Dachshund Binds p53 to Block the Growth of Lung Adenocarcinoma Cells

Ke Chen1,3, Kongming Wu1,3, Shaoxin Cai1,3, Wei Zhang1,3, Jie Zhou1,3, Jing Wang1,3, Adam Ertel1,3, Zhiping Li1,3, Hallgeir Rui1, Andrew Quong1, Michael P. Lisanti2,3, Aydin Tozeren3,4,5, Ceylan Tanes3,4,5, Sankar Addya1,3, Michael Gormley1,3, Chenguang Wang1,2,3, Steven B. McMahon1,3, and Richard G. Pestell1,3

Abstract

Hyperactive EGF receptor (EGFR) and mutant p53 are common genetic abnormalities driving the progression of non–small cell lung cancer (NSCLC), the leading cause of cancer deaths in the world. The Drosophila gene Dachshund (Dac) was originally cloned as an inhibitor of hyperactive EGFR alleles. Given the importance of EGFR signaling in lung cancer etiology, we examined the role of DACH1 expression in lung cancer development. DACH1 protein and mRNA expression was reduced in human NSCLC. Reexpression of DACH1 reduced NSCLC colony formation and tumor growth in vivo via p53. Endogenous DACH1 colocalized with p53 in a nuclear, extranucleolar location, and shared occupancy of ~15% of p53-bound genes in ChIP sequencing. The C-terminus of DACH1 was necessary for direct p53 binding, contributing to the inhibition of colony formation and cell-cycle arrest. Expression of the stem cell factor SOX2 was repressed by DACH1, and SOX2 expression was inversely correlated with DACH1 in NSCLC. We conclude that DACH1 binds p53 to inhibit NSCLC cellular growth. Cancer Res; 73(11): 3262–74. ©2013 AACR.

Introduction

Lung cancer is the leading cause of cancer mortality worldwide with approximately 160,000 deaths reported each year in the United States (1). Non–small cell lung cancer (NSCLC) constitutes approximately 80% of lung tumors and includes adenocarcinoma and squamous cell carcinoma, which are histologically distinct from small cell lung cancer (SCLC; ref. 2). Several oncogenes are important in lung cancer including Ras and EGF receptor (EGFR), which are mutated in 15% and 10% of NSCLCs. Other activating genetic changes include overexpression of SOX2 and fusion proteins, including ALK and ROS and other translocations, which induce tyrosine kinase activity (3–5). Inactivation of tumor suppressors occurs, including the inactivation of p53, in which loss of heterozygosity (LOH) is observed in 60% and mutations arise in 50% (6, 7).

p53 functions as a transcription factor that senses the favorability of the local microenvironment, regulating gene expression and, thereby, a variety of functions, including senescence, energy metabolism, DNA repair, cell-cycle arrest, and apoptosis (8). The function of p53 is mediated through binding proteins, altered cellular localization, and posttranslational modification (9). p53 activation in vivo involves stress-induced stabilization via loss of binding to its negative regulators, including Mdm2 and Mdmx, with sequential recruitment of cointegrator complexes. The mechanisms enhancing p53 function are fundamental to the biology of p53 and tumor suppression.

The Drosophila Dac gene is a key member of the retinal determination gene network (BDGN), including eyes absent (eya), ey, twin of eyeless (toy), teashirt (tsh), and sin oculis (so), which specify eye tissue identity (10, 11). Initially cloned as a dominant inhibitor of the hyperactive EGFR, Ellipse, in Drosophila, the mammalian DACH1 gene regulates gene expression of target genes, in part, through interacting with DNA-binding transcription factors (c-Jun, Smads, Six, and Eto), and in part through intrinsic DNA-sequence specific binding properties via Forkhead binding sequences (12–15). Clinical studies have shown a correlation between poor prognosis breast cancer and reduced DACH1 expression (15) and loss of DACH1 expression has been observed in prostate and endometrial cancer (14, 16). Given that Dac was identified as a dominant inhibitor of Ellipse and the importance of hyperactive EGFR in human lung cancer, we examined the role of DACH1 in lung cancer cellular growth. These studies identified DACH1 as a novel p53 binding partner that participates in p53-mediated induction of p21cip1 and cell-cycle arrest.
Materials and Methods

