The Endogenous Cell-Fate Factor Dachshund Restrains Prostate Epithelial Cell Migration via Repression of Cytokine Secretion via a CXCL Signaling Module

Ke Chen1,2, Kongming Wu1,2,3, Xuanmao Jiao1,2, Liping Wang1,2, Xiaoming Ju1,2, Min Wang1,2, Gabriele Di Sante1,2, Shaohua Xu1,2, Qiong Wang1,2, Kevin Li1,2, Xin Sun1,2, Congwen Xu1,2, Zhiping Li1,2, Mathew C. Casimiro1,2, Adam Ertel1, Sankar Addya1, Peter A. McCue2, Michael P. Lisanti2, Chenguang Wang1,2, Richard J. Davis4, Graeme Mardon5, and Richard G. Pestell1,2,6

Abstract

Prostate cancer is the second leading form of cancer-related death in men. In a subset of prostate cancer patients, increased chemokine signaling IL8 and IL6 correlates with castrate-resistant prostate cancer (CRPC). IL8 and IL6 are produced by prostate epithelial cells and promote prostate cancer cell invasion; however, the mechanisms restraining prostate epithelial cell cytokine secretion are poorly understood. Herein, the cell-fate determinant factor DACH1 inhibited CRPC tumor growth in mice. Using Dach1fl/fl/Probasin-Cre bitransgenic mice, we show IL8 and IL6 secretion was altered by approximately 1,000-fold by endogenous Dach1. Endogenous Dach1 is shown to serve as a key endogenous restraint to prostate epithelial cell growth and restrains migration via CXCL signaling. DACH1 inhibited expression, transcription, and secretion of the CXCL genes (IL8 and IL6) by binding to their promoter regulatory regions in chromatin. DACH1 is thus a newly defined determinant of benign and malignant prostate epithelium cellular growth, migration, and cytokine abundance in vivo. Cancer Res; 75(10); 1–13. © 2015 AACR.

Introduction

Adenocarcinoma of the prostate is the second leading cause of cancer-related death in American men (1). Androgens increase cellular proliferation of prostatic epithelial cells via the androgen receptor (AR). Androgen deprivation therapy (ADT) is an important form of treatment for most prostate cancer patients (2).

Although a majority of patients initially respond to ADT, most will eventually develop castrate resistance, defined as disease progression despite serum testosterone levels of <20 ng/dL. The cytokine/chemokine signaling pathway is important in promoting prostate cancer cell proliferation and migration. The AR is involved in both cytokine mRNA transcription, and secretion of the CXCL genes (IL8 and IL6) (14). Increased serum levels of IL6 also correlate with increased clinical stage, in turn induces prostate cancer cellular growth (14). Increased androgen resistance and progression of prostate cancer (13).

However, many tumors regrow after 12 to 18 months (3–6). Molecular genetic analysis in mice has confirmed clinical observation demonstrating key genes mediating the onset and progression of prostate cancer, including the AR, Pten, c-Myc, and Ras. Although a majority of patients initially respond to ADT, most will eventually develop castrate resistance, defined as disease progression despite serum testosterone levels of <20 ng/dL. The cytokine/chemokine signaling pathway is important in promoting prostate cancer cell proliferation and migration. The AR is believed to remain active in castrate-resistant prostate cancer (CRPC), and several new strategies to inhibit AR signaling have recently been developed (7, 8).

IL8 and IL6 are also thought to promote prostate cancer progression (9, 10). Tumor-derived IL8 activates epithelial cells to promote angiogenesis and to induce recruitment of neutrophils. Tumor-derived IL8 activates neutrophils and tumor-associated macrophages, which release IL6 to create a feed forward loop (10). The expression of IL8 is increased in human prostate cancer correlating with poor prognosis (11, 12) and IL8 promotes androgen resistance and progression of prostate cancer (13). Activation of the IL8 receptor promotes EGFR signaling, which in turn induces prostate cancer cellular growth (14). Increased serum levels of IL6 also correlate with increased clinical stage, hormone-refractory disease, and metastasis (15, 16). IL6 promotes conversion of prostate cancer cells to castrate resistance (17). In mouse models, increased expression of IL6 promotes progression of prostate cancer in transplantation models (18). Because IL8 and IL6 continue to the onset and prognosis of prostate cancer, and correlate with poor prognosis in some...
patients, it is important to understand the molecular mechanisms governing restraint of IL8 and IL6 production in the prostate. Prostate epithelial cells and prostate cancer cells produce IL8 and IL6, however, the mechanisms normally restraining prostate cancer epithelial cell production are poorly understood. Better understanding of the molecular mechanisms driving CRPC is required to define new therapeutic approaches.

The *Drosophila dac* gene is a key member of the retinal determination gene network, which also includes eyes absent (*eya*), *ey*, twin of eyeless (*toy*), teashirt (*ssh*), and *sin ocellis* (*so*), that specifies eye tissue identity (19, 20). The Dach1 gene is the mammalian homolog of the *Drosophila Dac* gene (21), which was cloned as a dominant inhibitor of the hyperactive EGFR, *ellipse* (22, 23). DACH1 regulates expression of target genes in part through interacting with DNA-binding transcription factors (c-Jun, Smads, Six, IRE6), and in part through intrinsic DNA sequence-specific binding to Forkhead binding sites (22, 24–26). Several lines of evidence suggest DACH1 may function as a tumor suppressor. DACH1 is phosphorylated and acetylated (27), and DACH1 acetylation mediates p53 association and thereby determines fine-tuning of p53 signal transduction (27, 28). Patient samples have demonstrated reduced DACH1 expression in a variety of malignances, including breast, prostate, non–small-cell lung cancer, endometrial, and brain cancer (25, 26, 28–30). A correlation has been shown between reduced DACH1 expression and poor outcome in breast cancer (26), and DACH1 inhibits breast cancer tumor metastasis (31). In prostate cancer, DACH1 expression is reduced (25) and DACH1 was shown to bind and inhibit AR activity via the AR acetylation site (25, 32).

