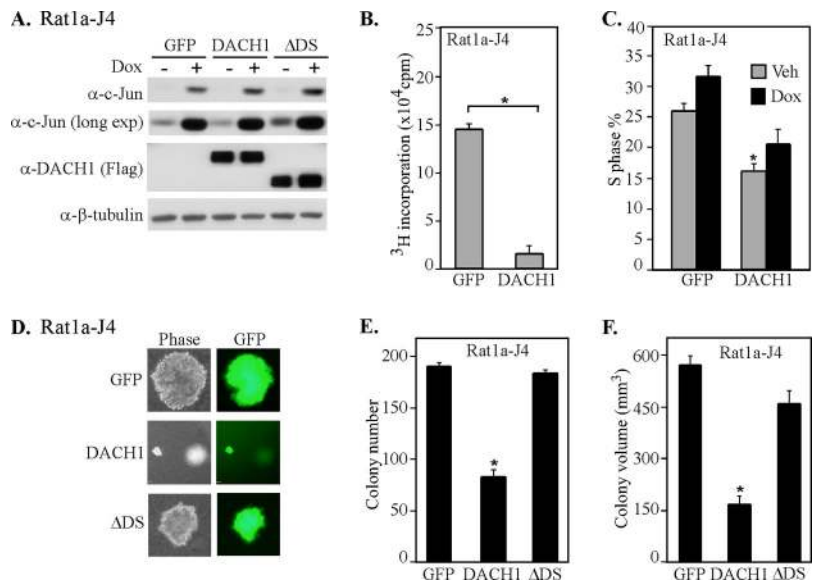
sion (Figure 3D) as described previously (Katabami *et al.*, 2005). Transduction of the inducible *c-Jun* stable cell line with a retroviral expression vector encoding DACH1 reduced the number of colonies by 40% (Figure 3E) and reduced the volume of individual colonies by >75% (Figure 3F). Expression of equal amount of a mutant DACH1 protein deleted of the DS domain ( $\Delta$ DS) failed to inhibit *c-Jun*-induced contact-independent growth (Figure 3F). Together, these results suggest that DACH1 inhibits *c-Jun* induced cellular proliferation and blocks *c-Jun*-mediated contact-independent growth. These results further suggest *c-Jun* is a key molecular target of DACH1 inhibition of contact-independent growth.

#### DACH1 Expression Inhibits Physiological Inducers of *c-jun* Expression and *c-jun* Target Gene Expression

The *c-jun* gene is induced by diverse physiological stimuli, including serum and growth factors. To determine whether DACH1 expression was capable of inhibiting the induction of *c-Jun* by physiological stimuli, NIH3T3 cells were treated with serum. Cells were transduced with retroviral expression vectors encoding either DACH1 or the DACH1 mutant ( $\Delta$ DS). Consistent with prior studies, serum induced *c-Jun* abundance approximately >10-fold. The *c-Jun* target genes cyclin A and  $\beta$ -PAK were also induced by serum (Figure 4A, lane 1 versus lane 2). NIH3T3 cells expressing DACH1 reduced serum-mediated expression of *c-Jun* by >50%. DACH1 expression reduced serum-induced expression of the *c-Jun* target genes cyclin A and  $\beta$ -PAK. Deletion of the DACH1 DS domain abrogated the DACH1-mediated inhibition of *c-Jun*, cyclin A, and  $\beta$ -PAK expression at 3 h. Expression of CDK4 and  $\beta$ -tubulin were unaffected by DACH1 expression, indicating the effect of DACH1, to inhibit *c-Jun* expression, is gene specific.

To examine further the mechanism by which DACH1 repressed the *c-jun* target gene cyclin A, we deployed the tetracycline-inducible *c-jun* stable Rat1a-J4 cell line (Figure 4B). Doxycycline addition induced *c-Jun* abundance, associated with the induction of the *c-jun* target genes cyclin A and  $\beta$ -PAK. Transduction of Rat1a-J4 cells with DACH1 abrogated *c-Jun*-mediated induction of cyclin A and  $\beta$ -PAK,

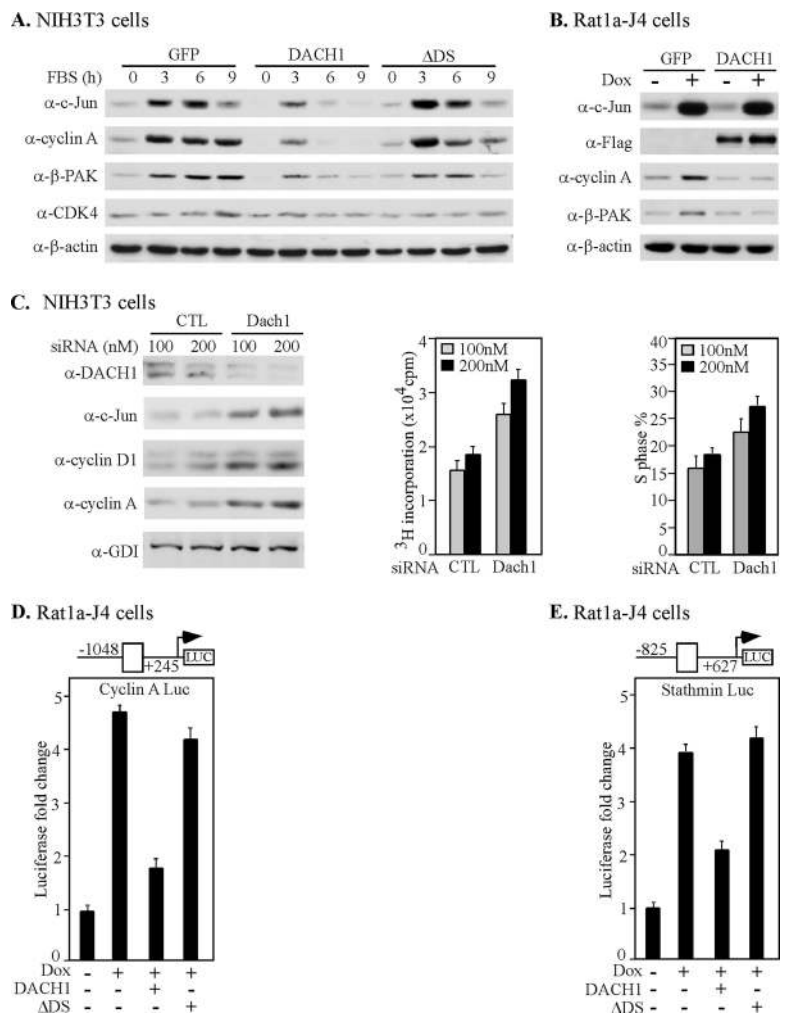
**Figure 3.** DACH1 inhibits c-Jun-mediated colony formation and cellular proliferation. (A) Rat1a cells stably expressing a doxycycline-inducible c-Jun cDNA were transduced with MSCV virus encoding either GFP, or DACH1 or  $\Delta$ DS domain. Western blot analysis of doxycycline-treated cells (Dox+) show the induction of c-Jun protein and presence of DACH1 by using the Flag epitope to the N terminus of DACH1.  $\beta$ -Tubulin is a loading control. (B) Rat1a-HA-c-Jun cells were analyzed for cellular proliferation by tritiated thymidine incorporation after 48 h. (C) The DNA synthetic phase determined by FACS analysis in presence or absence of 2  $\mu$ g/ml doxycycline. (D) Phase-contrast fluorescence microscopy of Rat1a-J4 cells infected with an MSCV vector encoding GFP, DACH1, or  $\Delta$ DS in presence of 2  $\mu$ g/ml doxycycline. Quantification of colony number (E) or colony volume (F) of Rat1a-J4 cells infected with MSCV vector encoding GFP, DACH1, or  $\Delta$ DS analyzed after 14 d. Data represent the mean  $\pm$  SEM of n = 6 separate experiments (\*p < 0.01).