Cell culture, plasmid construction, reporter genes, expression vectors, DNA transfection, and luciferase assays

Cell culture, DNA transfection, and luciferase assays using the Rad51-Luc, p21-Luc, and SOX2-Luc reporter genes were conducted as previously described (17, 18). The H1299, HEK293T, H460, and HCT116 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, 1% penicillin, and 1% streptomycin as previously described (15). The expression plasmids encoding an N-terminal FLAG peptide linked to the DACH1, DACH1 C-terminal domain alone (C-term), or DACH1 C-terminal domain deleted (∆C) were previously described (13–15). The DACH1 C-terminal (C-term) and DACH1 C-terminal deleted (∆C) were subcloned into the MSCV-IRES-GFP retrovirus vector. GFP-positive cells were selected by FACS. Cells were plated at 10^5 cells in a 24-well plate on the day before transfection with Superfect according to the manufacturer’s instructions (Qiagen). For reporter gene assays, 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-change effect of expression vector was determined in comparison with the effect of the empty expression vector cassette, and statistical analyses were carried out using the t test.

Cell-cycle analysis

Cell-cycle parameters were determined using laser scanning cytometry. Cells were processed by standard methods using propidium iodide staining of cellular DNA. Each sample was analyzed by flow cytometry with a FACSScan 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. Annexin V staining was conducted as described previously (19).

Cell proliferation assays

H1299 cells infected with MSCV-IRES-GFP, MSCV-DACH1-IRES-GFP, MSCV-DACH1 C-term-IRES-GFP, or MSCV-DACH1 ∆C-IRES-GFP 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. The tet-inducible p53 cell line H1299 was plated in growth medium in either the presence or absence of doxycycline overlaid on a 0.5% agarose base, also in complete growth medium. Two weeks after incubation, colonies more than 50 μm 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.

Immunohistochemistry

Immunohistochemical (IHC) analysis of human lung cancer was conducted using a polyclonal DACH1 antibody from Proteintech using the same approach as previously described (15). Human lung cancer tissue arrays were from Biomax.

Microarray, cluster, and chromatin immunoprecipitation analysis

DNA-free total RNA isolated from H1299 cells expressing p53 and/or DACH1 were used to probe human OneArray (Phalanx). RNA quality was determined by gel electrophoresis. Probe synthesis and hybridization were carried out as described previously (21). Analysis of the arrays was conducted using GeneSpring. Arrays were normalized using robust multi-array analysis, and the P value of 0.05 was applied as statistical criteria for differentially expressed genes. These genes were then grouped using hierarchical clustering with “complete” agglomeration, and each cluster was further analyzed on the basis of the known function of the genes contained in the cluster. Expression profiles are displayed using Treeview. Classification and clustering for pathway-level analysis were done by using gene sets Analysis of Sample Set Enrichment Scores (ASSESS), available online. ASSESS provides a measure of enrichment of each gene set in each sample. Gene set enrichment was dependent on a concordance of at least 2 samples within the replicates that was opposite between phenotypes (15).

IP and Western blot were: anti-p53 (DO-I) (SC-126), anti-FLAG (M2 clone, Sigma), and DACH1 (Proteintech, cat:10914-1-AP).