Although these studies suggest DACH1 may function as a prostate tumor suppressor, several key questions remain unresolved, including the role of DACH1 in AR-negative prostate cancer cells, and the function of DACH1 in prostate cancer cellular migration and invasion. As Dach1 gene deletion leads to a perinatal lethal phenotype, the role of endogenous Dach1 in prostate cellular growth in *vivo* was unknown. DACH1 binds to the androgen response element (ARE) of target genes in chromatin and reexpression of DACH1 in androgen therapy–resistant prostate cancer cell lines restored repression of AR signaling. DACH1 abundance was rate limiting in the recruitment of AR corepressors (NCoR, SIRT1, HDAC3; ref. 33) in the presence of the androgen antagonist, bicalutamide (25). The finding that DACH1 was essential for recruitment of NCoR to endogenous AREs is considered important as AR transcriptional repression by AR antagonists (bicalutamide, finasteride) is dependent upon NCoR (34, 35). Furthermore, androgen antagonists function as agonists in the absence of NCoR (34).

Given the potential importance of DACH1 as a tumor suppressor in human prostate cancer, we generated prostate cancer cell lines of distinct molecular genetic makeup, including AR-negative and AR-expressing cell lines, that reexpress DACH1. To determine the role of endogenous Dach1 in normal prostate cellular proliferation, we generated bitransgenic mice encoding a floxed allele of the Dach1 gene and a prostate targeted Cre recombinase. We found DACH1 blocks prostate tumor growth and migration by inhibiting cytokine secretion. Transgenic Dach1 gene deletion enhanced cytokine (IL8, IL6) secretion by approximately 1,000-fold, defining Dach1 as a key endogenous restraint to secretion of these cytokines, which thereby enhanced prostate epithelial cell migration. Dach1 is a key genetic determinant that restrains endogenous prostate epithelial cell cytokine secretion and invasion in *vivo*.

**Materials and Methods**

**Immunohistochemistry**

IHC analysis of prostate cancer cell lines was conducted using a polyclonal DACH1 antibody (26).

**Mice, chemicals reagents, and Western blotting**

Experimental procedures with transgenic mice and nude mice were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University (Philadelphia, PA). Dach1**+/** and Probasin-Cre mice were in the FVB strain and were intercrossed to form Dach1**+/**/Probasin-Cre bitransgenic mice. Comparison was made between the Dach1**+/**/Probasin Cre and Dach1**+/**/Probasin-Cre.

**Cell culture, plasmid construction, reporter genes, expression vectors, DNA transfection, luciferase assays, ChIP-Seq, and ChIP assays**

Cell culture, DNA transfection, and luciferase assays using the cyclin A-Luc and IL6-Luc and IL8-Luc reporter genes were performed as previously described (36). Primary prostate epithelial cells were cultured as previously described (37). The HEK293T, PC3, the isogenic oncogene–transformed prostate cancer cell lines (37), C4-2 and 22Rv1 cells were cultured in DMEM supplemented with 10% FCS, 1% penicillin, and 1% streptomycin as previously described (26). The expression plasmids encoding an N-terminal FLAG peptide linked to DACH1 and DACH1 deleted of the DNA-binding domain (ΔDS, aa 183–282) were previously described (24). Cells were plated at a density of 1 × 10⁵ cells in a 24-well plate on the day before transfection with Superfect according to the manufacturer’s protocol (Qiagen). A dose response was determined in each experiment with 50 and 200 ng of expression vector and the promoter reporter plasmids (0.5 μg). Luciferase activity was normalized for transfection efficiency using β-galactosidase reporters as an internal control. The fold effect of expression vector was determined with comparison to the empty vector as a control and statistical analyses were performed using a t test. Chromatin immunoprecipitation sequencing (ChIP-Seq) was previously conducted (31) using an anti-FLAG antibody to the N-terminal Tag of the DACH1-FLAG expression fusion protein (24, 27, 31, 38). The prior data were interrogated for significant DACH1 association in chromatin using genes identified as candidate targets of DACH1 in the current studies. MDAMB-231 cells stably expressing FLAG-tagged DACH1 were used for analysis with comparison made to MDAMB-231 cells stably expressing vector alone. Briefly, approximately 10⁷ cells were fixed with 1% formaldehyde for 10 minutes at room temperature. The chromatin template was fragmented to 200 to 500 bp with sonication. The quality-filtered 25 nt short sequence reads were aligned to the hg18 (NCBI Build 36) human genome sequence using ELAND software, allowing up to two mismatches with the genome sequence. We obtained approximately 5.9 million uniquely aligned short sequence reads each for DACH1-p901 and IgG control samples in breast cancer cells. As the 25 nt short sequence reads originate from the ends of approximately 300 bp ChIP fragments, we shifted the locations of short reads toward the center of ChIP fragments by 150 nt (sense and antisense strand reads are shifted in opposite directions) and
counted the resulting reads in 400 bp nonoverlapping consecutive windows along the human genome. As the total number of uniquely aligned reads in the two samples (DACH1-p901 and IgG) is very similar, it was meaningful to directly compare window read counts of the two samples and look for DACH1-enriched regions. Model-based analysis for ChIP-Seq (MACS) software was used to identify putative DACH1-binding sites in the DACH1 IP using IgG as control. Peaks were reported using a raw \( P \) value cutoff of 0.01 (default is \( 1e^{-5} \)). Default settings were used for all other parameters. The peak intervals identified for IL6, IL8, cyclin A2, and cyclin E1 were within this set, however, based on the MACS peak call the binding was weak. We therefore initiated ChIP experiments to verify Dach1 association with these genes of interest. The primers used for the IL6 and KC (mouse homolog of IL8) were previously described (39).

