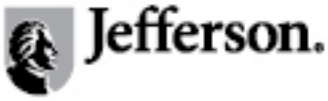

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The Endogenous Cell-Fate Factor Dachshund restrains Prostate Epithelial Cell Migration via Repression of Cytokine Secretion via a CXCL Signaling Module

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Abstract

Prostate cancer (PCa) is the second leading form of cancer death in men. In a subset of PCa patients increased chemokine signaling IL-8 and IL-6 correlates with androgen therapy-resistant prostate cancer (CRPC). IL-8 and IL-6 are produced by prostate epithelial cells and promote PCa cell invasion, however the mechanisms restraining prostate epithelial cell cytokine secretion are poorly understood. Herein the cell-fate determinant factor DACH1 inhibited androgen therapy-resistant prostate cancer (CRPC) tumor growth in mice. Using *Dach1*$^{fl/fl}$/Probasin-Cre bi-transgenic mice, we show IL-8 and IL-6 secretion was altered ~1000 fold by endogenous *Dach1*. Endogenous *Dach1* is shown to serve as a key endogenous restraint to prostate epithelial cell

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growth and restrains migration via CXCL signaling. DACH1 inhibited expression, transcription and secretion of the CXCL genes (IL-8, IL-6) 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*.

Keywords

prostate cancer; cell fate; Dach1

INTRODUCTION

Adenocarcinoma of the prostate is the second leading cause of 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). However, many tumors re-grow 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 PCa, 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).

IL-8 and IL-6 are also thought to promote prostate cancer progression (9, 10). Tumor derived IL-8 activates epithelial cells to promote angiogenesis and to induce recruitment of neutrophils. Tumor derived IL-8 activates neutrophils and tumor associated macrophages which release IL-6 to create a feed forward loop (10). The expression of IL-8 is increased in human prostate cancer correlating with poor prognosis (11, 12) and IL-8 promotes androgen resistance and progression of prostate cancer (13). Activation of the IL-8 receptor promotes EGFR signaling which in turn induces prostate cancer cellular growth (14). Increased serum levels of IL-6 also correlate with increased clinical stage, hormone refractory disease and metastasis (15, 16). IL-6 promotes conversion of prostate cancer cells to castrate resistance (17). In mouse models increased expression of IL-6 promotes progression of prostate cancer in transplantation models (18). Because IL-8 and IL-6 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 IL-8 and IL-6 production in the prostate. Prostate epithelial cells and PCa cells produce IL-8 and IL-6, however the mechanism normally restraining PCa epithelial cell production are poorly understand. 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 (RDGN), which also includes *eyes absent (eya), ey, twin of eyeless (toy), teashirt (tsh)* and *sin oculis (so)*, that specifics eye tissue identity (19, 20). The *Dach1* gene is the mammalian

homologue 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, ERα), and in part through intrinsic DNA-sequence specific binding to Forkhead binding sites (22, 24, 25, 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, 29, 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 re-expression 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 co-repressors (NCoR, SIRT1, HDAC3 (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. In order to determine the role of endogenous *Dach1* in normal prostate cellular proliferation, we generated bi-transgenic mice encoding a floxed allele of the *Dach1* gene and a prostate targeted Cre recombinase. We found DACH1 blocks prostate tumor growth and migration, inhibiting cytokine secretion. Transgenic *Dach1* gene deletion enhanced cytokine (IL-8, IL-6) secretion ~ 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

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

Mice, chemicals reagents, Western blotting

Experimental procedures with transgenic mice and nude mice were approved by the Institutional Animal Care and Use Committee (IACUC) of Thomas Jefferson University. *Dach1*$^{fl/fl}$ and Probasin-Cre mice mice were in the FVB strain and were intercrossed to form *Dach1*$^{fl/fl}$-Probasin-Cre bi-transgenic mice. Comparison was made between the *Dach1*$^{fl/fl}$/ Probasin Cre and *Dach1*^{wt} 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 CyclinA-Luc and IL-6-Luc and IL-8-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% fetal calf serum, 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 1×10^5 cells in a 24-well plate on the day prior to transfection with Superfect according to the manufacturer's protocol (Qiagen, Valencia, CA). 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 effect of the empty expression vector cassette and statistical analyses were performed using a t-test. ChIP-Seq (Chromatin Immunoprecipitation Sequencing) 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 was interrogated for significant DACH1 association in chromatin using genes identified as candidate targets of DACH1 in the current studies. MDA-MB-231 cells stably expressing FLAG-tagged DACH1 were used for analysis with comparison made to MDA-MB-231 cells stably expressing vector alone. Briefly, approximately 10^7 cells were fixed with 1% formaldehyde for 10 min 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 IL-6, IL-8, 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 IL-6 and KC (mouse homologue of IL-8) were previously described (39).