Protein–protein interaction by GST–pull-down assays

_In vitro_ protein–protein interactions were done as described previously (21). _In vitro_ translated proteins were prepared by coupled transcription–translation with a TNT-coupled reticulocyte lysate kit (Promega) using plasmid DNA (1.0 μg) in a total of 50 μL. GST fusion proteins were prepared from E. coli. _In vitro_ translated protein (15 μL) was added to 5 μg GST fusion protein of GST as control in 225 μL binding buffer [50 mmol/L Tris-HCl, 120 mmol/L NaCl, 1 mmol/L DTT, 0.5% NP40, 1 mmol/L EDTA, 2 μg/mL leupeptin, 2 μg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 2 μg/mL pepstatin] and rotated for 2 hours at 4°C. Glutathione-Sepharose beads slurry (50 μL) was added and the mixture was rotated for a further 30 minutes at 4°C. Beads were washed 5 times with washing buffer (1 mL), and binding buffer (30 μL) was added after the final wash. Sepharose beads were washed 5 times with lysis buffer and boiled in SDS sample buffer, and the proteins released were resolved by SDS-PAGE followed by Western blot.

Colony-forming assays

A total of 4.0 × 10^4 H1299 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. Two weeks after incubation, colonies more than 50 μm 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.
Binding sites for p53 in human lung fibroblasts were obtained from a chromatin immunoprecipitation sequencing (ChIP-Seq) analysis of chromatin occupancy by p53 following activation by 5-fluorouracil (5-FU; ref. 22). High-confidence ChIP-Seq peaks were identified as described previously (22). Genes regulated by p53 were identified by mapping ChIP-Seq peaks to gene coding regions (ChIP-Seq peak was located inside the transcribed region of the gene or within 20 kb of either end). Gene identifiers of p53-regulated genes were translated from gene symbol to Ensembl gene identifier for comparison with DACH1 regulated genes. DACH1 binding sites were identified from ChIP-Seq of a breast cancer cell line stably expressing DACH1. ChIP-Seq peaks from DACH1 ChIP-Seq were mapped to the nearest proximal Ensembl gene identifier. Significant overlap in p53 and DACH1 regulated genes was tested using the hypergeometric distribution with all Ensembl gene identifiers in Homo sapiens used as a reference set.

Mice study

About 1 × 10^6 H1299 cells expressing p53 and/or DACH1 were implanted subcutaneously into 4- to 6-week-old athymic female nude mice purchased from the National Cancer Institute, NIH. The tumor growth was measured every 5 days for 32 days by using a digital caliper. Tumor weight was measured when mice were sacrificed on day 35 after cell implantation (15).

Results

DACH1 abundance is reduced in NSCLC

IHC staining was conducted to detect DACH1 abundance in human lung cancer samples using an antibody from Proteintech, according to a previously described protocol (15). Quantification of the nuclear DACH1 staining among distinct human NSCLC tumors (n = 97) and in 10 normal control samples showed an approximately 50% decrease in DACH1 immunopositivity (Fig. 1A). DACH1 abundance was not reduced in SCLC or carcinoid tumors (Fig. 1A).

In a separate cohort, the abundance of DACH1 was examined and shown to be reduced in cancer tissues (Fig. 1B). DACH1 levels were decreased further in stage III versus stage I cancer (Fig. 1B). In normal lung, DACH1 was identified within the lung epithelial cells (Fig. 1C). Representative examples of IHC for DACH1 in different subtypes of lung cancer are shown in Fig. 1D.

In order to examine the mRNA expression of DACH1 in human lung cancer, Oncomine databases were interrogated (Supplementary Fig. S1). The relative abundance of DACH1 was reduced 2- to 3-fold in NSCLC, compared with normal lung, in each of the databases. In contrast, DACH1 mRNA was increased in carcinoid tumors compared with normal lung (Supplementary Fig. S1E; n = 144, normal: n = 157, NSCLC patients).