without affecting  $\beta$ -actin expression. Thus, DACH1 inhibits c-Jun-mediated induction of cyclin A. To determine whether endogenous DACH1 regulates c-Jun expression and c-Jun

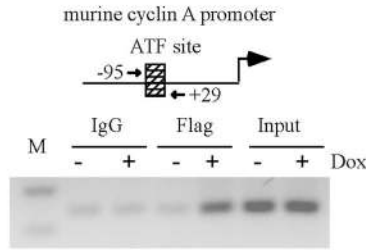
target gene expression, DACH1 siRNA was used. DACH1 siRNA reduced DACH1 abundance by  $\sim$ 75% associated with 2.5-fold induction of c-Jun abundance (Figure 4C). The

**Figure 4.** DACH1 inhibits serum-induced expression of c-Jun and c-Jun target genes. (A) Western blot analysis of NIH3T3 transduced with MSCV-GFP, MSCV-DACH1 or MSCV-DACH1 $\Delta$ DS. Cells were serum starved and then treated with serum for the time points indicated. Western blot, with the antibodies indicated, demonstrated DACH1 inhibits c-jun induction by serum. (B) The Rat1a-J4 cell line encoding tetracycline-inducible c-jun was treated with doxycycline for 48 h to induce c-Jun. Cells were transduced with retrovirus expressing GFP or DACH1. The M2 antibody detects the N-terminal FLAG-tag of DACH1. DACH1 inhibits c-Jun mediated induction of cyclin A and  $\beta$ -PAK.  $\beta$ -Actin is a loading control. (C) NIH3T3 cells were treated with DACH1 siRNA for 72 h. Western blot analysis was conducted with the antibodies indicated. Reduction in DACH1 abundance induces c-jun, and expression of the c-jun target genes cyclin D1 and cyclin A. [ $^3$ H]Thymidine incorporation and percentage of cells in S phase is shown. The cyclin A (D) and stathmin promoter (E), linked to a luciferase reporter, were assessed in Rat1a-J4 cells. Cells were transduced with either expression vector for DACH1 or DACH1 $\Delta$ DS. Data are shown as luciferase activity as mean  $\pm$  SEM for n > 5 separate transfections.

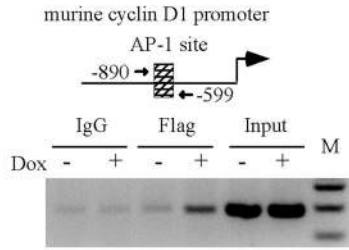




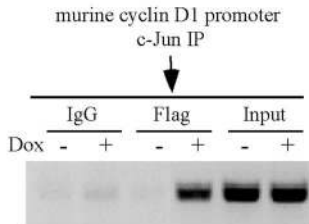
**A. 3T3 Tet-DACH1 stable**



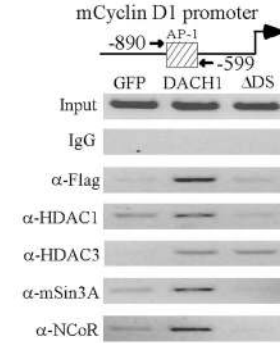
**B. 3T3 Tet-DACH1 stable**



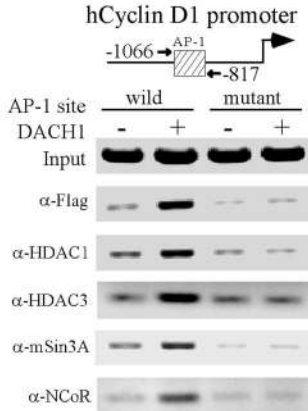
**C. Double ChIP**



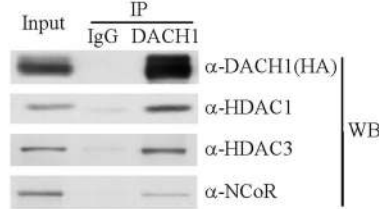
**D. 3T3 (ChIP)**



**E. 293T cells(ChIP)**



**F. HEK293T (IP)**



**Figure 5.** Chromatin immunoprecipitation assays demonstrate DACH1 within the AP-1 site of endogenous genes. ChIP assay conducted using oligonucleotides to the endogenous murine (A) cyclin A and (B) cyclin D1 promoter. Schematic representation of cyclin A and cyclin D1 promoter is shown. Amplification was conducted of products precipitated with indicated antibodies or control IgG. The cell line used is an NIH3T3 cell line expressing FLAG-tagged DACH1 under control of the Tet enhancer (Tet-DACH1). Doxycycline addition induced DACH1 protein abundance. A similar amount of DACH1 was used as a loading control. (C) The chromatin was first precipitated with antibody to c-Jun, and then the elution was diluted with IP buffer and immunoprecipitated with anti-FLAG. PCR amplification was conducted of the AP-1 site of the cyclin D1 promoter. (D) 3T3 cells were transduced with MSCV-GFP, MSCV-DACH1, and MSCV-DACH1ΔDS. ChIP assays were conducted of the endogenous murine cyclin D1 promoter AP-1 site with the indicated antibodies. The FLAG antibody is directed to the N-terminal tag of DACH1 or DACH1ΔDS. Equal amounts of DACH1 and DACH1ΔDS are detected by Western blot of the cells (data not shown). (E) ChIP assays of the transfected human cyclin D1 promoter, either wild type or mutant of the AP-1 binding site, was conducted using antibodies directed to endogenous HDAC1, HDAC3, mSin3A, and NCoR. (F) Immunoprecipitation Western blot analysis of DACH1 from HEK293T cells. The HA tagged DACH1 was used for immune-precipitation, with subsequent Western blot to the endogenous proteins indicated.