**Cell proliferation assays**

Cells infected with MSCV-IRES-GFP, MSCV-DACH1-IRES-GFP, or MSCV-DACH1ADs, were seeded into 96-well plates in normal growth medium, and cell growth was measured daily by MTT assays.

**Colony forming assays**

A total of \( 4 \times 10^3 \) cells 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 more than 50 \( \mu \)m in diameter were counted using an Omnicron 3600 Image Analysis System. The colonies were visualized after staining with 0.04% crystal violet in methanol for 1 to 2 hours.

**Tumor xenografts**

22Rv1 cells stably transduced with expression vectors encoding DACH1, DACH1ADs, or empty vector cassette were implanted by subcutaneous injection into the flank of 6- to 8-week-old nude mice. Comparisons were made between groups with 7 mice in each group. The tumor volume was calculated using the equation where \( a \) and \( b \) are tumor length and width, respectively. At the completion of the experiments, tumors were excised, weighed, and statistical significance of differences in tumor volume was analyzed using a linear mixed model.

**IHC staining**

Paraffin-embedded tissue blocks were used for IHC in the Sidney Kimmel Cancer Center Pathology Core Facility (Thomas Jefferson University, Philadelphia, PA) as previously described (40). The antibody to Dach1 (10914-1-AP, Proteintech), Ki67 (RM-9106-S1, Thermo Scientific), and TUNEL (TACS2 TdT DAB, Trevigen) was previously described (28).

**Cell-cycle analysis**

Cell-cycle parameters were determined using laser scanning cytometry. Cells were processed by standard methods using propidium iodide staining of cell DNA. Each sample was analyzed by flow cytometry with a FACScan Flow Cytometer (Becton Dickinson Biosciences) using a 488 nm laser. Histograms were analyzed for cell-cycle compartments using ModFit version 2.0 (Verity Software House). A minimum of 20,000 events were collected to maximize statistical validity of the compartmental analysis. Apoptosis was determined by Annexin V staining (41).

**Western blot analysis**

Western blot assays were performed in cells as indicated. Cells were pelleted and lysed in buffer (50 mmol/L HEPES, pH 7.2, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 0.1% Tween 20) supplemented with a protease inhibitor cocktail (Roche Diagnostics).

**Microarray and cluster analysis**

DNA-free total RNA isolated from DACH1-expressing PC3 stable lines were used to probe human OneArray (Phalanx). RNA quality was determined by gel electrophoresis. Analysis of the arrays was performed using GeneSpring. Arrays were normalized using robust multi-array analysis, and the \( P \) value of 0.05 was applied as a statistical criterion for differentially expressed genes. These genes were then grouped using hierarchical clustering with “complete” agglomeration, and each cluster was further analyzed based upon the known function of the genes contained in the cluster. Expression profiles are displayed using Treeview. Classification and clustering for pathway level analysis were performed by using gene sets ASSESS (Analysis of Sample Set Enrichment Scores), and DAVID. ASSESS provides a measure of enrichment of each gene set in each sample.

**Transwell migration, 3D Matrigel invasion, and migratory velocity assays**

In wound-healing assays, cells were grown to confluence on 12-well plates (42). Transwell migration assays were conducted as previously described (43). The assessment of migratory velocity and distance was conducted using time-lapse video images that were collected and stored as images using Metamorph, version 3.5, software as previously described (44).

**Cytokine array analysis**

Human cytokine arrays spotted on nitrocellulose membranes were obtained from Raybiotech (45). Conditioned medium was prepared from cells transduced with an expression vector encoding DACH1 or DACH1ADs by culturing cells in serum-free DMEM for 24 to 48 hours. The conditioned medium from Dach1\(^{+/+}\) and Dach\(^{+-}\) PEC was prepared from cells grown in serum-free media for 24 hours.

**Assessment of CXCL abundance**

To quantitate CXCL expression, real-time PCR analysis was conducted using equal amounts of purified RNA samples that were reverse transcribed using the Iscript Reverse transcriptase kit (Bio-Rad) to form cDNA, which was subjected to SYBR Green-based Real-Time PCR relative quantification method for amplification of IL6 and IL8 transcripts (43). The abundance of secreted IL6, human IL8, murine KC was determined by ELISA (Raybiotech).