DACH1 binds to p53 via its carboxyl terminus

In view of the importance of p53 in NSCLC growth (7, 23), we considered the possibility that DACH1 may associate with p53. IHC staining was conducted of a tet-inducible p53 H1299 NSCLC cell line. The parental H1299 cell line is p53 null due to a homozygous partial deletion of the TP53 gene. Induction of p53 by tetracycline in the p53 H1299 line restored p21<sup>WAF1</sup> gene expression and cell-cycle arrest (24). IHC staining of cells induced to express p53, showed nuclear p53 localized with DACH1 (Fig. 2A; Supplementary Fig. S2). Association of p53 with DACH1 was shown by IP-Western blot analysis (Fig. 2B). IP with an antibody directed to the DACH1 N-terminal FLAG tag coprecipitated p53. Similarly, IP with a p53 antibody and subsequent Western blot for DACH1 evidenced coprecipitated DACH1 (Fig. 2B). In order to determine whether endogenous p53 associated with DACH1, a cell line expressing p53 wild-type (wt) and DACH1 was assessed. DACH1 expression is reduced in NSCLC and many tumor cell lines. The human colon cancer HCT116 was found to express p53-wt and DACH1 (Fig. 2C; Supplementary Fig. S2B). IHC staining showed a colocalization of DACH1 with p53 in a nuclear, extranucleolar distribution (Fig. 2C). IP with an antibody directed toward endogenous p53 coprecipitated DACH1 (Fig. 2D). In view of the reduced abundance of DACH1 in H1299 NSCLC, we investigated possible mechanisms governing the reduced expression. Cells were treated with the DNA methylase inhibitor 5 azacytidine (AZT) for 96 hours, either alone or with the addition of the histone deacetylase (HDAC) inhibitor trichostatin A (TSA; Supplementary Fig. S3). DACH1 abundance was induced by the addition of 5 azacytidine, TSA, and a combination of both, indicating that endogenous DACH1 abundance is reduced in H1299 NSCLC because of DNA methylation and histone deacetylation.

In order to determine whether DACH1 bound directly to p53, the DACH1 protein was purified as a 6-His-GST protein and p53 was expressed from <i>in vitro</i> translation (IVT). GST pull-down was conducted upon co incubation of DACH1 with p53. Comparison was made between the binding of DACH1-wt and the DACH1 DBD (183–293), p53-bound 6-His DACH1 (Fig. 2E), but failed to bind the DACH1 DBD. The binding of DACH1-wt to p53 was greater despite the significantly lower abundance of 6-His DACH1-wt compared with DACH1 DBD by Western blotting to the 6-His epitope (Fig. 2E).

To identify the domains of DACH1 required for binding to p53, a series of DACH1 mutant expression vectors were transfected into HEK293T cells (Fig. 2F). p53 localized with DACH1 in the nucleus of HEK293T cells (Fig. 2G). The N-terminal FLAG epitope was used to immunoprecipitate DACH1. Transfection of HEK293T cells with a series of DACH1 mutants showed that DACH1 bound to p53 and that the DACH1 C-terminus was sufficient for binding to p53 (Fig. 2H). Deletion of the DACH1 C-terminus abolished binding to p53 (Fig. 2H). These studies indicated that the C-terminus of DACH1 is required for binding to p53.

The DACH1 C-terminus is required for the enhancement of p53-mediated inhibition of contact-independent growth and cell-cycle arrest

In order to determine the functional significance of DACH1 binding to p53 via its carboxyl terminus, colony-forming assays and cell proliferation studies were conducted.
Either DACH1-wt, DACH1ΔC, or the DACH1 C-terminus were stably introduced into the tet-inducible p53 H1299 cell line. Tetracycline was used to induce p53. p53 expression inhibited colony formation, reducing both the number and size of colonies (Fig. 3A). DACH1-wt expression inhibited colony formation to a similar level as p53 (Fig. 3B).

Figure 1. Reduced DACH1 abundance in non–small cell lung cancer (NSCLC). A and B, immunohistochemical staining for DACH1 in lung cancer samples was quantitated for each subtype of lung cancer and the data is shown as mean ± SEM for N as indicated in the figure in parentheses. C and D, representative examples of immunohistochemical staining for DACH1 in each of the histologically distinct types of lung cancer as indicated.
The DACH1 C-terminus was required for inhibition of colony formation in the presence of p53 (Fig. 3A). DACH1 enhanced p53-dependent inhibition of colony size and number (Fig. 3B–E). The DACH1 C-terminus was sufficient to enhance a p53-mediated inhibition of colony formation. In the presence of p53, the expression of DACH1ΔC was defective in reducing colony formation, compared with DACH1-wt (Fig. 3C), despite a relatively greater abundance of the ΔC protein expressed, based on Western blotting (data not shown).