Cell Proliferation Assays

Cells infected with MSCV-IRES-GFP, MSCV-DACH1-IRES-GFP, or MSCV-DACH1ΔDS, were seeded into 96 well plates in normal growth medium, and cell growth was measured daily by MTT assays using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide.

Colony forming assays

4×10^3 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 μg/ml doxycycline overlaid on a 0.5% agarose base, also in complete growth medium. 2 weeks after incubation, colonies more than 50 μm in diameter were counted using an Omnicon 3600 image analysis system. The colonies were visualized after staining with 0.04% crystal violet in methanol for 1 to 2 h.

Tumor xenografts

22Rv1 cells stably transduced with expression vectors encoding DACH1, DACH1 ΔDS 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 $\left(\frac{a \times b^2}{2}\right)$ 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.

Immunohistochemistry (IHC) staining

Paraffin-embedded tissue blocks were used for IHC in the Sidney Kimmel Cancer Center Pathology Core Facility as previously described (40). The antibody to Dach1 (10914-1-AP, Proteintech), Ki67 (RM-9106-S1, Thermo) 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, Mansfield, MA) using a 488 nm laser. Histograms were analyzed for cell cycle compartments using ModFit version 2.0 (Verity Software House, Topsham, ME). A minimum of 20,000 events were collected to maximize statistical validity of the compartmental analysis. Apoptosis was determined by Annexin V staining (41).

Western Blot

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

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 (Norcross, GA) (45). Conditioned medium was prepared from cells transduced with an expression vector encoding DACH1 or DACH1 ΔDS by culturing cells in serum free DMEM for 24–48 hrs. The conditioned medium from *Dach1*$^{+/+}$ and *Dach*$^{-/-}$ PEC was prepared from cells grown in serum free media for 24 hrs.

Assessment of CXCL abundance

To quantitate CXCL expression, real time PCR analysis was conducted using equal amounts of purified RNA samples which were reverse transcribed using the Iscript Reverse transcriptase kit (Bio-Rad, Hercules, CA) to form cDNA, which was subjected to SYBR Green based Real-Time PCR relative quantification method for amplification of IL-6 and IL-8 transcripts (43). The abundance of secreted IL-6, human IL-8, murine KC was determined by ELISA (Raybiotech, Nacross, GA).

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 19 metastasis samples. Pearson’s correlation

coefficient was calculated for these transcripts for all samples and within normal prostate and prostate cancer, and metastatic sample groups. The 29 matched tumor-normal pairs were used to investigate changes in mRNA expression for IL6 and IL8 versus DACH1 in tumor relative to normal baseline. Samples were divided into a subset showing a trend in prostate cancer samples vs normal adjacent prostate tissue for a decrease in DACH1 accompanied by an increase in IL6 or IL8, and a subset of other samples in order to illustrate the frequency of this relationship between IL6/IL8 and DACH1.

RESULTS

DACH1 inhibits CRPC contact-independent growth

In order to determine whether DACH1 was capable of inhibiting AR negative prostatic cancer cell contact-independent growth, the PC3 cell line was stably transduced with an expression vector encoding DACH1. In order 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 Figure. 1A is shown quantitatively for PC3 cells in Supplemental Fig. 1A. 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 which was contingent upon the DS domain (Supplemental Fig. 1B). 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 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, 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, G).

The C4-2 prostate cancer cells are a well characterized model of androgen-independent prostate cancer. The PI3-Kinase/Akt pathway is constitutively active in C4-2 due to the loss of the tumor suppressor phosphatase and tensin homolog (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 immunohistochemistry to the DACH1 protein. Western blot 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, J). DACH1 reduced proliferation ~50% at 6 days.

DACH1 inhibits CRPC and PEC growth *in vivo*

In order 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. Re-expression of DACH1 in CWR22Rv1 cells also reduced tumor growth in mice approximately 50% at 5 weeks post 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 vs. AR positive cells *in vivo*. The weight of the tumor was reduced >80% by DACH1 expression (Fig. 2D).