**Coexpression analysis in GSE21034**

A normalized mRNA expression dataset for Prostate Adenocarcinoma (46) was downloaded from the cbioPortal for cancer genomics and used to evaluate coexpression of IL6, IL8, and DACH1 transcript levels (46, 47). This dataset includes mRNA profiles for 131 primary tumor, 29 matched normal prostate, and...
Results

DACH1 inhibits CRPC contact-independent growth

To determine whether DACH1 was capable of inhibiting AR-negative prostate cancer cell contact-independent growth, the PC3 cell line was stably transduced with an expression vector encoding DACH1. To define the genetic cell-cycle targets of DACH1, microarray analysis was conducted of the cell lines (Fig. 1A). Interrogation of the cell-cycle control proteins demonstrated that, unlike several other cell types in which cyclin D1 is a direct-target of DACH1 repression, the cyclin A1, cyclin E1, cyclin E2, and cyclin A2 genes were repressed in PC3 cells (Fig. 1A, highlighted in red text). The mRNA data for all experiments shown by color display in Fig. 1A are shown quantitatively for PC3 cells in Supplementary Fig. S1A. We examined the possibility that the cyclin E1 gene was a direct transcriptional target of DACH1. The cyclin E1 promoter linked to a luciferase reporter gene was examined for responsiveness to DACH1. The DS domain (dac and ski/sno domain) (21) is required for transcriptional regulation and binding to gene targets in the context of local chromatin (24). Transfection of a DACH1 expression vector with the cyclin E1 promoter reporter in PC3 cells induced a dose-dependent repression that was contingent upon the DS domain (Supplementary Fig. S1B). Antibodies directed to the mutant of DACH1 (ΔDS) domain or the DACH1 protein demonstrated the presence of DACH1 in PC3 cells stably expressing DACH1 or the ΔDS domain (Fig. 1B). Western blot analysis directed to the N-terminal FLAG epitope demonstrate similar levels of exogenous DACH1 Wt or DACH1 ΔDS domain protein. Stable DACH1 expression reduced PC3 cell proliferation by approximately 50%, assessed by both the MTT assay and cell counting (Fig. 1C and D). PC3 cell growth, assayed by colony number in soft agar, was also inhibited approximately 80% by DACH1 (Fig. 1E). Inhibition of colony number by DACH1 was abrogated by deletion of the DS domain (Fig. 1F and G).

The C4-2 prostate cancer cells are a well characterized model of androgen-independent prostate cancer. The PI3K/Akt pathway is
Figure 2.
DACH1 inhibits prostate cancer tumor growth and cellular proliferation in mice. A and B, PC3 cells stably transduced with either control vector DACH1 or the DACH1 ΔDS mutant were introduced into nude mice and assessed by tumor size (A) or tumor weight (B) after implantation. C and D, 22RV1 cells stably expressing either DACH1, DACH1 ΔDS, or vector control were assessed after implantation into mice by the tumor size (C) or the tumor weight (D). (Continued on the following page.)
constitutively active in C4-2 due to the loss of the tumor suppressor PTEN, which is also deleted or inactivated in up to 70% of advanced androgen-independent prostate cancers (48). DACH1 or the DACH1 ΔDS domain mutant was expressed in the C4-2 cells (Fig. 1H), as evidenced by IHC to the DACH1 protein. Western blot analysis to the N-terminal tag of DACH1 demonstrated similar levels of the exogenous DACH1 or DACH1 ΔDS proteins (Fig. 1H). Expression of DACH1 reduced C4-2 cellular proliferation as assessed by the MTT assay and cell counting (Fig. 1I and J). DACH1 reduced proliferation approximately 50% at 6 days.

DACH1 inhibits CRPC and PEC growth in vivo

To determine whether DACH1 inhibited CRPC tumor growth in vivo, tumor size of DACH1-transduced PC3 cells was determined weekly after implantation (Fig. 2A). The tumor size increased in the vector control, but was reduced by the expression of DACH1 with an approximately 50% decrease in tumor volume at week 7. Deletion of the DS domain abrogated the reduction of tumor growth in nude mice. The tumor weight was similarly reduced, approximately 50% in vivo (Fig. 2B), whereas deletion of the DS domain abrogated the inhibition of tumor weight. The CWR22Rv1 line was derived from a relapsed human prostate cancer CWR22 xenograft (49). This line encodes a ligand-independent AR variant that arises by splicing of cryptic exons (50). The CWR22Rv1 cells express the AR variant and are p53 positive, unlike PC3 cells, which are AR negative and p53 negative. Reexpression of DACH1 in CWR22Rv1 cells also reduced tumor growth in mice approximately 50% at 5 weeks after implantation (Fig. 2C). Deletion of the DACH1 DS domain reduced, but did not abrogate the inhibition of cell growth, suggesting distinguishable domains of DACH1 may be involved in the inhibition of cellular growth in AR-negative versus AR-positive cells in vivo. The weight of the tumor was reduced >80% by DACH1 expression (Fig. 2D).

To determine whether the expression of DACH1 was reduced upon oncogenic transformation of prostate epithelial cells (PEC), we determined the abundance of DACH1 in isogenic oncogene-transduced PEC (37) (Fig. 2E). Western blot analysis demonstrated a reduction in DACH1 abundance in PEC transduced with distinct oncogenes (c-Myc, NeuT, Ha-Ras, v-Src) when normalizes to the protein loading control vinculin (Fig. 2E). Multiplicate analysis of the DACH1 mRNA abundance by quantitative PCR analysis demonstrated reduced DACH1 mRNA levels in each of the oncogene-transduced lines (Supplementary Fig. S1C).

