DACH1 Inhibits Transforming Growth Factor-β Signaling through Binding Smad4*[S]

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Kongming Wu‡, Ying Yang§, Chenguang Wang‡, Maria A. Davoli, Mark D’Amico, Anping Li‡, Kveta Cveklova§, Zbynek Kozmik, Michael P. Lisanti, Robert G. Russell‡, Ales Cvekl§**, and Richard G. Pestell‡ ‡‡

From the §Lombardi Cancer Center, Department of Oncology, Georgetown University, Washington, D. C. 20057, the $Department of Ophthalmology and Visual Sciences and Molecular Genetics, the ‡Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York 10461, and the ¶Institute of Molecular Genetics, 166 37 Prague 6, Czech Republic

The vertebrate homologues of Drosophila dachshund, DACH1 and DACH2, have been implicated as important regulatory genes in development. DACH1 plays a role in retinal and pituitary precursor cell proliferation and DACH2 plays a specific role in myogenesis. DACH proteins contain a domain (DS domain) that is conserved with the proto-oncogenes Ski and Sno. Since the Ski/Sno proto-oncogenes repress AP-1 and SMAD signaling, we hypothesized that DACH1 might play a similar cellular function. Herein, DACH1 was found to be expressed in breast cancer cell lines and to inhibit transforming growth factor-β (TGF-β)-induced apoptosis. DACH1 repressed TGF-β induction of AP-1 and Smad signaling in gene reporter assays and repressed endogenous TGF-β-responsive genes by microarray analyses. DACH1 bound to endogenous NCoR and Smad4 in cultured cells and DACH1 co-localized with NCoR in nuclear dotlike structures. NCoR enhanced DACH1 repression, and the repression of TGF-β-induced AP-1 or Smad signaling by DACH1 required the DACH1 DS domain. The DS domain of DACH was sufficient for NCoR binding at a Smad4-binding site. Smad4 was required for DACH1 repression of Smad signaling. In Smad4 null HTB-134 cells, DACH1 inhibited the activation of SBE-4 reporter activity induced by Smad2 or Smad3 only in the presence of Smad4. DACH1 participates in the negative regulation of TGF-β signaling by interacting with NCoR and Smad4.

The pleiotropic transforming growth factor-β (TGF-β)1 family of cytokines regulate diverse biological functions through transmembrane Ser/Thr kinase receptors. The inhibition of epithelial cell proliferation, production of extracellular matrix components, regulation of differentiation, and apoptosis by TGF-β involve a tightly coordinated signaling pathway (1). Numerous components of the TGF-β pathway are tumor suppressors that are functionally mutated in cancer (2), and TGF-β1, the first member of the TGF-β family, plays an important role in cancer, including breast cancer, functioning both as an antiproliferative factor and as a tumor suppressor (3, 4). Upon ligand binding, a heterodimeric complex forms between the type I and type II receptor, with the type II receptor transphosphorylating the type I receptor. The activated type I receptor interacts with an adaptor protein, SARA, which recruits Smad2 and Smad3 to serve as phosphorylation substrates of the type I receptor.

The Smad family of transcription factors participates in TGF-β signaling at multiple levels (3). Phosphorylated pathway-restricted Smad2 and Smad3 form heterodimers with the common mediator Smad4 in the cytoplasm and translocate into the nucleus, where they bind Smad-binding elements (SBEs) at specific promoters of genes regulating cell growth (5, 6). Alternatively, Smad complexes bind DNA in conjunction with other DNA-binding proteins such as FAST1 and FAST2. DNA-bound Smad complexes regulate transcription, either positively through recruitment of coactivators of the p300/CREB-binding protein class (7) or negatively by recruiting the Sno/Ski family (8, 9). Interaction of Smad3 with Ski and Sno allows formation of a DNA-binding complex that represses transcription of TGF-β-responsive genes (9, 10). Thus, overexpression of Ski antagonizes the normal response to TGF-β signaling (i.e., inhibition of cell growth) and enables the cells to grow in the presence of TGF-β (11).

Understanding the specificity of TGF-β signal transduction pathway is critically dependent upon identifying factors in the cellular environment and key cellular components within this signal transduction pathway. To identify and functionally characterize the candidate co-regulators contributing to TGF-β signaling, we hypothesized that proteins structurally related to important components mediating the TGF-β signal transduction pathway are likely to play significant roles. The Ski/Sno proteins share structural homology with the Dach protein (12). The founding member of the DACH subfamily of nuclear proteins, Drosophila dachshund (dac), is an essential gene regulating the development of eye and leg (13). The Dach N-BOX (the dac and sklsno DS domain) (12, 14, 15) consists of ~100

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1 The abbreviations used are: TGF-β, transforming growth factor-β; FACS, fluorescence-activated cell sorting; TBS, Tris-buffered saline; SBE, Smad-binding element; CREB, cAMP-response element-binding protein.

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Drosophila dac is a component of a genetic network, including eyeless (ey), sine oculis (so), and eyes absent (eya) that regulates proliferation and differentiation of the eye imaginal disk epithelium. Two vertebrate homologues (DACH1 and DACH2 in humans, Dach1 and Dach2 in mouse and chicken) have been cloned, and their expression patterns have been characterized (12, 14, 15, 18–22). It has been proposed that Dach1 and Dach2 are partially functionally redundant, since Dach1−/− mice survive to birth but exhibit postnatal lethality associated with a failure to suckle, cyanosis, and respiratory distress (19). Dach1 is a direct target gene for fibroblast growth factor signaling during limb skeletal development (23). Muscle development is regulated by a synergistic interaction of Dach2, Pax3, Eya2, and Six1 (18). This group of genes is composed of vertebrate genes structurally and functionally related to the Pax3, Eya2, and Six1 (18). This group of genes is composed of factor signaling during limb skeletal development (23). Muscle distress (19). 

DACH1 inhibits TGF-β signaling through binding Smad4. These studies thus identify DACH1 as a regulator of this complex pathway.