DACH1 inhibits cell proliferation and enhances p53-dependent cell-cycle arrest

In order to determine the mechanism by which DACH1 enhanced p53-mediated inhibition of colony formation, we assessed cell proliferation, cell-cycle changes, and cellular apoptosis. p53 expression inhibited H1299 cell proliferation assessed using the MTT assay (Fig. 3F). DACH1 expression inhibited cell proliferation and enhanced p53-mediated inhibition of cell proliferation (Fig. 3F). The p53 binding-defective DACH1 mutant (DACH1ΔC) exhibited reduced p53-dependent
inhibition of cell proliferation (Fig. 3G). In H460 cells with endogenous expression of p53, DACH1 inhibited cellular proliferation. However, the inhibition of cell proliferation by DACH1 was abolished when p53 expression was knocked down by p53 shRNA (Fig. 3H). The C-terminal deletion mutant of DACH1 failed to repress cell proliferation either in the presence or absence of p53 (Fig. 3I).

The effect of DACH1 on p53-dependent cell-cycle function was determined by fluorescence-activated cell sorting (FACS) analysis. Induction of p53 increased the proportion of cells in G0–G1 (Fig. 4A). DACH1 expression enhanced p53-dependent induction of G0–G1 but deletion of the DACH1 C-terminus abolished this effect (Fig. 4A). In the absence of p53, DACH1 had no effect on S phase but, in the presence of p53, it was associated with an inhibition of S phase. The induction of p53 reduced the proportion of cells in the S phase of the cell cycle at 24 hours (Fig. 4B). DACH1 expression in the presence of p53 enhanced the inhibition of S phase. Deletion of the DACH1 C-terminus abolished the p53-dependent inhibition of S phase (Fig. 4B). These findings suggest that DACH1 and p53 both inhibit S-phase cell-cycle progression and that DACH1 expression with p53 enhanced

Figure 3. DACH1 inhibition of NSCLC colony formation and cell proliferation requires the DACH1 p53-binding domain. A, H1299 colony-forming assays comparing DACH1-wt and DACH1 mutants are shown. B–E, colony size and number are shown as mean ± SEM for n > 5 separate experiments using stably transduced cell lines. DACH1 expression vectors were stably transduced into tet-inducible p53 H1299 cells. Colony number (B and C) and colony size (D and E), are shown as mean ± SEM for n > 5 separate experiments. F–I, H1299 cells and H460 cells were assessed for cellular proliferation using the MTT assay.

<table>
<thead>
<tr>
<th>H1299 cells</th>
<th>Vector</th>
<th>DACH1</th>
<th>DACH1-ΔC</th>
<th>DACH1-C term</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 (-)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53 (+)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Colony number</th>
<th>H1299</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 -</td>
<td>+</td>
</tr>
<tr>
<td>p53 +</td>
<td>+</td>
</tr>
<tr>
<td>DACH1 -</td>
<td>+</td>
</tr>
<tr>
<td>DACH1 +</td>
<td>+</td>
</tr>
<tr>
<td>ΔC -</td>
<td>+</td>
</tr>
<tr>
<td>ΔC +</td>
<td>+</td>
</tr>
<tr>
<td>C-term -</td>
<td>+</td>
</tr>
<tr>
<td>C-term +</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Colony size</th>
<th>H1299</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 -</td>
<td>+</td>
</tr>
<tr>
<td>p53 +</td>
<td>+</td>
</tr>
<tr>
<td>DACH1 -</td>
<td>+</td>
</tr>
<tr>
<td>DACH1 +</td>
<td>+</td>
</tr>
<tr>
<td>ΔC -</td>
<td>+</td>
</tr>
<tr>
<td>ΔC +</td>
<td>+</td>
</tr>
<tr>
<td>C-term -</td>
<td>+</td>
</tr>
<tr>
<td>C-term +</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>H1299 (MTT)</th>
<th>Fold change</th>
<th>Repression rate (100%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 -</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>p53 +</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DACH1 -</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DACH1 +</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ΔC -</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ΔC +</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C-term -</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C-term +</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>H460 (MTT)</th>
<th>Fold change</th>
<th>Repression rate (100%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 -</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>p53 +</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DACH1 -</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DACH1 +</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ΔC -</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ΔC +</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C-term -</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C-term +</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
a G0–G1 cell-cycle accumulation, requiring the DACH1 carboxyl terminus.