The isogenic murine prostate cancer lines were introduced into FVB mice, and after 6 weeks, the extirpated tumors examined for DACH1 expression with comparison made to benign murine PEC. Compared with the normal murine PEC, from which the isogenic oncogene-transformed lines were derived, DACH1 abundance was significantly reduced (Fig. 2G). To determine whether the cell-cycle target genes identified as DACH1 targets in cultured PC3 cells were altered in abundance in the isogenic oncogene-transformed cells, microarray analysis was conducted. In both tissue culture and in extirpated tumors grown in vivo, the expression of cyclin E1 and cyclin A2 was increased (Fig. 2H and I). In contrast, the mRNA levels of several other cyclins (cyclin D2, cyclin D3, cyclin A1, and cyclin B3) was unchanged (Fig. 2H and I). Cyclin D1 mRNA levels were induced in the NeuT and Src isogenic lines in tissue culture and in all lines in vivo (Fig. 2H and I). The mRNA data for all experiments shown by color display are shown quantitatively for in vitro tissue culture and in vivo tumors (Supplementary Fig. S1D and E).

Conditional Dach1 gene knockout in the prostate demonstrates a role for endogenous Dach1 as an inhibitor of cellular proliferation and an inducer of apoptosis

To determine the potential role for Dach1 in prostatic cellular proliferation in vivo, bitransgenic mice were generated (Supplementary Fig. S 2A). The Dach1/Ctfl mice were intercrossed with Probasin-Cre mice, in which Cre recombinase is expressed in the basal and luminal epithelial cells of the prostate. Immunohistochemical staining demonstrated loss of Dach1 in both the anterior and ventral prostates of the bitransgenic mice (Supplementary Fig. S2B–S2E). An analysis of cellular proliferation and apoptosis was conducted. Ki67 staining, a surrogate measure for cellular proliferation, was significantly increased in the PEC of the Dach1/Ctfl/Probasin-Cre mice (Fig. 2J and Supplementary Fig. S3A and S3B). TUNEL staining was conducted to assess the effect of endogenous Dach1 on cellular survival in vivo. The percentage of apoptotic cells was reduced approximately 3-fold in the Dach1/Ctfl- prostate (Fig. 2K and Supplementary Fig. S 3C and S3D). Consistent with the finding that Dach1 inhibited cyclin A1, A2, and cyclin E1 in the prostate cancer cell lines, immunohistochemical staining demonstrated that the abundance of cyclin A2 and cyclin E1 was increased in the Dach1/Ctfl- PEC (Fig. 2L and M and Supplementary Fig. S3E and S3F), as was the abundance of cyclin D1 and cyclin A1 (Supplementary Fig. S4).

DACH1 inhibits prostate cancer cellular migration and persistence of migratory directionality

To determine whether DACH1-regulated prostate cancer cellular migration, the effect of DACH1 on the ability of PC3 cells to traverse a membrane was assessed. DACH1 expression in PC3 cells reduced Transwell migration by >50% (Fig. 3A and B) and deletion of the DACH1 DS domain abrogated the effect on cell migration (Fig. 3B). To define further the distinct components of cell migration regulated by DACH1 expression, video microscopy was conducted to determine the distance and effect of DACH1 on prostate cancer cellular migratory directionality. DACH1 inhibited the distance traveled by PC3 cells. The effect was abrogated by the deletion of the DS domain (Fig. 3C). The velocity of migration was also reduced by DACH1, requiring the DACH1 DS domain (Fig. 3D).

(Continued) The data are shown as mean ± SEM for n > 7 throughout. E and F, Dach1 expression is detected by Western blot analysis in primary mouse (FVB) PEC and isogenic oncogene-transformed cell lines derived from FVB PEC. H, microarray gene expression of FVB PEC or isogenic oncogene transformed PEC with relative abundance of cyclins is shown. G, PEC line tumor grown in mice and primary prostate gland, from which lines were derived, was immunostained for Dach1 and the percentage of Dach1-positive cells determined. H and I, microarray analysis of cyclin gene expression in isogenic PEC grown in tissue culture or grown as tumors (H) and then extirpated from FVB mice (I). The changes in gene expression are shown calorimetrically. Gene names are shown to the right of the panels. Genes highlighted in red were induced in both tissue culture and in vivo and were followed for mechanistic analysis. J, analysis of bitransgenic mice encoding Dach1/Ctfl/Probasin-Cre by immunohistochemical staining for Ki67 in the ventral prostate shown at ×10 and ×40. Data is shown for mean ± SEM for n > 100 cells. K, apoptosis determined by TUNEL staining of the ventral prostate. Data shown as mean ± SEM, n > 100 cells. L and M, immunohistochemical staining for cyclin E1 (L) and cyclin A2 (M) in the ventral prostate of bitransgenic mice encoding Dach1/Ctfl/Probasin-Cre mice. Transgenic deletion of Dach1 in the prostate epithelial cells enhances cellular proliferation and reduces cellular apoptosis.
To define the mechanisms by which DACH1 inhibited cellular migration, microarray analysis was conducted. Interrogation of the microarray analysis was conducted to determine functional pathways using KEGG and GO. The enrichment score for each pathway, the name of the pathway, and the number of genes within each GO-defined functional pathway was determined and represented graphically for each parameter as a unique dimension in Fig. 3E. The cytokine/cytokine receptor interaction pathway was highly enriched. We therefore examined the cytokine/chemokine signaling pathway as it is known to promote cellular proliferation and migration. A module of cytokine signaling was selectively repressed by DACH1, including IL6, IL8, and CXCL6, 1, 2, 5 (Fig. 3F). Deletion of the DS domain abrogated DACH1-mediated repression of IL8 and IL6 expression (Fig. 3F). The mRNA data for all experiments in PC3 cells shown by color display are also shown quantitatively in Supplementary Fig. S5A.