Materials and Methods

Plasmid Construction—The full-length DACH1, DACH1 DS domain alone (DS), or DACH1 DS domain deleted (ΔDS) were cloned to pKW10 vector containing N-terminal FLAG peptide. The FLAG-tagged DACH1 cDNA was subcloned into the pIND vector (Clontech) to produce pN-D-FLAG-DACH1. The DACH1 cDNA was also subcloned into the vectors to form HA-DACH1. CS2-FLAG Smad2 was a gift from Dr. J. Massague, CMV2-FLAG Smad3 was from Dr. Chang (25), pCMV5-HA-Smad4 was from Dr. Bottinger (26), and the FLAG-tagged NCoR expression vector was from Dr. Rosenfeld (24). The reporter plasmids 3TP Lux and SBE-4 Luc were previously described (27). Ski and Sno cDNAs were obtained from Dr. Ishii (28) and subcloned into 3TP vector (Sigma).

Western Blotting and Immunohistochemistry—Western blot analysis was conducted as previously described with minor changes (29). Proteins were separated by electrophoresis in 6–10% graded polyacrylamide gel and transferred to nitrocellulose filters, immunoblotted with anti-Smad4, anti-Smad2/3, anti-NCoR, anti-mSin3A antibody (Santa Cruz Biotechnology), anti-phosphorylated Smad2/3 (Upstate Biotechnology, Inc., Lake Placid, NY), or anti-FLAG M2 antibody (Sigma). The bands were detected using the enhanced chemiluminescence detection system (Amersham Biosciences). Guanine nucleotide dissociation inhibitor antibody, a generous gift from Dr. Perry Bickel (Washington University, St. Louis, MO) (30) was used as an internal control for protein abundance. We generated anti-DACH1 antibody by hyperimmunizing rabbits with purified DACH1 DS domain peptide. For purification of DACH1 DS domain antibody, 2 ml of immune serum diluted to 40 ml was bound to 2 ml of protein-A agarose beads (Sigma) as a column overnight. The bound immunoglobulin was eluted with a high salt method using 2 M NaCl, and the immunoglobulin concentration was estimated using the spectrophotometer. This antibody was used for Western blotting. Immunostaining of the mouse embryo tissues was performed in a manner similar to a previous method (31) using a polyclonal DACH1 antibody that was a gift from Dr. G. Nuckolls (23).

Cell Culture, Luciferase Reporter Assays, and Fluorescence-activated Cell Sorting—HaCaT, MDA-MB-231, MCF10A, Panc-1, HTB-134, and 293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin/streptomycin. The cells were maintained in a humidified atmosphere with 5% CO2 at 37 °C. Human recombinant TGF-β1 is from Calbiochem. Transfections were performed using Superfect transfection reagent (Qiagen, Valencia, CA) according to the manufacturer’s protocol. Stable MDA-MB-231 cell lines were generated expressing the VgEcR/RXRα plasmid and the (EGRE)3 FLAG DACH1 plasmid. Colonies selected with Zeocin (400 µg/ml) and G418 (500 µg/ml) were analyzed for low expression of DACH1 vectors in 293T cells assessed by Western blotting. Guanine nucleotide dissociation inhibitor antibody (GDI) is a loading control. D, immunohistochemical staining for DACH1 of 15.5-day embryos showing DACH1 in cells of the cochlea duct, retina, and some staining of the ectoderm surrounding the eye. E, Western blot analysis detects DACH1 in 293T cells transfected with the DACH1 expression vector and in the epithelial cells lines.
basal and robust inducibility by Western blot analysis of the FLAG epitope.

For reporter gene assays, cells were transiently transfected with an appropriate combination of the reporter, expression plasmids, and control vector. In some experiments, cells were serum-starved for 36 h and stimulated with or without TGF-β for 12 h before collecting cells for

![Figure 2](image1.png)

**Fig. 2.** **DACH1 repression of AP-1 and Smad reporter activity.** A, HaCaT cells were transfected with 3TP Lux or SBE-4 Luc reporter, serum-starved for 36 h, and then stimulated with TGF-β for 12 h. Data is shown as mean ± S.E. for luciferase activity of n=9 separate transfections. B, HaCaT cells were transfected with 3TP Lux or SBE-4 Luc, and Ski or Sno expression plasmids were then incubated with TGF-β at 1 ng/ml for 12 h before luciferase assays. HaCaT cells were transfected with either 3TP Lux (C) or SBE-4 Luc (D) reporters, DACH1 expression vector, and treated with TGF-β for 12 h.

![Figure 3](image2.png)

**Fig. 3.** **DACH1 repression of TGF-β signaling.** MDA-MB-231 (A) or Panc-1 (B) cells were co-transfected with the SBE-4 Luc reporter and treated with TGF-β (1 ng/ml) for 12 h. The data represent mean ± S.E. for n=9.

![Graphs](image3.png)
luciferase assay. The transfection efficiency was normalized by co-transfection with 0.2 μg of pRL-CMV plasmid (Promega, Madison, WI) and was measured with the Promega dual-luciferase reporter assay system according to the manufacturer's protocol. Luciferase assays were performed at room temperature using an Autolumat LB 953 (EG&G Berthold) (32). Statistical analyses were performed using Student's t test, and significant differences were established as p < 0.05. FACS analysis was used to determine the proportion of cells in the sub-G1 or apoptotic phase as previously described (33).

Microarray Analysis—mRNA was prepared from MDA-MB-231 pIND-DACH1 stable lines, treated with either vehicle or ponasterone A (2 μg/ml) for serial time points using Trizol reagent (Invitrogen). Following DNase I treatment (Takara Bio Inc., Japan) according to the manufacturer's instructions, total RNA was amplified according to the Eberwine procedure (34) using the Ambion MessageAmp™ kit (Ambion). During in vitro transcription, biotin-11-CTP and biotin-16-UTP (Enzo Diagnostics, Farmingdale, NY) were incorporated. 20 μg of the biotinylated cRNA product was fragmented at 94 °C for 35 min. The
sample was used for each hybridization reaction. Hybridization to a set of two Affymetrix U133A GeneChips (representing ~22,000 open reading frames and at least 17,000 genes) was performed overnight, followed by staining and washing as per the manufacturer’s instructions. The clean processed chips were then scanned using Agilent GeneArray scanner. Grid alignment and raw data generation were performed using Affymetrix GeneChip 5.0 software. For quality control, oligonucleotide scanner. Grid alignment and raw data generation were performed using Affymetrix GeneChip 5.0 software. For quality control, oligonucleotide washing procedures were performed. Raw expression values, representing the average difference in hybridization intensity between oligonucleotides that perfectly match the transcript sequence and oligonucleotides containing single base pair mismatches, were measured. A noise value (Q) based on the variance of low intensity probe cells was used to calculate a minimum expression threshold (2.1\(\times\)Q) for each chip.