*c-jun* target gene cyclin D1 (Albanese *et al.*, 1995) was induced threefold and cyclin A was induced two- to fivefold (Figure 4C). Consistent with a key role for cyclin D1 in 3T3 cell DNA synthesis (Albanese *et al.*, 2003) the DACH1 siRNA-mediated induction of cyclin D1 was associated with an induction of DNA synthesis determined by [<sup>3</sup>H]thymidine uptake and S-phase distribution by FACS (Figure 4C).

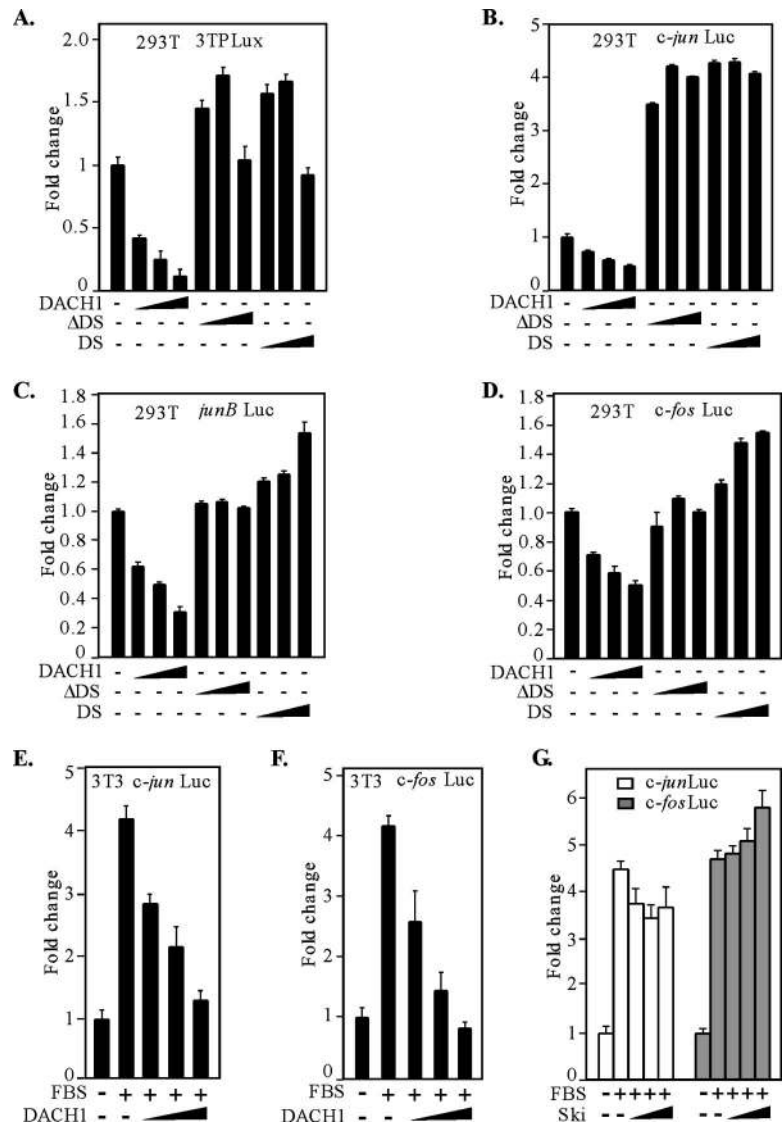
To determine whether DACH1 directly regulated *c-jun* target genes, the cyclin A and stathmin promoters were assessed. Induction of c-Jun expression in the Rat1a-J4 cells by addition of doxycycline induced the activity of the cyclin A promoter activity fourfold (Figure 4D). Expression of DACH1 inhibited c-Jun-mediated induction of cyclin A promoter activity. Deletion of the DACH1 DS domain abolished the DACH1 repression function (Figure 4D). Similarly the stathmin promoter was induced by c-Jun, and c-Jun induction of the stathmin promoter was inhibited by DACH1, requiring the DACH1 DS domain (Figure 4E).

**DACH1 Occupies the AP-1 Site of Endogenous *c-Jun* Target Genes in ChIP Assays**

Because DACH1 repressed *c-jun*-mediated induction of several AP-1 target genes (Wu *et al.*, 2003), we sought to determine whether DACH1 occupied AP-1 sites of endogenous

genes in the context of their local chromatin structure. For these studies, NIH3T3 cells were stably transduced with a FLAG-tagged DACH1 expression vector. The cyclin A promoter, a known c-Jun target gene, was examined. Using oligonucleotides directed to the endogenous murine cyclin A promoter AP-1/ATF-1 site, ChIP assays, conducted with the anti-FLAG antibody, demonstrated the presence of DACH1 in the context of the local chromatin structure of the endogenous cyclin A promoter (Figure 5A). Control IgG did not result in similar amplification. Analysis of the endogenous murine cyclin D1 AP-1 site by using oligonucleotides directed to the murine promoter demonstrated the presence of DACH1 at the cyclin D1 promoter AP-1 site (Figure 5B). The complex formation of c-Jun and DACH1 protein at the AP-1 site of the cyclin D1 promoter was further confirmed by sequential immune-precipitation with c-Jun and FLAG antibodies (Figure 5C). Further analysis of the endogenous proteins associated with DACH1 at the murine cyclin D1 promoter was conducted. Oligonucleotides directed to the murine cyclin D1 AP-1 site evidenced the presence of the DACH1-associated corepressor proteins HDAC1, HDAC3, mSin3A, and NCoR recruited to the murine cyclin D1 promoter (Figure 5D). To determine the role of the cyclin D1 promoter AP-1 site in recruitment of DACH1, ChIP assays