To determine whether DACH1-mediated repression of IL8 was oncogene specific, we assessed the isogenic oncogene transformed prostate cancer cell lines, in which DACH1 expression was reduced (Fig. 4A). Microarray analysis shown colorimetrically of the isogenic prostate cancer cell lines in tissue culture (Fig. 4A), or in vivo in tumors derived from FVB mice (Fig. 4B), demonstrated increased expression of IL6 in the Ha-Ras transformed line and increased IL8 (CXCL1) in each of the lines transformed by distinct oncogenes (c-Myc, NeuT, Ha-Ras, V-Src). IL6 and IL8 mRNA was increased in each of the cell lines when grown as tumors in mice. Similar data are shown quantitatively in Supplementary Fig. S5B and S5C. Immunohistochemical staining of the extirpated tumors demonstrated a significant inverse correlation between DACH1 and the abundance of CXCL1 (KC), IL6, and CXCR2 (the receptor for CXCL1; 4C–E).

To determine whether a clinical correlate existed for DACH1-mediated repression of cellular migration, we hypothesized that DACH1 expression may be lost in metastatic prostate cancer. Interrogation of clinical databases demonstrated the relative abundance of DACH1 was reduced in prostate cancer compared with benign prostate disease, with significant further reduction in metastatic prostate cancer samples (Fig. 4F). We next examined the prostate cancer sample and the adjacent normal prostate tissue of individual patient samples. Individual patient samples of patients matched showed a decrease in Dach1 levels associated with an increase in IL6 and IL8 mRNA level in the prostate tumor compared with adjacent normal prostate tissue. This decrease in DACH1 (DACH1 expression) correlated significantly with an increase in IL6 and IL8 (Fig. 4G and H; t test, P < 0.05). The mRNA level in individual patient matched samples is shown in Supplementary Fig. S6A.

To determine the mechanisms by which DACH1 reduced the cytokine signaling module, we considered the possibility that DACH1 may inhibit the transcription of CXCL genes. We examined the IL6 and IL8 promoters. Both IL6 and IL8 were repressed in a dose-dependent manner by DACH1, and repression required the DS domain (Fig. 4I and J). Western blot analysis of the transfected PC3 cells using a FLAG antibody directed to the N-terminal of DACH1 protein demonstrated a dose-dependent increase in the abundance of transfected wild-type or mutant DACH1 protein when normalized to vinculin protein as a loading control (Fig. 4K).

To determine at a higher level of resolution the mechanism by which DACH1 repressed IL6 and IL8 mRNA and promoter activity, we determined whether DACH1 was recruited in the context of chromatin to the promoters of regulatory regions of these genes. Genome-wide chromatin immunoprecipitation (ChIP-Seq) analysis previously conducted in MDA-MB-231 cells expressing FLAG-Tagged DACH1 (24, 27), identified enrichment of Tag density for DACH1 at the cell-cycle DACH1-regulated target genes (cyclin E1, cyclin A2, cyclin A1) and the cytokine target genes IL6 and IL8 (Supplementary Fig. S6B). The ChIP analysis of the cyclin E1, cyclin A2, IL6, and IL8 promoter (in 293T cells) using oligonucleotide primer targeted to the AP-1 regulatory region demonstrated recruitment of DACH1, but not DACH1 ΔDS, to the cyclin E1, cyclin A2, IL6, and IL8 promoters at the AP-1 sites (Fig. 4L and Supplementary Fig. S6C and S6D).

**Dach1 inhibition of cellular migration involves secreted cytokines (IL6, CXCL1)**

To determine whether endogenous DACH1 regulated the secretion of the cytokine signaling mRNA module identified in human prostate cancer cells in tissue culture, the prostatic epithelium of the bitransgenic mice (Dach1fl/fl/Probasin-Cre) was analyzed. A cytokine array analysis demonstrated increased secretion of CXCL signaling in the Dach1fl/fl/Probasin-Cre bitransgenic mice PECs in culture (Supplementary Fig. S7A–S7D). The increased abundance of IL6 and KC (homolog of human IL8) in Dach1fl/fl–PEC was confirmed by quantitative ELISA (Fig. 5A and B, Supplementary Fig. S8A and S8B). The abundance of IL6 was increased approximately 1,000-fold (Fig. 5A and Supplementary Fig. S8A) and the abundance of KC was also increased by approximately 1,000-fold (Fig. 5B and Supplementary Fig. S8B) when assayed by ELISA. The Dach1fl/fl–PEC derived from the anterior prostate showed a similar increase in cellular migratory distance and velocity (Supplementary Fig. S8C and S8D). The mRNA levels for IL6 and IL8 determined by quantitative PCR were increased by approximately 6-fold in Dach1fl/fl–versus Dach1+/+–PEC (Supplementary Fig. S9A and S9B). The circulating serum levels for IL6 and IL8 determined by quantitative ELISA in the Dach1 wild-type versus knockout mice show a 3-fold difference in abundance (Fig. 5D and E).