In order 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 normalized to the protein loading control vinculin (Fig. 2F). Multiplicate analysis of the DACH1 mRNA abundance by quantitative PCR analysis demonstrated reduced DACH1 mRNA levels in each of the oncogene transduced lines (Supplemental Fig. 1C)

The isogenic murine PCa lines were introduced into FVB mice, and after 6 weeks the extirpated tumors examined for DACH1 abundance 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). In order 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, I). In contrast, the mRNA levels of several other cyclins (cyclin D2, cyclin D3, cyclin A1, and cyclin B3) was unchanged (Fig. 2H, 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, I). The mRNA data for all experiments shown by color display is shown quantitatively for *in vitro* tissue culture and *in vivo* tumors (Supplemental Fig. 1D–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

In order to determine the potential role for *Dach1* in prostatic cellular proliferation *in vivo*, bi-transgenic mice were generated (Supplemental Fig. 2A). The $Dach1^{fl/fl}$ 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 the loss of Dach1 in both the anterior and ventral prostates of the bi-transgenic mice (Supplemental Fig. 2B–E). 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*$^{fl/fl}$/Probasin-Cre mice (Fig. 2J, Supplemental Fig. 3A, B). TUNEL staining was conducted to assess the effect of endogenous *Dach1* on cellular survival *in vivo*. The percentage of apoptotic cells was reduced ~3-fold in the *Dach1*$^{-/-}$ prostate (Fig. 2K, Supplemental Fig. 3C, D). 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*$^{-/-}$ PEC (Fig. 2L, M, Supplemental Fig. 3E, F), as was the abundance of cyclin D1 and cyclin A1 (Supplemental Fig. 4).

DACH1 inhibits prostate cancer cellular migration and persistence of migratory directionality

In order 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 >90% (Fig. 3A, B) and deletion of the DACH1 DS domain abrogated the effect on cell migration (Fig. 3B). In order 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).

In order 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 IL-6, IL-8 and CXCL6, 1, 2, 5 (Fig. 3F). Deletion of the DS domain abrogated DACH1-mediated repression of IL-8 and IL-6 expression (Fig. 3F). The mRNA data for all experiments in PC3 cells shown by color display is also shown quantitatively in Supplemental Figure. 5A.

In order to determine whether DACH1-mediated repression of IL-8 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 IL-6 in the Ha-Ras transformed line and increased IL-8 (CXCL1) in each of the lines transformed by distinct

oncogenes (c-Myc, NeuT, Ha-Ras, V-Src). IL-6 and IL-8 mRNA was increased in each of the cell lines when grown as tumors in mice. Similar data is shown quantitatively in Supplemental Fig. 5B, C. Immunohistochemical staining of the extirpated tumors demonstrated a significant inverse correlation between DACH1 and the abundance of CXCL1 (KC), IL-6 and CXCR2 (the receptor for CXCL1) (Fig. 4C–E).

In order 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 IL-6 and IL-8 mRNA level in the prostate tumor compared with adjacent normal prostate tissue. This decrease in DACH1 (DACH1 expression) correlated significantly with an increase in IL-6 and IL-8 (Fig. 4G, H, **t test, $p<0.05$**). The mRNA level in individual patient matched samples is shown in Supplemental Fig. 6A.

In order 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 IL-6 and IL-8 promoters. Both IL-6 and IL-8 were repressed in a dose-dependent manner by DACH1, and repression required the DS domain (Fig. 4I, 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).

In order to determine at a higher level of resolution the mechanism by which DACH1 repressed IL-6 and IL-8 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 IL-6 and IL-8 (Supplemental Fig. 6B). The ChIP analysis of the cyclin E1, cyclin A2, IL-6 and IL-8 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, IL-6 and IL-8 promoters at the AP-1 sties (Fig. 4L, Supplemental Fig. 6C–D).

Dach1 inhibition of cellular migration involves secreted cytokines (IL-6, CXCL1)

In order 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 bi-transgenic mice (*Dach1*$^{fl/fl}$/probasin Cre) was analyzed. A cytokine array analysis demonstrated increased secretion of CXCL signaling in the *Dach1*$^{fl/fl}$/Probasin-Cre bi-transgenic mice prostatic epithelial cells (PEC) in culture

(Supplemental Fig. 7A–D). The increased abundance of IL-6 and KC (homologue of human IL-8) in *Dach1*$^{-/-}$ PEC was confirmed by quantitative ELISA (Fig. 5A, B, Supplemental Fig. 8A, B). The abundance of IL-6 was increased ~1,000 -fold (Fig. 5A, Supplemental Fig. 8A) and the abundance of KC was also increased ~1,000 fold (Fig. 5B, Supplemental Fig. 8B) when assayed by ELISA. The *Dach1*$^{-/-}$ PEC derived from the anterior prostate showed a similar increase in cellular migatory distance and velocity (Supplemental Fig. 8C–D). The mRNA levels for IL-6 and IL-8 determined by QT-PCR were increased ~6–fold in *Dach1*$^{-/-}$ vs *Dach1*$^{+/+}$ PEC (Supplemental Fig. 9A, B). The circulating serum levels for IL-6 and IL-8 determined by quantitative ELISA in the Dach1 wild-type vs. knockout mice show a 3-fold difference in abundance (Fig. 5D, E).