**Figure 6.** DACH1 inhibits serum-induced activation of AP-1 activity. (A) HEK293T cells were transfected with luciferase reporter plasmids encoding either a multimeric AP-1 site (A), the *c-jun* promoter (B), the *junB* promoter (C), or the *c-fos* promoter (D) and DACH1 mammalian expression vectors. A  $\beta$ -galactosidase report gene driven by a  $\beta$ -actin promoter was used to normalize the transfection deficiency. Data are shown as -fold change in luciferase activity. Data are mean  $\pm$  SEM of  $n = 6$  separate transfections. (E–G) 3T3 cells were transfected with a reporter plasmid encoding either the *c-jun* promoter (E) or *c-fos* promoter (F) and a DACH1 mammalian expression vector. Cells were serum starved and stimulated with 15% FBS for 6 h before the luciferase activity was determined. 3T3 cells were transfected with the luciferase reporter plasmid for the *c-jun* or *c-fos* promoter and a mammalian expression vector encoding Ski (G). Cells were treated as shown in E and F. The expression vector encoding Ski does not significantly reduce reporter activity. Data are mean  $\pm$  SEM of  $n = 6$  separate transfections.

were conducted comparing the wild-type and mutant cyclin D1 AP-1 site promoters (Figure 5E). Expression of FLAG-tagged DACH1 enhanced recruitment of DACH1 (FLAG), together with HDAC1, HDAC3, mSin3A, and NCoR. Mutation of the cyclin D1 AP-1 site reduced recruitment of each of these proteins (Figure 5E). Immune-precipitation Western blot indicated these proteins form a cellular complex (Figure 5F).

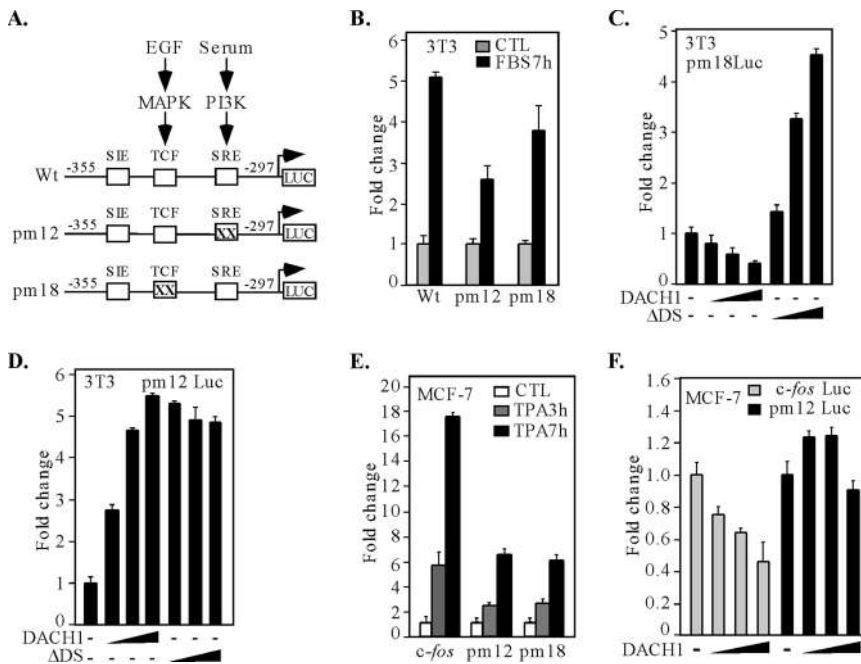
#### DACH1 Inhibition of AP-1 Family Members

To determine the domains of DACH1 involved in inhibiting c-Jun function, an AP-1-responsive reporter gene (3TP-LUX) was assessed in HEK293T cells. The activity of the AP-1 reporter gene was inhibited by DACH1 expression (Figure 6A). The deletion of the DACH1 DS domain abrogated repression of AP-1 reporter activity, and the DS domain alone was insufficient for repression of AP-1 activity (Figure 6A). We assessed the effect of DACH1 on the transcriptional activity of the key AP-1 genes, *c-jun*, *junB*, and *c-fos*. The transcriptional activity of the *c-Jun* promoter was repressed by DACH1 expression, and this transcriptional repression was abrogated by the deletion of the conserved DS domain

(Figure 6B). Similarly, the *junB* and *c-fos* promoters were repressed by DACH1 expression, and the DS domain was required for repression (Figure 6, C and D).

AP-1 activity is serum inducible, and serum induction of the *c-jun* promoter was inhibited by DACH1 expression (Figure 6E). Similarly, the *c-fos* promoter induction by serum was inhibited by DACH1 expression (Figure 6F). DACH1 shares homology with Ski (Kozmik *et al.*, 1999). To determine whether Ski functions as a transcriptional repressor of *c-fos* or *c-jun* promoter activity, serum induction experiments were conducted. Ski expression failed to repress serum-induced *c-jun* and *c-fos* promoter activity (Figure 6G). Thus, *c-fos* was repressed by DACH1 and not by Ski. Together, these results demonstrate distinct transcriptional repression profiles of DACH1 and the related Ski protein.

We examined further the DNA sequences of the *c-fos* promoter required for transcriptional repression by DACH1. A point mutant of the SCF binding site and the TCF binding site of the *c-fos* promoter were compared (Figure 7A). Consistent with previous findings (Hill *et al.*, 1995), mutation of either the *c-fos* SRE or TCF binding site reduced serum-induced activation of the *c-fos* promoter (Figure 7B). The



**Figure 7.** DACH1 inhibition of *c-fos* promoter activity requires the SRE, but not TCF site. (A) Schematic representation of *c-fos* promoter luciferase reporter vectors, indicating the TCF and SRE sites. (B) 3T3 cells were transiently transfected with a *c-fos* wild-type or mutant promoter reporter constructs and stimulated by serum for 7 h as shown. 3T3 cells were transiently transfected with *c-fos* promoter point mutant PM18 (C) or PM12 (D) luciferase reporter genes, and expression vectors encoding DACH1 wild-type or mutant. MCF-7 cells were transiently transfected with either wild-type or mutant *c-fos* promoter luciferase reporters and treated with TPA for 3 or 7 h (E) or cotransfected with a mammalian expression vector for DACH1 (F). All changes showing mean  $\pm$  SEM of  $n = 6$  separate transfections.