The Dach1fl/fl–PEC of the ventral prostate showed an approximately 250% increase in both cellular migratory distance (data not shown) and velocity (Fig. 5C and Supplementary Fig. S10A). The addition of media from the Dach1fl/fl–PEC enhanced migration of Dach1+/+–PEC (Fig. 5C, lanes 1 vs. 2) and media from Dach1+/+–PEC reduced migration of Dach1fl/fl–PEC (Fig. 5C, lanes 9 vs. 10). This finding suggested that endogenous Dach1 inhibits the secretion of factors that promote PEC cellular migration (Fig. 5F). The addition of IL6 or CXCLI (KC, murine homolog of IL8) enhanced migration of Dach1+/+–PEC. Addition of an immunoneutralizing antibody to IL6 or CXCLI (KC, murine homolog of IL8) enhanced migration of Dach1+/+–PEC (Fig. 5C and Supplementary Fig. S10A).

We considered potential mechanisms contributing to the reduction in DACH1 abundance in prostate cancer cell lines. DNA methylation of gene regulation may contribute to reduced expression. Treatment of the PC3 cell line with the DNA methyltransferase inhibitor 5-aza-cytidine demonstrated a substantial induction of DACH1 abundance by Western blot analysis (Supplementary Fig. S11), suggesting that DNA methylation may contribute to the reduction of DACH1 abundance in prostate cancer cell lines.

To determine whether the inverse relationship between DACH1 and IL8/IL6 expression was found in human prostate cancer, we examined a database in which matched prostate cancer
Figure 3.
DACH1 inhibits Transwell migration and cytokine gene expression via the DS domain. A, PC3 cells stably expressing either vector, DACH1, or ΔDS were stained after Transwell migration. B, the relative migration is shown for $n > 5$ separate experiments. C, the PC3 stable cell lines were analyzed by video microscopy for distance traveled. Representative examples of individual cell tracking are shown for stable PC3 cells coincubated with the media from cells expressing either DACH1 or DACH1 ΔDS. D, the cellular migratory velocity for the PC3 cells is shown as mean ± SEM for three separate experiments. E, microarray analysis of PC3 cells stably expressing either DACH1, DACH1 ΔDS, or vector control were analyzed for functional pathways using KEGG. The Gene Ontology terms, the enrichment score, and number of gene enhanced for each Gene Ontology term are shown. F, the relative abundance of genes significantly altered by DACH1 via the DS domain in the cytokine–cytokine receptor interaction categories is shown. DACH1 inhibits CXCL gene expression as shown by the blue color, indicating repression in the presence of DACH1 expression. Genes highlighted in red were repressed by DACH1 expression via the DACH1 ΔDS domain and used for further analysis.
DACH1 Determines CXCL Signaling in Prostate Cancer

**A** In vitro tissue culture

<table>
<thead>
<tr>
<th>PEC</th>
<th>c-Myc</th>
<th>NeuT</th>
<th>H-Ras</th>
<th>v-Src</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR2</td>
<td>CXCL1(KC)</td>
<td>IL6</td>
<td>IL6ST</td>
<td>IL6RA</td>
</tr>
</tbody>
</table>

**B** In vivo tumors

<table>
<thead>
<tr>
<th>Normal prostate</th>
<th>c-Myc</th>
<th>NeuT</th>
<th>H-Ras</th>
<th>v-Src</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR2</td>
<td>CXCL1(KC)</td>
<td>IL6</td>
<td>IL6ST</td>
<td>IL6RA</td>
</tr>
</tbody>
</table>

**C**

\[ R = -0.81 \]

**D**

\[ R = -0.46 \]

**E**

\[ R = -0.58 \]

**F**

<table>
<thead>
<tr>
<th>Relative DACH1 level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign (N = 6)</td>
</tr>
<tr>
<td>Primary (N = 7)</td>
</tr>
<tr>
<td>Metastatic (N = 6)</td>
</tr>
</tbody>
</table>

**G**

GSE21034 tumor-normal pairs

\[ N = 13 \]

**H**

GSE21034 tumor-normal pairs

\[ N = 17 \]

**I**

IL6-Luc

PC3 cells

Luciferase reporter activity

DACH1

\[ \Delta \text{DACH1 expression} \]

**J**

IL8-Luc

PC3 cells

Luciferase reporter activity

DACH1

\[ \Delta \text{DACH1 expression} \]

**K**

PC3 cells

DACH1

\[ \Delta \text{DACH1 expression} \]

α-Vinculin

α-FLAG (DACH1)

**L**

IL6 binding site

293T

Relative binding

DACH1

\[ \Delta \text{DACH1 expression} \]

AP-1 CTL AP-1 CTL AP-1 CTL

IL8 binding site

293T

Relative binding

DACH1

\[ \Delta \text{DACH1 expression} \]

AP-1 CTL AP-1 CTL AP-1 CTL

*P < 0.001
and adjacent normal tissue had been interrogated by microarray gene expression (Supplementary Fig. S12A and S12B). The data showed substantial variation in gene expression between patient samples, but an overall trend for reduced DACH1 mRNA and increased IL8 and IL6 mRNA in the tumor compared with adjacent normal tissue. Although matched data for progression of primary to metastatic samples were not available, a similar trend was observed comparing metastatic prostate cancer lesions with non-transformed adjacent prostate tissue (Supplementary Fig. S12A and S12B).

**Discussion**

Using bitransgenic mice, the current studies demonstrate for the first time that the endogenous cell fate determination factor pathway defined by Dach1 restrains prostate epithelial cell proliferation, migration, and cytokine secretion. IL8 and IL6 are known to promote prostate cancer progression (51–53); however, the mechanisms governing the restraint of IL8 and IL6 mRNA expression in the prostate were previously unknown. Herein, Dach1 genetic deletion increased IL6 and KC abundance by approximately 1,000-fold, illustrating Dach1 is a key endogenous determinant of IL8 and IL6 production. Oncomine analysis of mRNA abundance for IL6 and IL8 demonstrated increased expression in metastatic prostate cancer, compared with normal prostate in some (Fig. 4F), but not in all clinical datasets (Supplementary Fig. S12), likely due to the heterogeneous nature of prostate cancer as shown in the wide spread of individual patient data (Supplementary Fig. S12). The loss of DACH1 expression in metastatic prostate cancer suggests that DACH1 may serve as a clinically relevant prostate cancer metastasis suppressor in a subset of prostate cancers.