The *Dach1*$^{-/-}$ PEC of the ventral prostate showed a ~250% increase in both cellular migratory distance (data not shown) and velocity (Fig. 5C, Supplemental Fig. 10A). The addition of media from the *Dach1*$^{-/-}$ PEC enhanced migration of *Dach1*$^{+/+}$ PEC (Fig. 5C **lanes 1 vs. 2**) and media from *Dach1*$^{+/+}$ PEC reduced migration of *Dach1*$^{-/-}$ 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 IL-6 or CXCLI (KC, murine homologue of IL-8) enhanced migration of *Dach1*$^{+/+}$ PEC. Addition of an immunoneutralizing antibody to IL-6 or CXCLI reduced the migration of *Dach1*$^{+/+}$ PEC (Fig. 5C, Supplemental Fig. 10A).

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-azacytidine, demonstrated a substantial induction of DACH1 abundance by Western blot (Supplemental Fig. 11), suggesting that DNA methylation may contribute to the reduction of DACH1 abundance in prostate cancer cell lines.

In order to determine whether the inverse relationship between DACH1 and IL-8/IL-6 expression was found in human prostate cancer we examined a data base in which matched prostate cancer and adjacent normal tissue had been interrogated by microarray gene expression (Supplemental Fig. 12A, B). The data showed substantial variation in gene expression between patient samples but an overall trend for reduced DACH1 mRNA and increased IL-8 and IL-6 mRNA in the tumor compared with adjacent normal tissue. Although matched data for progression of primary to metastatic samples was not available, a similar trend was observed comparing metastatic prostate cancer lesions with non-transformed adjacent prostate tissue (Supplemental Fig. 12A, B).

DISCUSSION

Using bi-transgenic 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. IL-8 and IL-6 are known to promote prostate cancer progression (51, 52, 53) however the mechanisms governing the restraint of IL-8 and IL-6 mRNA expression in the prostate were previously unknown. Herein, *Dach1* genetic deletion increased IL-6 and KC abundance ~1000-fold, illustrating

Dach1 is a key endogenous determinant of IL-8 and IL-6 production. Oncomine analysis of mRNA abundance for IL-6 and IL-8 demonstrated increased expression in metastatic PCa, compared with normal prostate in some (Fig. 4F), but not in all clinical data sets (Supplemental Fig. 12) likely due to the heterogeneous nature of prostate cancer as shown in the wide spread of individual patient data (Supplemental Fig. 12). The loss of DACH1 expression in metastatic prostate cancer suggests that DACH1 may serve as a clinically relevant prostatic 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 immune competent 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 re-expression of DACH1 reduced tumor growth in immune-competent mice. These studies extend the prior findings in mice that showed PTEN and $p27^{Kip1}$ restrain prostate epithelial cell proliferation *in vivo* (54). The current studies also extend prior observations that DACH1 represses AR expression in PCa Cells (25). Herein, the PC3, CWR22Rv1 and C4-2 cell lines were each repressed by DACH1 expression. The genetic make up 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 positive, indicating DACH1 inhibition of PCa doesn't 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 anti-proliferative 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 DS domain of DACH1. The repression of cyclin A1 also required the DS 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 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 co-regulator protein CA150 (54). The mechanism by which DACH1 inhibited AR-ve 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 $Dach1^{fl/fl}$/Probasin-Cre prostate epithelial cells, we demonstrated that endogenous *Dach1* restrains a homotypic pro-migratory signal in the murine prostate epithelium via repression of IL-6 and IL-8. Deletion of endogenous *Dach1* reduced IL-6 and IL-8 protein