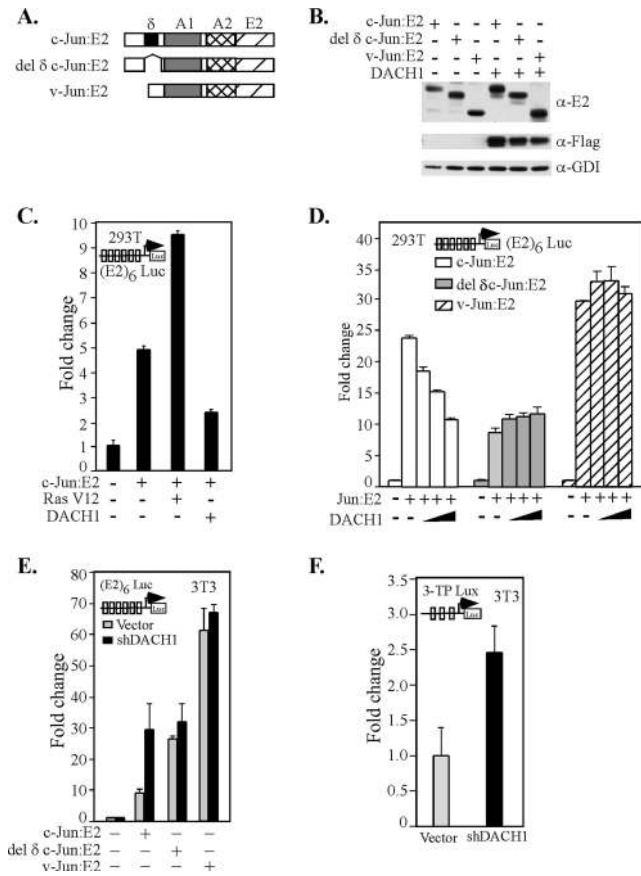
*c-fos* promoter encoding a point mutation of either the SRE or the TCF site was compared for repression by DACH1. DACH1 repressed activity of the *c-fos* promoter, encoding a mutation of the TCF site (*c-fos* PM18) (Figure 7C). In contrast, point mutation of the SRE binding site (*c-fos* PM12) abrogated repression by DACH1, inducing transactivation rather than repression by DACH1 (Figure 7D). These results demonstrate the SRE site of the *c-fos* promoter is a transcriptional target of DACH1 repression in 3T3 cells. To determine whether these effects were similar in other cell types, MCF-7 cells were assessed. The *c-fos* promoter was activated sixfold by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Figure 7E). Point mutation of either the TCF or SRE site of the *c-fos* promoter reduced activation by TPA. Consistent with findings in NIH3T3 cells, DACH1 repressed the *c-fos* promoter and point mutation of the SRE site abrogated repression (Figure 7F). Collectively, these experiments demonstrate DACH1 inhibits the physiological induction of the promoters encoding AP-1 response genes, including *c-jun*, *junB*, and *c-fos*. The mechanism by which DACH1 inhibits gene expression is distinct from the related protein Ski.

#### DACH1 Inhibition of *c-Jun* Transactivation Requires the $\delta$ Domain

To examine further the mechanisms by which DACH1 inhibited *c-Jun* transactivation, mammalian two-hybrid studies were undertaken. Comparison was made between either wild-type *c-Jun* or *c-Jun* proteins encoding either a deletion of the  $\delta$  domain (del  $\delta$  *c-Jun*) or the *v-Jun* protein. The *v-Jun* protein differs from the wild-type *c-Jun* primarily by the deletion of the  $\delta$  domain. Previous studies with these expression vectors have demonstrated the presence of a *c-Jun* inhibitor functioning through the  $\delta$  domain (Baichwal and Tjian, 1990). The heterologous transactivation domains of *c-Jun* linked to the E2 DNA binding domain were assessed using a multimeric E2 DNA binding domain, linked to a luciferase reporter gene (Figure 8A). The wild-type and mutant chimeric *c-Jun* proteins were expressed equally in DACH1-transfected cells (Figure 8B). Activity of the *c-Jun*

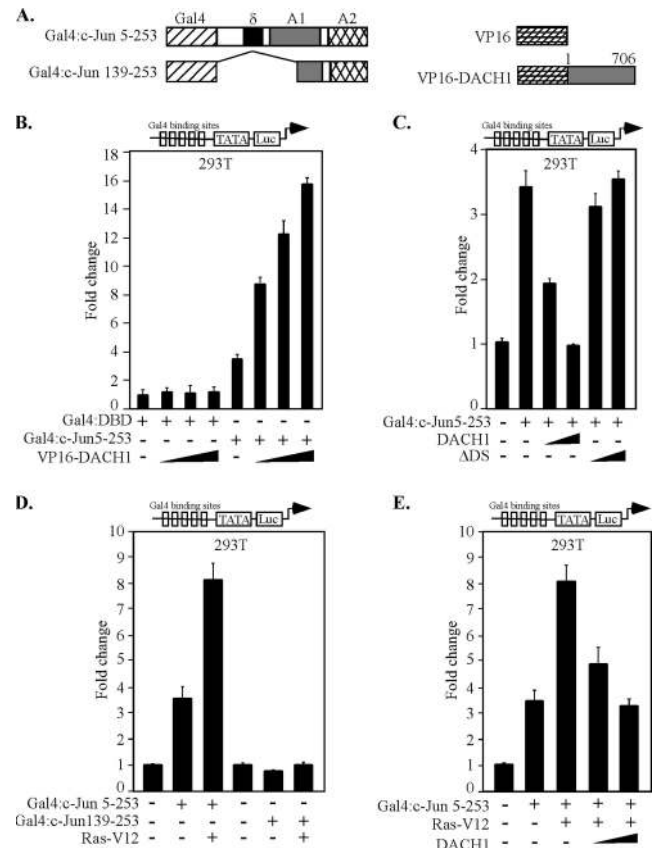
protein was enhanced by oncogenic Ras (Ha-RasV12) consistent with previous findings that Ras alleviates repression of a *c-Jun* inhibitor (Baichwal *et al.*, 1991). DACH1 expression inhibited *c-Jun* transactivation by Ha-RasV12 (Figure 8C). To determine the domains of *c-Jun* repressed by DACH1, comparison were made between *c-Jun* and the mutant *c-Jun* proteins. *c-Jun* E2 activity was repressed by DACH1 in a dose-dependent manner. In contrast, the del  $\delta$  *c-Jun* and *v-Jun* activities were not repressed by DACH1 (Figure 8D). To determine the role of endogenous DACH1 as an inhibitor of *c-Jun* transactivation function, DACH1 shRNA was used in 3T3 cells. The transactivation function of *c-Jun* was enhanced by expression of DACH1 shRNA (Figure 8E). Activity of both the *c-Jun* protein deleted of the  $\delta$  domain and *v-Jun* was constitutively more active than the corresponding *c-Jun* protein, and they were unaffected by depletion of DACH1 abundance (Figure 8E). Similarly, shRNA expression to DACH1 enhanced AP-1 activity using on AP-1-responsive reporter gene (Figure 8F). These findings suggest the  $\delta$  domain of *c-Jun* is required for endogenous DACH1 repression of *c-Jun* transactivation activity.