In two separate prostate cancer models of castrate-resistant prostate cancer, we demonstrated DACH1 blocks prostate tumor growth in vivo. DACH1 inhibited prostate cancer cell growth in immunocompetent mice in four murine prostate cancer cell lines transformed by distinct oncogenes (Ha-Ras, c-Myc, v-Src, NeuT). The isogenic prostate cancer cell lines resemble prostate adenocarcinoma cancer by histology, by gene expression, and by genomic rearrangements (37). DACH1 mRNA and protein levels were reduced in the isogenic prostate cancer cell lines and reexpression of DACH1 reduced tumor growth in immunocompetent mice. These studies extend the prior findings in mice that showed PTEN and p27Kip1 restrain prostate epithelial cell proliferation in vivo (54). The current studies also extend prior observations that DACH1 represses AR expression in prostate cancer cells (25). Herein, the PC3, CWR22Rv1, and C4-2 cell lines were each repressed by DACH1 expression. The genetic makeup of these cell lines involves distinct mutations, PC3 cells are AR-negative, p53-negative, PTEN-negative, and p38-negative (55), indicating the inhibition of cell proliferation by DACH1 is p53-independent. The CWR22Rv1 cells are AR-positive and p53-positive, but are PTEN-negative, indicating DACH1 inhibition of prostate cancer does not require PTEN inactivation. In the AR-mutant LNCaP cells, DACH1 inhibited prostatic cancer cellular DNA synthesis and growth in colony forming assays and blocked contact-independent growth in soft agar. Together, these studies demonstrate that DACH1 has antiproliferative functions in both AR-positive, AR-negative, and AR-mutant prostate cancer cell lines.

The current studies demonstrate DACH1 inhibits human and murine prostate tumor growth in vivo. The inhibition of AR-negative PC3 cells, proliferation, and colony formation, required the D8 domain of DACH1. The repression of cyclin A1 also required the D8 domain. DACH1 repressed cyclin E1 and cyclin A1 in PC3 cells, whereas cyclin E1 and cyclin A2 were repressed in each of the isogenic prostate cancer cell lines. In LNCaP cells, the cell-cycle signaling module repressed by DACH1 was not determined (25); however, induction of DACH1 expression in LNCaP cells repressed cyclin D1 abundance (data not shown). Together, these findings suggest DACH1 targets distinct components of the cell cycle to reduce cell proliferation in different cell lines. DACH1 conveys transcriptional repression directly via DNA binding to FKHR sites (24), indirectly through association with other transcription factors (c-Jun, So/SIX, AR), or through binding the coregulator protein CA150 (54). The mechanism by which DACH1 inhibited AR-negative prostate cancer cell growth correlated with repression of cyclin E1 and DACH1-bound cyclin E1, cyclin A1, and cyclin A2 promoters in CHIP assays, consistent with a direct repression mechanism.

Using Dach1/Probasin-Cre prostate epithelial cells, we demonstrated that endogenous Dach1 restrains a homotypic promigratory signal in the murine prostate epithelium via repression of IL6 and IL8. Deletion of endogenous Dach1 reduced IL6 and IL8 protein abundance by approximately 1,000-fold. The normal mechanisms governing prostate epithelial cell proliferation of IL6 and IL8 are important because these cytokines have profound effects on prostate cancer onset and progression. DACH1 inhibited prostate cancer cellular migration across a membrane and inhibited migratory distance and velocity via homotypic secretion of cytokines. Pathway analysis of microarray gene expression profiling from tumor xenografts demonstrated DACH1 repressed a cytokine module including IL6 and IL8 (KC). Immunoneutralizing antibodies...
demonstrated the importance of DACH1-mediated inhibition of IL6 and IL8 in determining prostate cancer cellular migration directionality. DACH1 repressed transcription of the IL6 and IL8 promoters and DACH1 was associated in the local chromatin by ChIP at the IL6 and IL8 promoters. DACH1 reduced IL6 and IL8 mRNA abundance by approximately 6-fold, indicating DACH1 governs both transcriptional and nontranscriptional mechanisms. Given the importance of IL8 and IL6 in
human prostate cancer progression, the current studies are important in identifying a key endogenous restraint of cytokine production that is lost during prostate cancer development.

Disclosure of Potential Conflicts of Interest

R.G. Pestell is a founder of Prostgene, has ownership interest in pending patents, and is a consultant/advisory board member for Prostgene. No potential conflicts of interest were disclosed by the other authors.

Disclaimer

The Pennsylvania Department of Health disclaims responsibility for any analysis, interpretations, or conclusions of this article.

Authors’ Contributions


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Chen, K. Wu, X. Jiao, L. Wang, X. Ju, C. Xu, Q. Wang, S. Addya, P.A. McCue, R.J. Davis


Writing, review, and/or revision of the manuscript: K. Chen, K. Wu, X. Jiao, K. Li, X. Sun, M.P. Lisanti, G. Mardon, R.G. Pestell

Conflict of Interest

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