abundance ~1000-fold. The normal mechanisms governing prostate epithelial cell production of IL-6 and IL-8 is important because these cytokines have profound effects on prostate cancer onset and progression. DACH1 inhibited PCa 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 IL-6 and IL-8 (KC). Immunoneutralizing antibodies demonstrated the importance of DACH1-mediated inhibition of IL-6 and IL-8 in determining prostate cancer cellular migration directionality. DACH1 repressed transcription of the IL-6 and IL-8 promoters and DACH1 was associated in the local chromatin by ChIP at the IL-6 and IL-8 promoters. DACH1 reduced IL-6 and IL-8 mRNA abundance ~6-fold, indicating DACH1 governs both transcriptional and non-transcriptional mechanisms. Given the importance of IL-8 and IL-6 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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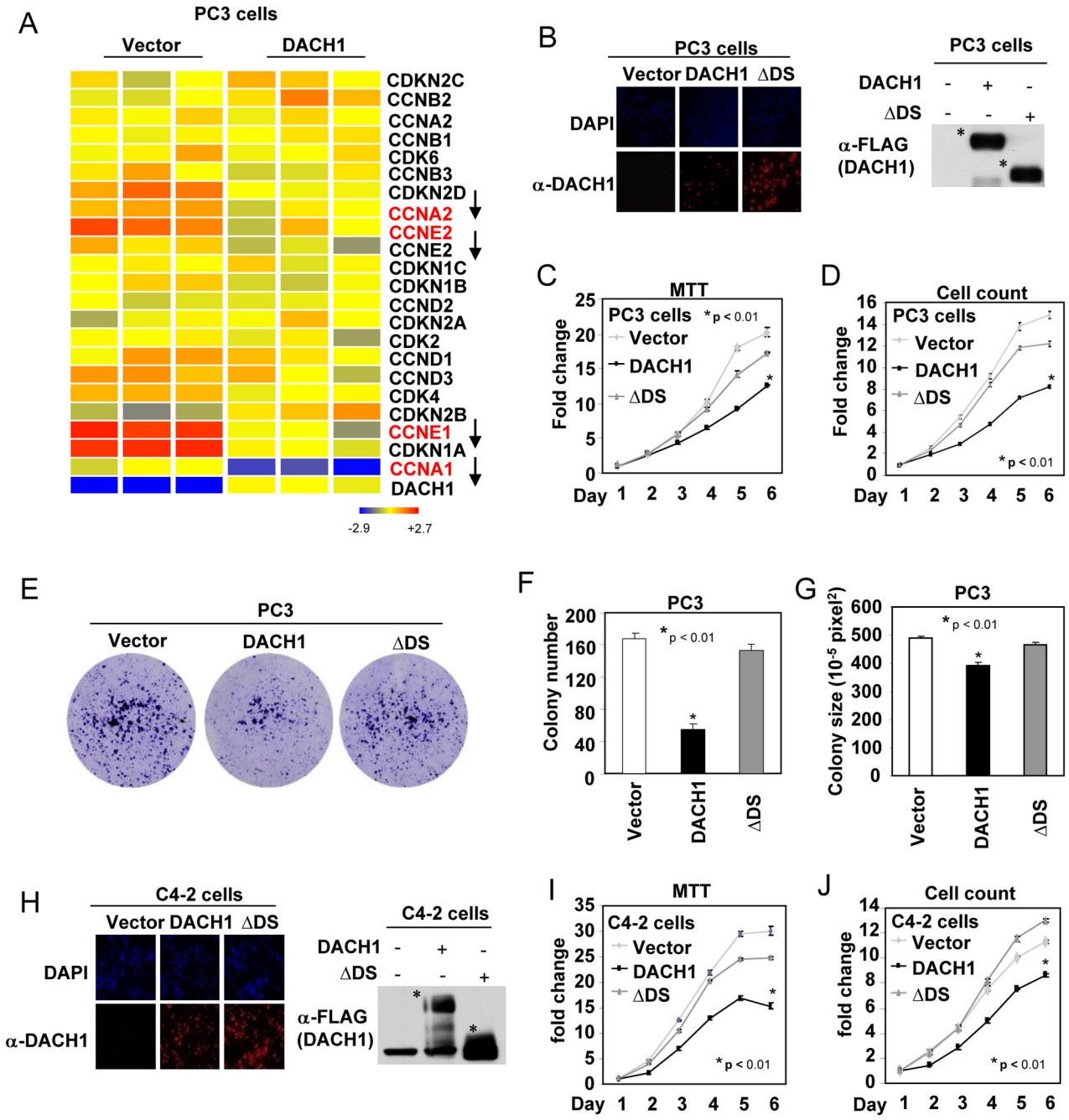

Figure 1. DACH1 inhibits AR-negative prostate cancer cell proliferation and contact independent growth by the DS domain