p53 induces apoptosis in H1299 cells when assessed by the sub-G1 fraction of the cell cycle by FACS or by Annexin V staining (25). Expression of p53 increased the proportion of cells in the sub-G1 fraction at 24 hours, which was enhanced by DACH1 (Fig. 4C). Deletion of the DACH1 C-terminus abolished DACH1-dependent enhancement of p53 apoptosis (Fig. 4C). A similar observation was made when apoptosis was assessed by Annexin V staining (Fig. 4D). In the distinct H460 NSCLC line that expresses p53, DACH1 inhibited S phase, requiring the DACH1 C-terminus. p53 short hairpin RNA (shRNA)
abolished DACH1 inhibition of S phase showing the requirement of p53 for DACH1–cell-cycle inhibition (Fig. 4E). Similarly, DACH1 induction of apoptosis assessed by Annexin V staining required the DACH1 C-terminus and was abolished by p53 shRNA (Fig. 4F).

**DACH1 inhibition of Rad51 and induction of p21<sub>CIP1</sub> requires endogenous p53**

We next examined the functional significance of DACH1 on p53-dependent gene expression using the tet-H1299 cells and the known p53 target genes, p21<sub>CIP1</sub> and Rad51. An analysis of gene expression was conducted upon induction of either p53, DACH1, or the combination of both. The expression of p53 induced (Fig. 5A, in red) and repressed (Fig. 5A, in green) clusters of genes (representative example Fig. 5A). DACH1 expression augmented p53-mediated induction of gene clusters (Genes 1; Fig. 5A) and augmented repression of p53-repressed genes (Genes 2; Fig. 5A). Thus, the combination of DACH1 and p53 expression augmented expression of common target genes shown. p21<sub>CIP1</sub> is an important target of p53-mediated cell-cycle arrest (26). Induction of DACH1 increased p21<sub>CIP1</sub> abundance approximately 3-fold (Fig. 5B). Expression of DACH1 in the presence of p53 enhanced p21<sub>CIP1</sub> induction by p53 (Fig. 5B). The expression of p53 enhanced p21<sub>CIP1</sub> promoter activity, which was augmented by the expression of DACH1, but not DACH1ΔC (Fig. 5C). Expression of the DACH1-binding defective mutant p53-R248Q did not enhance p21<sub>CIP1</sub> transcriptional activity and DACH1 did not affect p21<sub>CIP1</sub> promoter activity in the presence of p53-R248Q, suggesting the effect of DACH1 on p21<sub>CIP1</sub> requires p53 association (Fig. 5D). DACH1 induced p53 activity in cells. In order to determine the mechanism by which DACH1 induced p21<sub>CIP1</sub> and reduced Rad51 abundance, we examined the ability of DACH1 to regulate transcription using the promoter-regulatory regions of these 2 target genes assessed in luciferase reporter assays. DACH1 expression inhibited Rad51 promoter reporter activity in the presence of p53 (Fig. 5E). In addition, the DACH1 DBD mutant repressed Rad51 Luc reporter activity (Fig. 5F). DACH1ΔC failed to repress Rad51 reporter activity (Fig. 5G). As DACH1 bound p53 and induced p53 target expression, we considered the possibility that DACH1 may enhance p53-dependent transcriptional activity. We deployed a synthetic multimeric canonical p53 response element reporter gene (Fig. 5H). In order to consider the possibility that DACH1 and p53 may bind to common genes in the context of chromatin, sets of genes binding p53 in IMR90 lung fibroblasts in ChIP Seq (22) were compared with genes binding DACH1, in ChIP Seq (22). A total of 743 binding sites for p53 and 1844 binding sites for DACH1 were identified. 112 genes regulated by both DACH1 and p53 were identified ($P < 1 \times 10^{-18}$; Supplementary Table S1). Collectively, these studies show that DACH1 directly associates with the p53 tumor suppressor to enhance p53-dependent apoptosis associated with increased transcriptional activity at the p21<sub>CIP1</sub> target gene.