To examine further the mechanisms by which DACH1 repressed oncogenic Ras-induced activity of *c-Jun*, a series of Gal4-*c-Jun* expression vectors were used (Baichwal and Tjian, 1990) (Figure 9A). The interaction between DACH1 and *c-Jun* was assessed using a mammalian two-hybrid system, and activity of *c-Jun* was determined using a multimeric Gal4 DNA binding site linked to a luciferase reporter gene (Figure 9B). VP16-DACH1 demonstrated no functional interaction with the Gal4-DBD. Cointroduction of VP16-DACH1 enhanced transactivation with Gal4-*c-Jun*, indicating functional interaction between DACH1 and *c-Jun* and the induction of transactivation by the recruitment of the potent acidic transactivation surface of VP-16 to the minimal E1B promoter. Thus, DACH1 functionally interacts with *c-Jun* in a mammalian two-hybrid assay (Figure 9B). To determine the effect of DACH1 expression on *c-Jun* activity, DACH1 was coexpressed with an expression vector encoding Gal4-*c-Jun* 5-253 and *c-Jun* activity determined using



**Figure 8.** DACH1 inhibits c-Jun transactivation, requiring the  $\delta$  domain. (A) Schematic representation of c-Jun expression vectors. (B) The HEK 293T cells transiently transfected with expression vectors encoding either wild-type or mutant c-Jun as an E2 fusion proteins or a DACH1 mammalian expression vector. Western blot analysis is shown for antibodies to either c-Jun (E2) or DACH1 (Flag). GDI is used as a protein loading control. (C and D) HEK293T cells were transiently transfected with a luciferase reporter encoding a multimeric E2 binding site linked to a luciferase reporter [(E2)<sub>6</sub> LUC] and the mammalian expression vectors encoding either c-Jun-E2, Ha-Ras V12, or a DACH1 mammalian expression vector as indicated. Luciferase activity is shown as -fold change normalized to a  $\beta$ -galactosidase reporter gene used to normalize for transfection efficiency. Data are expressed as mean  $\pm$  SEM for  $n = 7$  separate transfections. (E) 3T3 cells were transfected with the vectors described in D, and the effect of shRNA to endogenous Dach1 was assessed. (F) Activity of an AP-1-responsive reporter gene was determined upon reduction of endogenous Dach1 through shRNA.

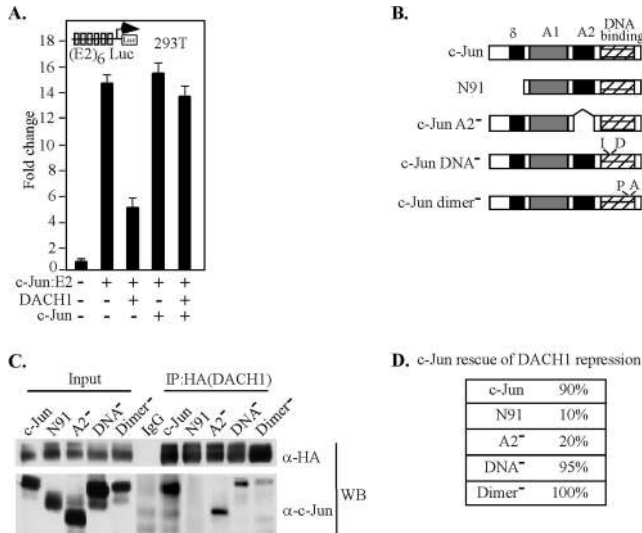
luciferase reporter activity. DACH1 repressed Gal4-c-Jun activity (Figure 9C). The DACH1 mutant  $\Delta$ DS, however, was incapable of repressing Gal4-c-Jun activity (Figure 9C). These findings are consistent with the previous studies, in which DACH1 inhibition of AP-1 activity required the DS domain. The activity of Gal4-c-Jun was enhanced by oncogenic Ras, however, deletion of amino acids 1-139 abrogated Ras-induced transactivation (Figure 9D), consistent with previous findings (Baichwal *et al.*, 1991). DACH1 inhibited c-Jun activation by Ras (Figure 9E). Together, these results demonstrate, first, that DACH1 inhibits oncogenic Ras-induced transactivation of c-Jun; second, that DACH1 associates with and inhibits c-Jun activity; third, that physical association of DACH1 with c-Jun is necessary but insufficient for repression; and fourth, that the c-Jun  $\delta$  domain is required for DACH1 repression.



**Figure 9.** DACH1 and c-Jun interact in mammalian two-hybrid system. (A) Schematic representation of c-Jun protein linked to a Gal4 binding domain or DACH1, linked to the VP16 transactivation domain. HEK293T cells were transfected with a multimeric luciferase reporter gene UAS<sub>5</sub> E1B TATA-LUC and mammalian expression vectors encoding the Gal4 DNA-binding domain linked to c-Jun with VP16-DACH1 (B) or DACH1 (C). HEK293T cells were transiently transfected with a luciferase reporter UAS<sub>5</sub> E1BTATA-LUC and mammalian expression vectors for c-Jun, Ras V12, or DACH1 as shown (D–E). Data are mean  $\pm$  SEM of  $n = 7$  separate transfections normalized to the  $\beta$ -galactosidase for transfection efficiency.