**Gene Selection**—Data generated after scanning were subjected to comparison analysis to select change calls at 100% increase or decrease compared with vehicle control at each time point. The data selected after comparison analysis were further filtered based on absolute analysis using the Mann-Whitney test and detection calls, and 422 genes were selected for multidimensional scaling and hierarchical clustering.

**Multidimensional Scaling and Cluster Analysis**—Multidimensional scaling (Matlab) was used to visualize the differences between control and treated samples selected above (see “Gene Selection”). To measure distance, the Pearson correlation coefficient was applied to each pair of genes, those that were selected above (see “Gene Selection”) and treated (●), control (●), and treated (●, ▲) samples. To visualize expressions of genes, those that were selected above (see “Gene Selection”) and intra- and intersample pairs, hierarchical clustering was performed using Cluster 3.0 (Stanford University). A gene list corresponding to clusters was generated using the data mining tool from Affymetrix.

**Immunoprecipitation and Immunoblotting**—293T cells and HaCaT cells were used for the detection of protein-protein interaction in vivo. Cells were transfected with the expression plasmids, cultured for 2 days, washed, scraped, and lysed in a buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, and proteinase inhibitor mixture (Sigma). Lysates were cleared by centrifugation at 4°C for 15 min. The protein concentration was measured by the Bio-Rad assay. 500 µg of total protein was incubated with anti-FLAG M2 antibody (Sigma) or anti-Smad4 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

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### Table I

List of genes with altered expression by DACH1 and known response to TGF-β

| Probe | Name      | Function                  | DACH1 | Ref. 48 | Ref. 47 | Ref. 4 |
|-------|-----------|---------------------------|-------|---------|---------|-------|
| 204451| FZD1      | Signal transduction       | ↓     | ↓       | ↑       | ↓     |
| 203897| FZD6      | Signal transduction       | ↓     | ↑       | ↓       | ↓     |
| 202454| ERBB3     | Signal transduction       | ↓     | ↓       | ↑       | ↓     |
| 209457| DUSP5     | Signal transduction       | ↓     | ↓       | ↑       | ↓     |
| 20905 | CSF-1     | Signal transduction       | ↓     | ↑       | ↓       | ↓     |
| 206277| P2Y       | Signal transduction       | ↓     | ↑       | ↓       | ↓     |
| 202393| TIEG      | Signal transduction       | ↓     | ↑       | ↓       | ↓     |
| 203508| TNFRSF1B  | Signal transduction       | ↓     | ↑       | ↓       | ↓     |
| 201382| SISP      | Signal transduction       | ↓     | ↓       | ↑       | ↓     |
| 204189| RARG      | Signal transduction       | ↓     | ↓       | ↑       | ↓     |
| 206277| P2RY2     | Signal transduction       | ↓     | ↑       | ↓       | ↓     |
| 202672| ATF3      | Transcription factor      | ↓     | ↑       | ↓       | ↓     |
| 201882| B4GALT1   | Transcription factor      | ↓     | ↑       | ↓       | ↓     |
| 202081| ETR101    | Transcriptional regulation| ↑     | ↑       | ↓       | ↓     |
| 209189| FOS       | Transcriptional regulation| ↑     | ↑       | ↓       | ↓     |
| 205251| PER2      | Transcriptional regulation| ↑     | ↑       | ↓       | ↓     |
| 32088 | JEM-1     | Transcriptional regulation| ↑     | ↑       | ↓       | ↓     |
| 209290| MECP2     | Transcriptional regulation| ↑     | ↑       | ↓       | ↓     |
| 210392| NR6A1     | Transcriptional regulation| ↑     | ↑       | ↓       | ↓     |
| 216330| POU6F1    | Transcriptional regulation| ↑     | ↑       | ↓       | ↓     |
| 204536| REST      | Transcriptional regulation| ↑     | ↑       | ↓       | ↓     |
| 210765| CSE1L     | Cell division             | ↑     | ↑       | ↓       | ↓     |
| 201372| Culin     | Cell growth               | ↑     | ↑       | ↓       | ↓     |
| 200983| Cyclin D2 | Cell cycle regulator      | ↑     | ↑       | ↓       | ↓     |
| 205899| Cyclin A2 | Cell cycle regulator      | ↑     | ↑       | ↓       | ↓     |
| 210550| Cdc25A    | Cell cycle regulator      | ↑     | ↑       | ↓       | ↓     |
| 208937 |Id1       | Cell cycle regulator      | ↑     | ↑       | ↓       | ↓     |
| 204200| PDGF      | Cellular communication    | ↑     | ↑       | ↓       | ↓     |
| 203725| GADD45    | Cellular communication    | ↑     | ↑       | ↓       | ↓     |
| 204542| STHM      | Adhesion/Matrix           | ↑     | ↑       | ↓       | ↓     |
| 219213| JAM       | Adhesion molecule         | ↑     | ↑       | ↓       | ↓     |
| 205032| ITGA2     | Adhesion/Extracellular matrix| ↑ | ↑       | ↓       | ↓     |
| 202837| ICAM1     | Adhesion/Extracellular matrix| ↑ | ↑       | ↓       | ↓     |
| 204475| MMP1      | Matrix remodeling         | ↑     | ↑       | ↓       | ↓     |
| 205871| PLGL      | Matrix remodeling         | ↑     | ↑       | ↓       | ↓     |
| 206924 |IL1       | Cytokine                  | ↑     | ↑       | ↓       | ↓     |
| 218995| EDN1      | Secreted                  | ↑     | ↑       | ↓       | ↓     |
| 205067| IL1B      | Secreted                  | ↑     | ↑       | ↓       | ↓     |
| 211259| BMP7      | Secreted                  | ↑     | ↑       | ↓       | ↓     |
| 219561| COP2Z     | Vesical endocytosis       | ↑     | ↑       | ↓       | ↓     |
| 204734| KRT15     | Cytoskeleton              | ↑     | ↑       | ↓       | ↓     |
| 214122| ENIGMA    | Cytoskeleton scaffolding  | ↑     | ↑       | ↓       | ↓     |
| 201586| SFPQ      | RNA processing            | ↑     | ↑       | ↓       | ↓     |
| 208673| SFRS3     | RNA processing            | ↑     | ↑       | ↓       | ↓     |
| 202525| PRSS8     | Proteolysis               | ↑     | ↑       | ↓       | ↓     |
| 209240| OGT       | Metabolism                | ↑     | ↑       | ↓       | ↓     |
| 214307| HGD       | Metabolism                | ↑     | ↑       | ↓       | ↓     |
| 221765| UCCG      | Metabolism                | ↑     | ↑       | ↓       | ↓     |
| 205681| BCL2A1    | Miscellaneous             | ↑     | ↑       | ↓       | ↓     |
| 221566| NOL3      | Miscellaneous             | ↑     | ↑       | ↓       | ↓     |
| 204351| S100P     | Miscellaneous             | ↑     | ↑       | ↓       | ↓     |