A) Treeview display of microarray gene expression studies conducted on PC3 cells stably expressing either vector control or DACH1. The cell cycle control proteins are shown with the relative abundance, demonstrated with a color scale as shown. DACH1 inhibits cyclin E1, E2, A1, A2 expression. B) Phase contrast using immunofluorescent microscopy for PC3 stable cell lines for DACH1 and DAPI as a nuclear stain. Western blot of PC3 stable cell line with antibody to the N-terminal FLAG tag of DACH1. C) The cellular proliferation rate determined by MTT assay or D) cell counting. E) Colony forming assays were conducted

with PC3 stable cell lines expressing control vector, DACH1 or ΔDS with colonies stained using crystal violet and F) colony number or G) colony size determined using N>5 separate experiments. H) C4-2 cells expressing either control vector DACH1 or the DACH1 ΔDS mutant were assessed for DACH1 abundance by immuno-histochemistry. DAPI and immunofluorescence for DACH1 is shown. Western blot is shown of the cells with an antibody directed to the N-terminal FLAG tag. I) The cellular proliferation rate of C4-2 cells expressing DACH1 or mutant DS was determined by either MTT assay or J) cell counting. Data are mean ±SEM for N>5 throughout.

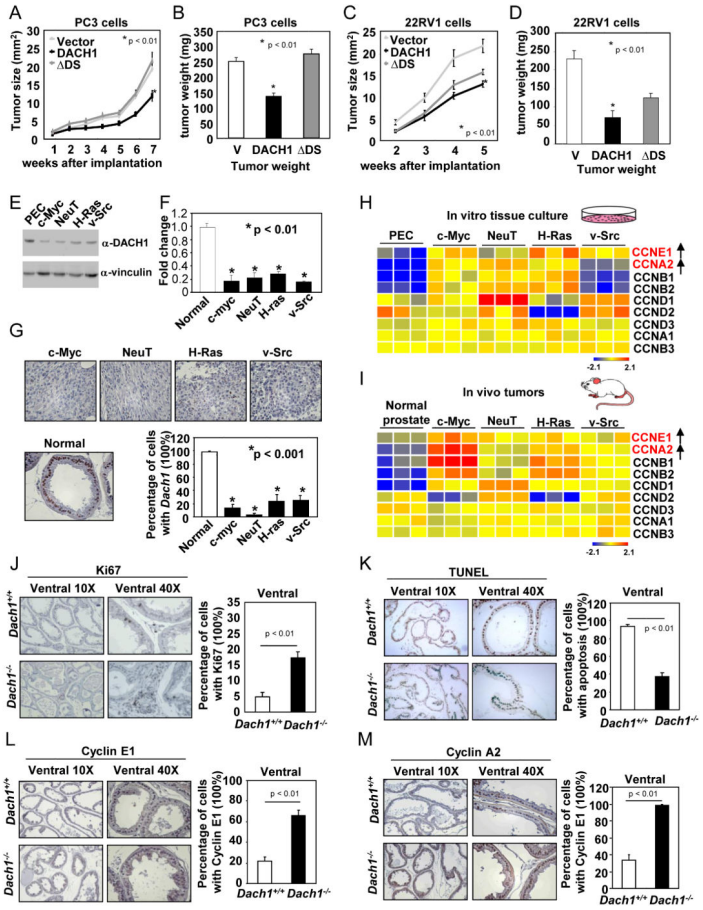

Figure 2. DACH1 inhibits prostate cancer tumor growth and cellular proliferation in mice

A–B) PC3 cells stably transduced with either control vector DACH1 or the DACH1 ΔDS mutant were introduced into nude mice and assessed by A) tumor size or B) tumor weight after implantation C–D) 22RV1 cells stably expressing either DACH1, DACH1 ΔDS or vector control were assessed after implantation into mice by C) the tumor size or D) the tumor weight. The data are shown as mean ±SEM for N>7 throughout. E–F) Dach1 expression is detected by Western blot 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 shown. G) PEC line tumor grown in mice and primary prostate gland from which lines were derived, were immunostained for Dach1 and the % of Dach1 positive cells determined. H) Microarray analysis of cyclin gene expression in isogenic PEC grown in tissue culture or I) grown as tumors and then extirpated from FVB mice. The changes in gene expression are shown calorimetrically. Gene names are shown to the right of the panels. Genes highlighted in red are induced in both tissue culture and *in vivo* and were followed for mechanistic analysis. J) Analysis of bi-transgenic mice encoding *Dach1*$^{fl/fl}$/Probasin-Cre by immunohistochemical staining for Ki67 in the ventral prostate shown at 10x and 40x. Data is shown for mean ±SEM N>100 cells. K) Apoptosis determined by TUNEL staining of the ventral prostate. Data shown as mean ±SEM N>100 cells. L) Immunohistochemical staining for cyclin E1 and M) cyclin A2 in the ventral prostate of bi-transgenic mice encoding *Dach1*$^{fl/fl}$/Probasin-

Cre mice. Transgenic deletion of *Dach1* in the prostate epithelial cells enhances cellular proliferation and reduces cellular apoptosis.