To examine further the mechanisms by which DACH1 represses c-Jun, we compared the properties of DACH1-mediated repression of c-Jun, with the properties of a previously described inhibitor of c-Jun. Previous studies using *in vivo* competition assays identified an inhibitor of c-Jun activity (Baichwal and Tjian, 1990; Baichwal *et al.*, 1991). In these prior studies, coexpression of the mammalian expression vector encoding either the wild-type or mutant c-Jun was used to identify the domains of c-Jun required for interaction with the  $\delta$  domain inhibitory factor (Baichwal and Tjian, 1990; Baichwal *et al.*, 1991). In our experiments, the ability of DACH1 to repress activity of c-Jun-E2 was overcome by c-Jun expression in *trans* (Figure 10A, lane 3 versus lane 5). To determine the domains of c-Jun capable of binding DACH1, immunoprecipitation Western blot analysis were conducted using truncated or mutant c-Jun (Figure 10B). The c-Jun mutants were detectable with a c-Jun antibody directed to the carboxy terminus of c-Jun. Immunoprecipitation of DACH1 with an antibody directed to the HA epitope of DACH1 coprecipitated c-Jun. Deletion of the N-terminal 91 amino acids of c-Jun abolished the binding of DACH1 to c-Jun. A c-Jun protein deleted of the A2 domain





**Figure 10.** DACH1 repression of c-Jun activity is rescued in trans by c-Jun requiring the N terminus. (A) HEK293T cells were transiently transfected with a multimeric E2 binding site linked to a luciferase reporter gene (E2)<sub>6</sub>-LUC and mammalian expression vectors encoding c-Jun-E2, DACH1 or c-Jun. (B) Schematic representation of mammalian c-Jun expression vectors. (C) Western blot analysis of HEK293T cells transiently transfected with c-Jun wild-type or mutant expression vectors with DACH1. HA epitope of DACH1 expression vector was used to immunoprecipitate DACH1. Coprecipitation of c-Jun is shown using a c-Jun-specific antibody. (D) Rescue of DACH1 repression by mammalian c-Jun mutant expression vectors. Rescue is shown as a percentage of wild-type c-Jun for each mutant. Data are mean  $\pm$  SEM of  $n = 6$ .

or mutated of either the DNA binding or dimerization domains remained capable of binding DACH1 (Figure 10C). Mutated of either the DNA binding domain or the dimerization domain, each rescued DACH1 repression. But deletion of the amino-terminal 91 amino acids (N91) and A2 domain resulted in a mutant that failed to overcome DACH1 repression (Figure 10D). Together, these findings are consistent with a model in which DACH1 repression involves an interaction in trans, which requires the c-Jun  $\delta$  and A2 domains but is independent of the c-Jun dimerization and DNA binding domains. DACH1 binding to c-Jun is insufficient for repression as DACH1 bound a c-Jun mutant deleted of the A2 domain; yet, this mutant failed to rescue DACH1 repression in trans. Together, these findings indicated the c-Jun  $\delta$  and A2 domains interact in trans to mediate DACH1 repression of c-Jun.

## DISCUSSION

In the current study, DACH1 inhibited DNA synthesis and cellular proliferation in 3T3 cells. Deletion of the c-Jun gene by using *c-jun*<sup>fl/fl</sup> 3T3 cells and Cre recombinase demonstrated a requirement for c-Jun in DACH1-mediated inhibition of cellular proliferation. c-Jun overexpression rescued DACH1-mediated inhibition of DNA synthesis. c-Jun promotes G<sub>1</sub> phase progression and c-Jun is necessary for G<sub>0</sub>/G<sub>1</sub>- and S-phase progression (Ryseck *et al.*, 1988; Kovary and Bravo, 1991). The finding that DACH1 inhibited the DNA synthetic phase of the cell cycle is consistent with the known role of c-Jun to promote G<sub>1</sub>/S-phase transition. The mechanism by which DACH1 inhibited c-Jun expression involved inhibition of AP-1 activity. c-Fos and c-Jun, which contribute to

AP-1 activity, were transcriptionally repressed by DACH1. c-Jun abundance is regulated in part by *c-Jun* gene transcription (Angel *et al.*, 1988; Treier *et al.*, 1994; Fuchs *et al.*, 1996; Musti *et al.*, 1997). The *c-Jun* promoter, which is autoregulated by AP-1 activity mediated through an AP-1 binding site (Angel *et al.*, 1988; Kayahara *et al.*, 2005) was repressed by DACH1. Thus, c-Jun expression and transcription were both inhibited by DACH1.

The current study identifies DACH1 as a c-Jun repressor protein, extending prior observations that c-Jun binds JDP1 and JDP2 (Piu *et al.*, 2001). Recent studies using genome-wide microarray analysis suggested a potential new mechanism controlling AP-1 activity (Wu *et al.*, 2003). In these studies, inducible expression of the cell fate determination factor DACH1 inhibited expression of the genes governing cellular migration, adhesion, and growth, many of which are AP-1 responsive (Wu *et al.*, 2003). The abundance and activity of c-Jun is a critical determinant of tumor progression and contact independent growth. Compelling evidence for a key role of c-Jun in tumor progression was the finding that disruption of the *c-jun* gene in murine hepatocytes prevents the emergence of hepatocellular carcinoma (Eferl *et al.*, 2003). DACH1 repression of c-Jun expression provides an additional mechanism controlling c-Jun abundance and function. DACH1 protein is lost during tumor progression, providing a mechanism by which c-Jun activity may be increased, contributing to contact-independent growth. Thus, the current study suggests an additional mechanism by which c-Jun transformation function is kept in check through the endogenous inhibitory protein DACH1.

c-Jun is sufficient for the induction of anchorage-independent growth of Rat1a cells (Schutte *et al.*, 1989; Katabami *et al.*, 2005). Herein, c-Jun-induced contact-independent growth was blocked by the cell fate determination factor DACH1. DACH1 inhibition of c-Jun-induced cellular growth required a conserved domain (DS domain). c-Jun acts in conjunction with Ras-V12 to transform rodent fibroblasts (Alani *et al.*, 1991; Binetruy *et al.*, 1991). c-Jun is required for transformation by several collaborative oncogenes, including *Ras*, *c-Fos*, *Raf*, *c-Myc*, *Mos*, and *Abl* (Rapp *et al.*, 1994; Johnson *et al.*, 1996). The finding that DACH1 blocks c-Jun function raises the possibility that DACH1 may inhibit multiple distinct oncogenic signaling pathways that converge on c-Jun. The mechanism by which c-Jun induces cellular growth and tumor progression is not well understood (Maeda and Karin, 2003). It has been proposed that c-Jun contributes to the onset and progression of tumorigenesis, in part through c-Jun inhibition of apoptosis via a p53-dependent cellular pathway (Eferl *et al.*, 2003). The defect in proliferation of *c-jun*-deficient cells has been attributed to elevated expression of p53 and p21<sup>CIP1</sup>, and several downstream targets of c-Jun are required, but not sufficient, for induction of contact-independent growth, including stathmin, HMG 1/Y, and cyclin A (Kinoshita *et al.*, 2003; Hommura *et al.*, 2004; Katabami *et al.*, 2005). DACH1 inhibited the physiological induction by serum of the c-Jun target genes cyclin A and  $\beta$ -PAK. The expression of c-Jun with a doxycycline-inducible stable cell line (Rat1a-J4) induced expression of cyclin A. DACH1 inhibited c-Jun induction of cyclin A expression. DACH1 inhibited c-Jun-mediated induction of several known endogenous c-Jun target genes, including cyclin A and stathmin; and DACH1 was identified within the context of local chromatin of the endogenous AP-1 sites at the known c-Jun-responsive promoter of cyclin A and cyclin D1. DACH1 siRNA reduced DACH1 abundance and induced expression of the c-Jun responsive genes c-Jun and cyclin D1. Collectively, these studies demonstrate that