*See Ref. 37.*
DACH1 inhibits TGF-β Signaling through Binding Smad4

RESULTS

DACH1 Represses TGF-β Signaling—The amino acid sequence of Dachshund has significant homology with the Ski and Snø family of proto-oncogenes, greatest in the region of DACH box-N (DS domain) (28% sequence identity with the vertebrate Ski and Snø proteins) (Fig. 1A). This domain of Ski has been implicated in transformation and the induction of myogenesis. Additional weak homology is found between DACH1 and NCoR (28). A mixture of 1.5 µg of FLAG-NCoR expression plasmid and 1 µg of the plasmid encoding DACH1 was transfected into HaCaT cells. Forty hours after transfection, cells were fixed and stained with anti-FLAG polyclonal antibodies (Sigma) and the anti-HA monoclonal antibody (Santa Cruz Biotechnology). The DACH1 and NCoR signals were visualized by Cy3- and FITC-conjugated donkey anti-secondary antibodies (Jackson Immunoresearch), respectively, and analyzed by confocal microscopy.

followed by incubation with protein G-Sepharose beads. The beads were washed five times with buffer containing 0.5% Tween 20 instead of 1% Triton X-100. The immunoprecipitates were eluted by boiling for 5 min in SDS sample buffer (100 mM Tris-Cl, 10 mM dithiothreitol, 4% SDS) and subjected to SDS-gel electrophoresis.

Protein Purification and Immunodepletion—500 µg of anti-FLAG M2 gel (Sigma) was equilibrated in TBS (50 mM Tris, pH 7.4, 0.15 mM NaCl). 1 ml of whole cell extract derived from 293T cells, transfected with expression vector-encoding FLAG-DACH1, was incubated with the gel in TBS/1% Triton X-100, washed five times with buffer containing 0.5% Tween 20 instead of 1% Triton X-100. The supernatant was saved as immunoprecipitated DACH1 whole cell extract for further Western blot and EMSA. The gel was washed five times with 1 ml of TBS/1% Triton X-100, followed by a 30-min incubation of TBS/EDTA (TBS containing 2 mM EDTA) at room temperature. FLAG-tagged DACH1 was incubated and eluted by five sequential 50-µl TBS/EDTA elutions. 10 µl of FLAG-DACH1-transfected 293T whole cell extract, 10 or 20 µl of immunoprecipitated DACH1 whole cell extract, and 5, 10, or 20 µl of purified FLAG-DACH1 was loaded on 4–15% precast protein gel (Bio-Rad). Electrophoresis was conducted at 150 V for 1.5 h at 4 °C, and samples were transferred to the 0.2-µm nitrocellulose membrane (Bio-Rad) at 100 V for 2 h at 4 °C. Blocking with 5% nonfat milk (Bio-Rad) at room temperature for 2 h and a blot with 1:1000 diluted anti-FLAG M2 monoclonal antibody (Sigma) were performed. Membranes were washed and blotted with anti-mouse antibody for 1 h at room temperature. The fluorescence signal was detected after SuperSignal treatment (Pierce).

Electrophoretic Mobility Shift Assays—2 µl of 293T whole cell extracts prepared from cells transfected with Smad4, 1 µl of whole cell extract transfected with DACH1, 1 µl of immunoprecipitated DACH1, and 1 µl of purified DACH1 were incubated with 1 µg of poly(D-I-C) in the presence or absence of specific self-oligo competitor on ice for 10 min. The FAST-ISEB oligonucleotide (5' GTCCCTAAAAGTGTATCTCATGGAAATGTCTGCCCTTCTCTCAG-3') (35) was end-labeled with polynucleotide kinase using [γ-32P]ATP and incubated with whole cell extracts of 293T cells at room temperature for 10 min. Protein-DNA complexes were separated by 5% native polyacrylamide gels, running at 150 V for 1.5 h at 4 °C. The gel was fixed and incubated with Amplifier (Amersham Biosciences) for 30 min to enhance the signal and dried by autoradiography.

In Vitro Expression of Protein—In vitro [35S]methionine-labeled protein was prepared by coupled transcription-translation with a Promega TNT coupled reticulocyte lysate kit (Promega, Madison, WI) using 1 µg of Smad4 expression plasmid DNA in a total of 50 µl. GST, GST-DACH1 DS/EYAD, GST-DACH1 EYAD/C-end were expressed in E. coli BL21 DE3 and purified using glutathione-Sepharose 4B. In vitro protein-protein interactions were performed as described (36). The in vitro translated protein (45 µl of Smad4) and 5 µg of purified GST protein were incubated with glutathione-Sepharose 4B beads in binding buffer at 4 °C for 6 h and then washed five times; 50 µl of binding buffer and 10 µl of 6× SDS loading buffer were added after the final wash; and the samples were denatured at 95 °C and subjected to electrophoresis on 8% SDS-polyacrylamide gel. The gel was fixed and incubated with Amplifier (Amersham Biosciences) for 30 min to enhance the signal and dried by autoradiography at −80 °C.