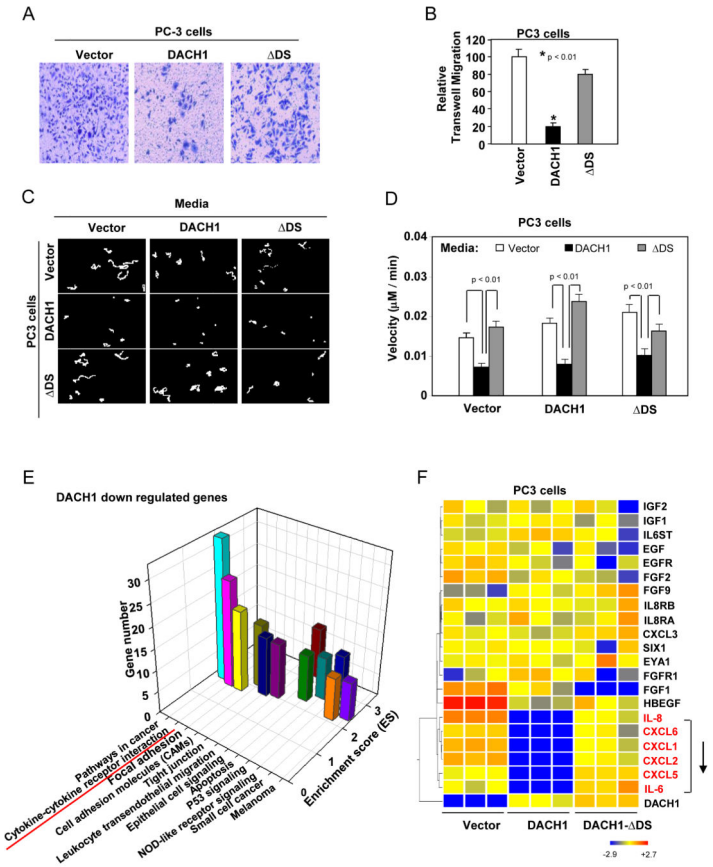

Figure 3. DACH1 inhibits transwell migration and cytokine gene expression via the DS domain

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 co-incubated 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 GO terms, the enrichment score, and number of gene enhanced for each GO 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 are repressed by DACH1 expression via the DACH1 DS domain and used for further analysis.

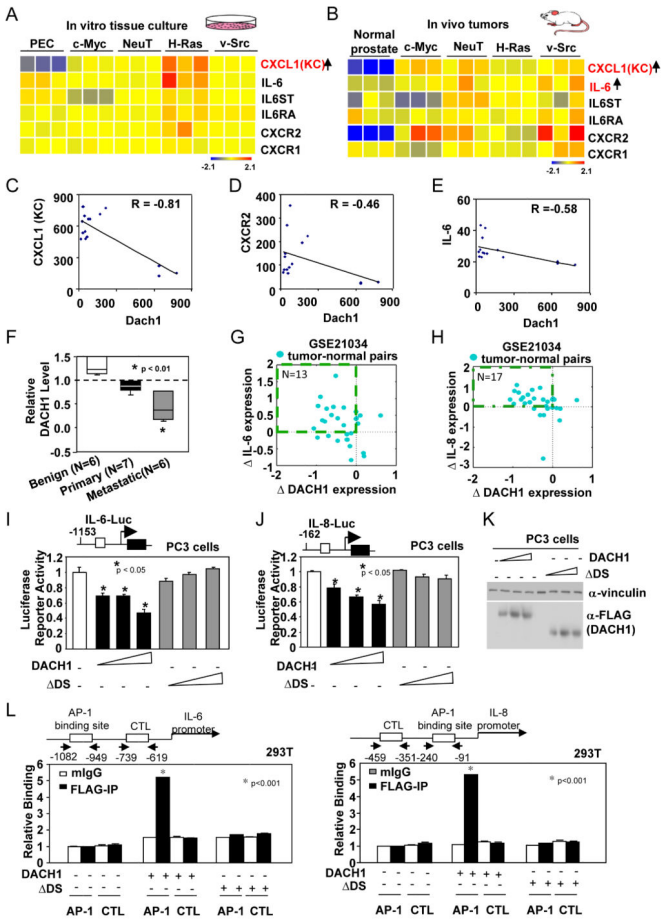

Figure 4. *Dach1* expression is reduced in oncogenic transformed PEC and inversely co-related with cytokine production