DACH1 is a physiological regulator of endogenous c-Jun function, inhibiting c-Jun expression and c-Jun target gene expression.

The binding and inhibition of c-Jun transactivation by DACH1 but not the structurally related Ski protein, suggests these proteins have evolved to regulate distinct subsets of transcriptional responses. Herein, the interaction of DACH1 with c-Jun was evidenced by mammalian two-hybrid and by immunoprecipitation Western blotting. DACH1 inhibited c-Jun transactivation when c-Jun was linked to a heterologous DNA binding domain. Deletion of the DS domain of DACH1 abrogated interaction with c-Jun. Similarly, deletion of the c-Jun N-terminal amino acids 1-91 abrogated binding of c-Jun to DACH1. Coexpression of c-Jun titrated the inhibitory function of DACH1, consistent with the model in which the relative abundance of c-Jun and DACH1 determined c-Jun transactivation.

In the current studies, DACH1 repression of c-Jun required the c-Jun  $\delta$  domain. Activity of the v-Jun E2 fusion protein, which deletes the  $\delta$  domain, was resistant to DACH1 repression. The c-Jun  $\delta$  domain engages cell-specific inhibitors of c-Jun (Baichwal and Tjian, 1990). Putative inhibitors of this domain include catalytically inactive JNK (Dai *et al.*, 1995; Ljungdahl *et al.*, 1997). JNK binds and phosphorylates c-Jun at serine 63 and 73. The role of the  $\delta$  domain itself versus JNK phosphorylation within the N terminus of c-Jun for cellular DNA synthesis and transformation remains controversial. C-Jun promotion of G<sub>1</sub> phase progression seems to be independent of its phosphorylation (Wisdom *et al.*, 1999). The JNK binding site of c-Jun can also be uncoupled from its transformation capabilities in same studies (Sprowles and Wisdom, 2003). Thus, mutation of the JNK phosphorylation sites did not affect the transformation activity of c-Jun in chicken embryo fibroblasts (Vogt, 2001). Furthermore, JNK-dependent phosphorylation of the c-Jun N terminus may not be required for Ras-induced cellular transformation (Johnson *et al.*, 1996; Kennedy *et al.*, 2003). In contrast with these studies, mice and cells encoding a mutant allele of c-jun in which the JNK phosphorylation site has been mutated demonstrated an important role for these residues in c-jun-dependent transformation (Behrens *et al.*, 2000). Furthermore, although mutation of serine 63 and 73 in v-Jun did not alter transforming activity of v-jun, mutation of these residues in c-jun reduced cooperation with Ha-Ras in oncogenic transformation (Binetruy *et al.*, 1991). The current study is consistent with a role for DACH1 as an inhibitor of the c-Jun  $\delta$  domain.

The transactivational potential of c-Jun and its oncogenic activity in cooperation with Ras are often correlated (Alani *et al.*, 1991; Smeal *et al.*, 1991), although exceptions exist in avian and yeast systems (Vogt, 2001). In the current study, DACH1 inhibited c-Jun-mediated transactivation. DACH1 binding to the N-terminal domain of c-Jun may repress c-Jun activity by recruiting a corepressor or interfering with coactivator binding. CBP binds to the N-terminal region of c-Jun (Bannister *et al.*, 1991; Mayr and Montminy, 2001) as does RH2/Gu-RNA-helicase (Westermarck *et al.*, 2002). The corecruitment of histone deacetylase proteins HDAC1 and HDAC3 by DACH1, to c-Jun and thereby to an AP-1 binding site, provides one mechanism by which DACH1 may regulate AP-1 signaling. Recent studies suggested that the c-Jun  $\delta$  domain may interact with a yet unidentified protein that recruits HDAC3 (Weiss *et al.*, 2003). The relative importance of DACH1 in recruitment of histone deacetylase to c-Jun remains to be further explored.

Herein, DACH1 repressed the *c-fos* promoter. DACH1 transcriptional repression was abolished by point mutation

of the SRE site. In the current study, serum-induced *c-fos* promoter activity was reduced by point mutation in either the TCF or SRE binding site. Extracellular stimuli enhance TCF transcriptional activity through the MAPK family of extracellular regulated kinases (extracellular signal-regulated kinases, JNKs, and p38s), which phosphorylate the TCF transactivation domain (Whitmarsh *et al.*, 1995, 1997). The SRF site of the *c-fos* promoter is induced by RhoA independently of MAPKs (Sotiropoulos *et al.*, 1999; Miralles *et al.*, 2003). RhoA induction of SRF involves ROCK and phosphorylation of LIM kinase (Hill *et al.*, 1995). Activation of SRF involves a decrease in the cellular pool of monomeric actin (Sotiropoulos *et al.*, 1999). LIM kinase-dependent phosphorylation of cofilin induces the stabilization of polymerized actin (F-actin), which is sensed by SRF to induce its activity (Sotiropoulos *et al.*, 1999; Miralles *et al.*, 2003). DACH1 regulates cytoskeletal proteins and their function as evidenced by genome-wide analysis of molecular genetic targets (Wu *et al.*, 2003). Although speculative, the finding that DACH1 inhibits *c-fos* promoter activity through the SRF site is consistent with a role for DACH1 in regulating a RhoA/ROCK/LIM kinase cytoskeletal signaling pathway.

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