Subcellular Localization of DACH1 and NCoR—Subcellular localization of DACH1 and NCoR was essentially examined as described (28). A mixture of 1.5 µg of FLAG-NCoR expression plasmid and 1 µg of the plasmid encoding DACH1 was transfected into HaCaT cells. Forty hours after transfection, cells were fixed and stained with anti-FLAG polyclonal antibodies (Sigma) and the anti-HA monoclonal antibody (Santa Cruz Biotechnology). The DACH1 and NCoR signals were visualized by Cy3- and FITC-conjugated donkey anti-secondary antibodies (Jackson Immunoresearch), respectively, and analyzed by confocal microscopy.

DACH1 Represses TGF-β Signaling—The amino acid sequence of Dachshund has significant homology with the Ski and Snø family of proto-oncogenes, greatest in the region of DACH box-N (DS domain) (28% sequence identity with the vertebrate Ski and Snø proteins) (Fig. 1A). This domain of Ski has been implicated in transformation and the induction of myogenesis. Additional weak homology is found between DACH box-C and the C-terminal domain of the Ski/Snø proteins, which are believed to share an α-helical structure capable of forming coiled-coil structures upon homodimerization (Fig. 1A) (14). To examine the role of the DACH1 DS domain in DACH1 function, expression constructions encoding full-length DACH1, a DS domain-deleted mutant (ADS), and the DS domain alone (DS) were assessed in cultured cells. Western blot analysis demonstrated that all three constructs were expressed well using either the anti-FLAG antibody or an antibody to the DACH1 DS domain. The DACH1 antibody (see “Materials and Methods”) showed no cross-immunoreactivity with Ski (Fig. 1C). Immunohistochemical study was performed of the murine 15.5-day embryo. Consistent with previous observations (20), DACH1 immunoreactivity was identified within cells of the cochlea duct and retinal epithelial cells with some staining of...
experiments. 

Since the Snp and Ski proteins regulate TGF-β signaling (10), we assessed the role for DACH1 in this pathway. TGF-β increased activity of both the AP-1-responsive reporter 3TP Lux (38) and an artificial promoter containing the Smad-binding elements (SBE-4 Luc). TGF-β induced reporter activity in the human keratinocyte cell line HaCaT (Fig. 2A). Coexpression of Ski or Snp repressed TGF-β-induced activity of both reporters (Fig. 2B), consistent with previous studies (8–10). To investigate the role of DACH1 in TGF-β signaling, DACH1 expression constructs were assessed for activity on the 3TP Lux and SBE-4 Luc reporter genes. DACH1 repressed both reporters in HaCaT cells. Deletion of the DACH1 DS domain (ΔDS) activated both the 3TP Lux and SBE-4 Luc reporters (Fig. 2, C and D). Expression of the DACH1 DS domain alone (DS) abrogated transcriptional repression of these reporter genes. DACH1 also repressed TGF-β signaling to the Smad and AP-1 pathways in the human breast cancer cell line MDA-MB-231 (Fig. 3A) and the human pancreatic cancer cell line Panc-1 (Fig. 3B). Deletion of the DACH1 DS domain again converted DACH1 from a repressor to an activator of TGF-β signaling. These studies demonstrated DACH1 repressed TGF-β-regulated reporter gene activity.

To determine whether DACH1 expression antagonized TGF-β function at a cellular level, ponasterone A-inducible DACH1 stable lines were generated in MDA-MB-231 cells. The addition of ponasterone A induced expression of DACH1 as assessed by Western blotting of the FLAG epitope of the DACH1 cDNA (Fig. 4A). FACS determination of apoptosis using the sub-G1 fraction evidenced the TGF-β-mediated increase in apoptosis (140 ± 9%, n = 6). The induction of DACH1 by ponasterone A abrogated TGF-β-induced apoptosis (Fig. 4B), suggesting that at least one function of TGF-β is antagonized by DACH1 expression.

To examine whether DACH1 expression regulated endogenous AP-1- or Smad-regulated genes, genome-wide expression profiling was conducted. Global gene expression profiling has proven useful in identifying clusters of genes, representative of histological tumor subtypes, indicative of therapeutic responses (39–41), and we have thereby identified clusters of genes coordinately induced by specific oncogenes including ErbB2 and Myc (42). The inducible DACH1 cell line was analyzed by global expression profiling, and mRNA was assessed after 0, 18, or 36 h of DACH1 expression induced by ponasterone A. Experiments were conducted on three separate occasions with highly reproducible findings (Fig. 4C). Tree view analysis demonstrated that 422 genes, of ~17,000, were regulated by DACH1. Of these genes, 194 were induced, and 228 were repressed (Fig. 4C). Dachshund (Drosophila) homologue mRNA was induced by ponasterone A 25-fold at 18 h and 11-fold at 36 h. Genes that were repressed by DACH1 include well characterized AP-1 or Smad-responsive genes including c-fos, Egr1, cyclin E2, neuregulin, tumor necrosis factor-α-induced protein 3, cdc25A, FGF5, GRO3, MEF2C, ETR101, and BMP4 (Table I). To determine whether global patterns of gene expression were regulated by DACH1, pairwise distances between ponasterone A-treated and -untreated cells, assessed using the Pearson correlation coefficient, were visualized in three-dimensional space using multidimensional scaling. These approaches are similar to those used in the past to represent global relationships between tumors at the level of gene expression (43) and to understand mammary gland development (44). These analyses of DACH1-mediated gene expression demonstrated that the induction of DACH1 led to discrete regions of gene expression space that were separable from other survey points. A clear trend in these points was observed from 0 to 18 and 36 h (Fig. 4D).