A) Microarray based gene expression of cytokines/chemokines in isogenic PEC in tissue culture or B) from tumors grown in FVB mice. The relative expression levels from the tumors *in vivo* are shown in C-E. F) Relative DACH1 mRNA levels were determined from Oncomine in either prostate cancer samples or benign prostate disease. DACH1 levels are significantly reduced in metastatic prostate cancer samples. G–H) mRNA abundance for DACH1 and either IL-6 or IL-8 in adjacent normal prostate tissue vs prostate cancer samples for individual patients was determined. The relative changes in expression for normal adjacent prostate tissue vs cancer is shown. Samples with a decrease in DACH1 mRNA abundance and increase in IL-6 or (H) IL-8 abundance are shown in the left upper quadrant. This quadrant expressing a decrease in DACH1 mRNA and corresponding increase in mRNA levels in individual patient cancer samples vs adjacent normal prostate epithelium was significantly enriched. I) The relative luciferase reporter activity of the IL-6 and J) IL-8 promoter was normalized to 1 for the vector control. The luciferase promoter reporters were transfected into PC3 cells, together with equimolar amounts of either vector, DACH1 expression vector or the ΔDS expression vector. Data are mean ± SEM for N>5 separate experiments. K) Western blot of transfected PC3 cells showing relative abundance of the exogenous DACH1 assessed by FLAG epitope. Vinculin is shown as a protein

loading control. L) ChIP of the IL-6 and IL-8 promoter with oligonucleotide primers directed to the regulatory region of the genes as shown.

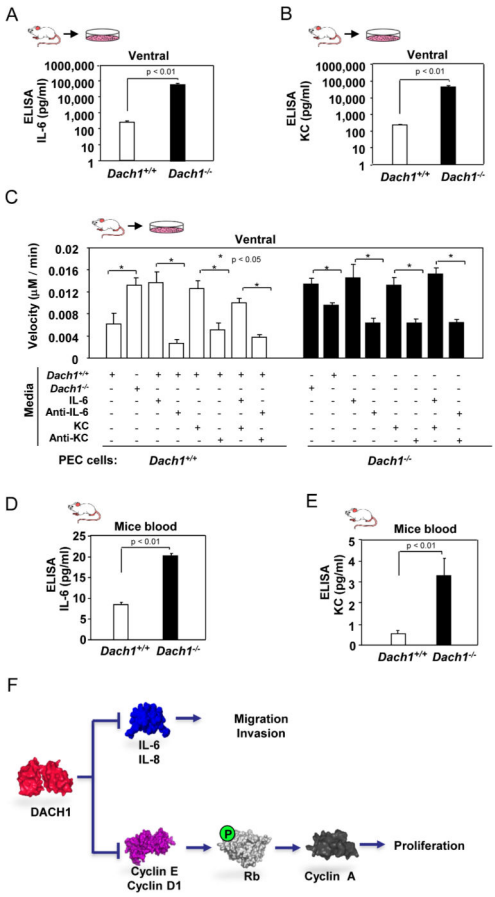

Figure 5. *Dach1* is a dominant endogenous restraint of prostate epithelial cell cytokine production and thereby cellular migration

The *Dach1*$^{fl/fl}$ Probasin-Cre prostate epithelial cells were cultured and comparison was made to *Dach1*$^{+/+}$. A) ELISA was used to determine the relative abundance of cytokines in the supernatant (ventral prostate epithelial cells) in culture for IL-6 and B) KC in pg/mL (Note: Log scale). C) Analysis of migratory cell velocity for prostate epithelial cells derived from *Dach1*$^{+/+}$ or *Dach1*$^{-/-}$ PECs. Cells were co-incubated with media derived from either *Dach1*$^{+/+}$ or *Dach1*$^{-/-}$, incubated with IL-6 or anti-IL-6 antibody. KC or anti-KC antibody is indicated in the Figure. P value is indicated (significance <0.05). Data is shown as mean ± SEM of three separate experiments. D) ELISA was used to determine the relative abundance for cytokines in the *Dach1* wild-type vs. knockout mice in circulating serum of IL-6 and E) KC in pg/mL (Note: Log scale). F) Schematic representation of *Dach1* as key determinant of prostate cellular cytokine secretion and cellular proliferation.