**DACH1 enhances p53-dependent suppression of NSCLC tumor growth in vivo**

In order to determine the significance of DACH1 inhibition of NSCLC in vivo, H1299 cells were stably transduced with an expression vector encoding DACH1 and/or p53. Tumors in nude mice were measured every 5 days, with terminal analysis at day 32 (Fig. 6). Tumor weight was reduced by DACH1 or p53 and DACH1 expression further augmented p53-mediated growth suppression (Fig. 6A and B). Expression of either DACH1 or p53 inhibited tumor growth and DACH1 enhanced p53-dependent tumor suppression (Fig. 6C and D). Immunohistochemistry showed the induction of DACH1 and p53 by the inducible systems; however, DACH1 did not enhance p53 abundance. DACH1 expression enhanced p53-mediated induction of p21<sub>CIP1</sub>, and enhanced p53-mediated repression of RAD51 and Ki67 (Fig. 6E–J).

**DACH1 restrains SOX2 expression and transcription in NSCLC**

In order to further examine the potential mechanisms by which DACH1 inhibited NSCLC, we considered SOX2 as an additional target. We examined expression profiles of DACH1, SOX2, and p53 in NSCLC ($n = 351$) by creating a NSCLC meta-database, as previously described, for breast cancer (21). A comparison was made with expression in normal lung ($n = 74$). DACH1 expression was reduced in NSCLC (Fig. 7A and C) compared with normal lung and SCLC ($n = 38$). Reduced DACH1 expression correlated with increased SOX2 in NSCLC. Low DACH1 expression was found in NSCLC of all stages (Fig. 7B). In contrast DACH1 was increased in SCLC (Fig. 7A and B). Reduced DACH1 correlated with increased EGFR expression in a subset NSCLC patient samples (Supplementary Fig. S4). Patients with Low DACH1 and high EGFR predicted an approximately 1-year worse prognosis (Supplementary Fig. S4E), compared with other tumors. In order to examine the mechanism by which DACH1 may repress SOX2 expression, we conducted transcriptional analysis using the SOX2 promoter linked to a luciferase reporter gene in H1299 NSCLC cell lines. DACH1 repressed SOX2, requiring the DACH1 C-terminus (Fig. 7D), p53 repressed SOX2 and DACH1 enhanced p53 repression (Fig. 7D).

**Discussion**

The current studies provide evidence that the cell-fate-determination factor DACH1 directly binds and enhances several functions of p53 in NSCLC. The abundance of DACH1 was reduced in NSCLC, correlating with clinical stage. Reduced DACH1 abundance was shown in NSCLC, as measured by both RNA and protein abundance. Prior studies of DACH1 had shown reduced abundance of DACH1 in human malignancies, including breast cancer, prostate cancer, and endometrial cancer (14–16). We examined expression profiles of DACH1 and p53 in NSCLC by creating a NSCLC meta-database, as previously described, for breast cancer (21). A comparison was made with expression in normal lung, DACH1 expression was reduced in NSCLC (Fig. 7), confirming oncomine analysis (Supplementary Fig. S1). The current studies extend the spectrum of human tumors in which DACH1 expression is reduced.