Because interaction of the Ski protein with Smads was previously shown to be required to regulate TGF-β signaling, we assessed the role of Smad proteins in DACH1-mediated repression of TGF-β signaling. Smad2 and Smad4 induced 3TP Lux reporter and expression vectors for DACH1 and Smad with or without Smad. The luciferase data represent mean ± S.E. for n = 6 separate experiments.
activity 4-fold in HaCaT cells. Coexpression of DACH1 or Ski repressed AP-1 activity, and deletion of the DACH1 DS domain abrogated repression by Smad2/Smad4 (Fig. 5A). The induction of AP-1 activity by Smad2 and Smad3 was reduced by greater than 90% by either DACH1 or Ski, consistent with previous studies of Ski (10). The same activity was found using the SBE-4 reporter, in which Smad2/Smad4 or Smad3/Smad4 activated transcription while deletion of the DACH1 DS Domain abolished repression (Fig. 5B). Upon stimulation, the receptor-regulated R-Smads accumulate in the nucleus with the co-

Fig. 7. p300 and NCoR in DACH1 repression of AP-1. A, HaCaT cells were transfected with 3TP Lux and expression vectors for DACH1 and p300 and treated with TGF-β. The luciferase data represent mean ± S.E. for n = 6. B, MDA-MB-231 or HaCaT cells were transfected with the 3TP Lux reporter (500 ng) together with expression vectors for other DACH1 (75 ng) or NCoR (150 ng) or both vectors. Cells were treated either with or without TGF-β for 12 h. C, MDA-MB-231 cells cotransfected with an SBE-4 reporter and Ski or DACH1 expression constructs. After serum starvation for 36 h, cells were stimulated with TGF-β for 12 h, and luciferase assays were performed. D, HaCaT cells transfected with the p21CIP1 and c-jun promoter luciferase reporters and DACH1 expression vectors and treated with TGF-β as indicated. Data represent mean ± S.E. of n = 9 separate transfections. E, HaCaT cells were transfected with DACH1 and treated with or without TGF-β, and FACS-sorted transfected cells were detected with the indicated antibodies by Western blot.
To examine the roles of p300 and NCoR, we first evaluated the activator and disengagement of the NCoR/mSin3 co-repressors. DACH1 inhibits TGF-β signaling through binding Smad4. In previous studies, the p21CIP1 and c-jun genes were examined as targets of DACH1 by genome-wide expression profiling (Fig. 4C). Consistent with these findings in HaCaT cells, the p21CIP1 promoter was induced by TGF-β. DACH1 coexpression inhibited TGF-β-induced p21CIP1 promoter activity. Deletion of the DACH1 DS domain abrogated repression of basal p21CIP1 promoter activity and substantially reduced repression of TGF-β-induced promoter activity. The c-jun promoter was induced 6-fold by TGF-β, and coexpression of DACH1 abrogated both basal and TGF-β-induced activation of the c-jun promoter. To determine the effects of DACH1 on TGF-β-induced p21CIP1 and c-jun expression, Western blot analysis was conducted of HaCaT cells sorted by FACS (Fig. 7E). TGF-β induced the phosphorylation of Smad2 and increased the abundance of p21CIP1 and c-Jun. Co-expression of DACH1 reduced TGF-β-induced p21CIP1 and c-Jun abundance; there was no change of the expression of mSin3A. Together, these studies demonstrate that DACH1 inhibits TGF-β-induced activation of endogenous and transfected TGF-β-responsive genes.

The DACH1 DS Domain Binds NCoR—In previous studies in 293 cells, epitope-tagged Ski was shown to bind transfected Smad4 and NCoR/SMRT (6). To examine proteins physically associated with DACH1 in cultured cells, FLAG-tagged
DACH1 expression vectors were transfected into 293T cells and immunoprecipitated with anti-FLAG (M2) antibody. The immunoprecipitates were analyzed for co-associated endogenous proteins with antibodies to NCoR, mSin3A, Smad4, Smad3, and FLAG (M2). Full-length DACH1 co-precipitated endogenous NCoR and mSin3A Smad3 and transfected Smad4. The DACH1ΔDS mutant bound mSin3A, Smad4, and Smad3 but failed to bind NCoR (Fig. 8A). The DS domain alone bound to NCoR and Smad3 but failed to bind mSin3A or Smad4. Consistent with previous studies, Ski bound to NCoR, mSin3A, Smad4, and Smad3. The immunoprecipitation efficiency was confirmed by anti-FLAG M2 antibody (Fig. 8A). Since these studies suggested that NCoR bound the DACH1 DS domain but that Smad4 bound outside the DACH1 DS domain, reciprocal immunoprecipitations were conducted with the anti-Smad4 antibody. The Smad4 immunoprecipitates were reprobed with the anti-FLAG M2 antibody to assess the DACH1 binding domain. The full-length DACH1 and the DACH1ΔDS construct co-precipitated with Smad4. The DACH1 DS domain alone did not bind Smad4. Ski was co-precipitated with Smad4 (Fig. 8B). In view of the finding that DACH1 co-precipitates endogenous NCoR (Fig. 8A), further studies were conducted to confirm the specificity of this interaction. The DACH1 cDNA was epitope-tagged with the HA vector and co-expressed in 293T cells with FLAG-tagged NCoR. Immunoprecipitation Western blotting with anti-HA antibody demonstrated the presence of DACH1 and the co-precipitation of NCoR using the FLAG epitope antibody (Fig. 9A). Confocal microscopy of NCoR- and DACH1-transfected cells demonstrated the co-localization of DACH1 with the NCoR in dot-like structures (27) (Fig. 9B). These studies suggested DACH1 and NCoR might participate in a common complex.

The co-precipitation studies suggested that Smad4 bound to DACH1 but did not determine whether this interaction was direct or through co-associated proteins. To determine whether Smad4 can bind directly to DACH1, equal amounts of bacterially expressed purified recombinant proteins were incubated with Smad4 (in vitro translated Smad4 protein (Fig. 9C)). The pull-down products were electrophoresed on an SDS-PAGE. The autoradiogram showed that DACH1 DS/EYAD bound to Smad4, that the DACH1 DS domain alone did not bind Smad4, and that the binding of EYAD/C-end to Smad4 was detectable but less than DACH1 DS/EYAD (Fig. 9C). Together these studies suggest Smad4 has the capacity to bind DACH1 through the EYAD domains.

**Fig. 10.** DACH1 within FAST/SBE DNA complexes. A, γ-32P-labeled FAST1/SBE oligonucleotide described under “Materials and Methods” was incubated total protein from 293 cells transfected with either Smad4 or DACH1 expression plasmid with or without competitor. B, whole cell protein from cells transfected with a DACH1 expression vector and immunoprecipitated with anti-FLAG M2 antibody. The supernatant was immunodepleted (IP DACH1), and then the immunoprecipitated DACH1 (IP) was eluted by TBS/EDTA, and the Western blot was shown using anti-FLAG antibody. C, electrophoretic mobility shift assay using γ-32P-labeled FAST/SBE oligonucleotide and protein from 293 cells transfected with Smad4, DACH1, Smad4 and DACH1, or purified DACH1.
DACH1, Smad3, and Smad4 Form a Ternary Complex with a Smad/FAST1 Binding Element (SBE)—Electrophoretic mobility shift assays were conducted using the FAST1/SBE oligonucleotide probe (35). In the presence of cell extracts transfected with Smad4, three complexes were formed that were competed with a 50-fold excess of cold oligonucleotide probe (Fig. 10A, lane 2 versus lane 3). DACH1-expressing cells also formed complexes that retarded the probe’s migration (Fig. 10A, lane 4). Using extracts enriched for both Smad4 and DACH1, a slowly migrating specific polycrystal was detected (Fig. 10A, lane 6), which was competed with cold oligonucleotide probe. We immunodepleted DACH1 from the cellular extracts and purified DACH1 using the anti-FLAG M2 gel (Fig. 10B). These purified proteins were used in electrophoretic mobility shift assay experiments with the FAST1 oligonucleotide probe (Fig. 10C). The whole cell extract from DACH1-expressing cells formed complexes 3 and 4, which were abrogated by immunodepletion of DACH1 (Fig. 10C, lane 3 versus lane 4). Immunopurified DACH1 did not bind the probe (Fig. 10C, lanes 5 and 6); however, the polycrystal, formed in whole cell extracts from cells transfected with DACH1 and Smad4 expression plasmids, was significantly reduced after DACH1 was immunodepleted (ID DACH1) (Fig. 10C, lanes 7 and 8) and partially restored by the addition of purified DACH1 (lanes 9 and 10). These studies suggest that DACH1 forms a complex in the presence of Smad4 at a FAST1/SBE site.

DISCUSSION

In the current studies, for the first time, DACH1 was shown to function as a transcriptional repressor of TGF-β signaling. DACH1 repressed TGF-β-induced activity of both SBE and AP-1 activity and inhibited TGF-β-induced apoptosis in MDA-MB-231 cells. NCoR enhanced repression of TGF-β signaling by DACH1. Repression by DACH1 required Smad4, being abrogated in Smad4-deficient cells and restored by Smad4 coexpression. Repression by DACH1 required a conserved DS domain that bound the transcriptional co-repressor NCoR. DACH1 and NCoR co-localized in a substantial proportion of cells that bound the transcriptional co-repressor NCoR. Together, these findings suggest NCoR may participate in DACH1-mediated repression of gene expression.

DACH1 was detectable in MDA-MB-231 cells by Western blotting, and genome-wide analysis of DACH1-responsive genes in these cells indicated that 422 genes of 17,000 were regulated >2-fold by DACH1 expression. Consistent with the reporter gene analysis demonstrating DACH1 inhibition of AP-1 activity, several AP-1-responsive genes were repressed by DACH1 expression, including c-fos, Egr1, cyclin E2, neuregulin, tumor necrosis factor α-induced protein 3, cdc25A, FGF5, GRO3, MEF2C, ETR101, and BMP4. A comparison between genes regulated significantly by DACH1 (Student’s t test, p < 0.05) with recent studies of TGF-β signaling using a similar approach demonstrated that genes induced by TGF-β in other cell types were repressed by DACH1 (ATF3, interleukin-11, P2RY2) and several genes repressed by TGF-β were induced by DACH1 (ID1 and interleukin-1-β) (Table I). Comparison between genome wide analysis “fingerprints” must be considered with caution; however, it is of interest that of 70 genes regulated by TGF-β (47), 22 of those genes were also significantly regulated by DACH1 expression; similarly, there is overlap with TGF-β response genes in recent publications (4, 48) (Table I). The functions of these genes are diverse and include cell division, transcriptional regulation, cellular adhesion, extracellular matrix remodeling, and signal transduction. The use of genome-wide expression studies to identify clusters of genes representing a molecular signature of DACH1-regulated activity suggests a normal function for DACH1 in the inhibition of AP-1-regulated genes. The current studies suggest DACH1 may function to regulate aberrant TGF-β signals that play important roles in human breast cancer progression (3, 4). TGF-β itself plays an important role in cancer progression by functioning both as an antiproliferative factor and as a tumor promotor. The numerous components of the signal conduction pathway are tumor suppressors that are functionally mutated in cancer (2, 46).

DACH1 was found within a complex bound to a FAST1/SBE DNA binding site with Smad4. Immunopurified DACH1, however, did not bind DNA directly, suggesting that Smad4 serves as a DNA-bound platform to recruit DACH1. The DACH1 DS domain alone was insufficient for Smad4 binding, which required the RYAD domain and was defective in SBE and AP-1 repression. DACH1 co-immunoprecipitated with Smad4 from cultured cells, and the association of DACH1 with Smad4 was observed in reciprocal immunoprecipitation. DACH1 associated with Smad4 in vitro using GST pull-down experiments, and, like Ski, multiple domains in DACH1 were required, including both the DS and EYA domains. Using saturating immunoprecipitation, the relative amount of co-precipitated Smad4 was greater for Ski than DACH1 (data not shown). In contrast, the relative abundance of NCoR coprecipitating with DACH1 was relatively greater than that associated with Ski. The finding that the DACH1DS domain mutant abrogated Ski-mediated repression of SBE activity suggests that DACH1 and Ski may function in a similar pathway.

DACH1, like Ski, repressed Smad3-regulated transactivation of either SBE or AP-1 activity. Our findings with Ski are similar to previous findings (49) but contrast with the effect of Sno-N, which has little effect on Smad3 transactivation (9). Sno-N is degraded rapidly in response to Smad3 or TGF-β, whereas Ski expression and DACH1 expression (data not shown) were not affected greatly by TGF-β. These findings suggest distinct roles for Sno-N versus Ski-DACH1 signaling.

DACH1 inhibited TGF-β- and Smad-induced AP-1 activity. Inhibition of TGF-β and Smad-induced AP-1 activity required the DACH1 DS domain. TGF-β induction of several genes, including PAI-1, clusterin, monocyte chemoattractant protein-1 (JE/MCP-1), type I collagen, and TGF-β itself depends on AP-1 DNA-binding sites in the promoter region of these genes (50–53). Induction of AP-1 activity by TGF-β involves interactions between Smads and AP-1 transcription factors (53). Smads bind directly to the Jun family, and both Smad3 and Smad4 can bind JunB, c-Jun, and JunD. Since the regions of DACH1 that bound Smads were required for repression of TGF-β-induced AP-1 activity, it is likely that DACH1 mediates AP-1 repression through Smad4 association.

Herein DACH1 antagonized TGF-β signaling. It is interesting in this regard that several nuclear receptors also inhibited TGF-β signaling, although this remains an area of controversy. Thus, the glucocorticoid receptor interacts with Smads, inhibiting TGF-β induction of the type-1 plasminogen activator inhibitor gene promoter (54). TGF-β with Smad3 inhibited androgen receptor transactivation of androgen receptor-responsive genes (55), and TGF-β induction of Smad-responsive promoters were repressed by the estrogen receptor-a in the presence of estrogen (45). Given the role of NCoR/mSin3 in regulating nuclear receptor function, it will be of interest to determine the role of DACH1 in nuclear receptor signaling.

The identification of DACH1 as a new co-repressor of TGF-β signaling extends our understanding of this key pathway. The role of TGF-β in cancer includes a complex function as both an antiproliferative activity and as a tumor promotor. DACH1, like Sno-N and v-Ski oncogenes (9), bind directly to NCoR/
SMRT and mSin3. TGF-β controls a plethora of cellular functions and regulates development and homeostasis. Since DACH1 and SKI have only partially overlapping expression patterns (available on the World Wide Web at www.ncbi.nlm.nih.gov/UniGene), with DACH1 expressed in neuroblastosas (12) and in cell lines derived from pancreas and breast cancer cell lines, it is possible that DACH1 contributes in a cell type-specific manner to regulate TGF-β signaling.

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Appendix 1.

E 15.5-day embryo tissues stained with anti-DACH1 polyclonal antibody

**Inner Ear**

**Eye**
422 genes regulated by DACH1 in MDA-MB-231 cells
422 genes regulated by DACH1 in MDA-MB-231 cells

|               | Ponasterone A 18h | Control | Ponasterone A 36h | Probe number |
|---------------|-------------------|---------|-------------------|-------------|
| Probe number  | 1                 | 2       | 3                 | 1           |
|               | 2                 | 3       | 1                 | 2           |
|               | 3                 | 1       | 2                 | 3           |

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422 genes regulated by DACH1 in MDA-MB-231 cells

| Ponasterone A 18h | Control | Ponasterone A 36h | Probe number |
|-------------------|---------|-------------------|--------------|
| 1                 | 2       | 3                 | 1            |
| 1                 |         |                   | 1            |
| 2                 |         |                   | 2            |
| 3                 |         |                   | 3            |

[Image of heatmap with probe numbers]
### 422 genes regulated by DACH1 in MDA-MB-231 cells

| Probe number | Control | Ponasterone A 18h | Ponasterone A 36h |
|--------------|---------|-------------------|-------------------|
| 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 216155_at | 216062_at | 214319_at | 205156_s_at | 222024_s_at | 37547_at | 214449_s_at | 216657_at | 203425_s_at | 216480_at | 211204_at | 217676_at | 217683_at | 202236_s_at | 2044135_at | 205165_at | 215987_at | 206449_at | 207143_at | 207232_s_at | 207360_at | 206420_at | 204575_s_at | 202643_s_at | 202644_s_at | 2099010_s_at | 205659_at | 204035_at | 207528_at | 207528_s_at | 209921_at | 209921_at | 217678_at | 207818_s_at | 212384_at | 203998_at | 205554_at | 213199_at | 202111_at | 204359_at | 205806_s_at | 219208_at | 219546_at | 217796_s_at | 214764_at | 203409_at | 208870_at | 203638_at | 214693_at | 205705_at | 208707_at | 91617_at | 216056_at | 210953_at | 203708_at | 213309_at | 200924_at | 207338_s_at | 213570_s_at | 203378_at | 213519_s_at | 222250_s_at | 201159_s_at | 207173_s_at | 216350_s_at | 222335_at | 201083_s_at | 203235_at | 202586_at | 205704_s_at | 206777_s_at | 202469_at | 202632_at | 214144_at | 206583_at | 212437_at | 208863_s_at | 218998_at | 219410_at | 202286_s_at | 202417_at | 2064932_at | 208706_s_at | 218203_at | 213376_at | 204080_at | 201586_s_at | 208705_at | 212291_s_at |
### 422 genes regulated by DACH1 in MDA-MB-231 cells

| Probe number | Ponasterone A 18h | Control | Ponasterone A 36h | Probe number |
|--------------|-------------------|---------|-------------------|--------------|
| 1            | 2                 | 3       | 1                 | 2            |
| 2            | 3                 | 1       | 2                 | 3            |
| 3            |                   |         | 1                 | 2            |
| 4            |                   |         | 2                 | 3            |

[Image of a heatmap showing gene expression levels]
422 genes regulated by DACH1 in MDA-MB-231 cells

| Ponasterone A 18h | Control | Ponasterone A 36h | Probe number |
|------------------|---------|------------------|-------------|
| 1                | 2       | 3                | 1           |
| 2                | 3       | 1                | 2           |
| 3                | 1       | 2                | 3           |
DACH1 Inhibits Transforming Growth Factor-β Signaling through Binding Smad4
Kongming Wu, Ying Yang, Chenguang Wang, Maria A. Davoli, Mark D'Amico, Anping Li, Kveta Cveklova, Zbynek Kozmik, Michael P. Lisanti, Robert G. Russell, Ales Cvekl and Richard G. Pestell

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