The molecular mechanisms by which DACH1 inhibits contact-independent growth are poorly understood. The current studies show the importance of p53 in DACH1-mediated inhibition of NSCLC tumor formation in vivo and...
Figure 5. DACH1 enhances p53-dependent transcriptional regulation of Rad51 and p21CIP1. Microarray-based gene expression analysis (A), Western blot (B), and Luciferase reporter assays (C–H) were conducted in H1299 cells stably expressing p53 and DACH1 wt. In the microarray analysis, expression direction is color coded (red, increased; blue, decreased). Genes 1 represent a cluster of genes that are induced by p53 and that are further enhanced in their expression through expression of DACH1. Genes 2 is a cluster of genes that are repressed by p53 in which repression is enhanced by expression of DACH1. B, Western blot analysis of cells that were transduced with the DACH1-expressing retrovirus and p53 abundance was induced by tetracycline. Western blot was conducted with antibodies as indicated, with longer exposure (L.E.) or shorter exposure (S.E.) shown. C–H, in the reporter assays, DACH1, DACH1ΔDBD, or the DACH1ΔC mutant were compared using either the Rad51 or p21CIP1 promoter-luciferase reporters. The relative induction of luciferase activity was normalized to β-galactosidase and shown as fold change. Data are mean ± SEM for n ≥ 5 separate transfections. Activity of the multimetric canonical p53 response element luciferase reporter gene was assessed in transient expression studies after cotransfection with either DACH1-wt or the DACH1 mutants. Data are mean ± SEM for n > 5 separate transfections.
Figure 6. DACH1 enhances p53-dependent NSCLC growth suppression in mice. Serial measurements were conducted every 5 days of H1299 tumors stably expressing either DACH1 and/or p53, injected into nude mice. The data are ± SEM for n = 12 separate tumors for each group (total of 48 tumors analyzed). The data are mean for tumor terminal weight, raw data (A), tumor weight fold change compared with vector control (B), tumor size (C), and tumor size measured as fold change versus vector (D; data are shown as mean ± SEM). E–J, immunohistochemical analysis of extirpated tumors. Data for immunohistochemical analysis are quantitated as mean ± SEM for n = 4 tumors in each group.
Figure 7. DACH1 constrains SOX2 expression. A, gene expression values for DACH1 and SOX2 in subjects with NSCLC (n = 251) and SCLC (n = 38) divided by the sample means. The tumor types are shown as adenocarcinomas (ADC), large cell carcinomas (LCC), squamous cell carcinomas (SCC), and small-cell lung cancer (SCLC). B, SOX2 versus DACH1 expression in NSCLC and SCLC patients divided by the sample means annotated with cancer stage information. C, correlation between SOX2 and DACH1 expression by tumor types, showing positive correlation between reduced DACH1 and increased SOX2 abundance. D, SOX2 promoter luciferase reporter assays conducted in H1299 cells. H1299 cells were transfected with SOX2-Luc and expression vectors encoding DACH1 or DACH1 mutants. Data are mean ± SEM of n > 5 separate transfection.
the inhibition of colony number and size. DACH1 bound p53 to inhibit NSCLC cellular proliferation and colony formation. The carboxyl terminus of DACH1 was required for p53 binding, and was required for p53-dependent inhibition of colony formation and cellular proliferation. DACH1 inhibited S phase requiring the presence of p53. DACH1-induced p21^{CIP1} and inhibited Rad51, requiring the p53 binding region of DACH1. Furthermore, DACH1 expression enhanced p53 signaling via a canonical p53 binding site. Collectively, these studies are consistent with a model in which DACH1 functions as an accessory tumor suppressor in the p53 pathway.

Recent studies showed the ability of DACH1 to inhibit the expression of tumor-initiating cells (18, 27). Genome-wide expression analysis of mammary tumors showed DACH1 expression repressed a gene signature associated with stem cells, including Sox2, Nanog, and Klf4 (18). As SOX2 amplification is a feature of NSCLC, we examined the possibility that DACH1 may repress SOX2, contributing to NSCLC tumor suppression. SOX2 is an amplification of lineage-survival oncogene in lung squamous cell carcinoma (3) that is oncogenic in the lung (28), with mutations identified in both NSCLC and SCLC. SOX2 expression is essential for lung development, can reprogram cells to pluripotency, and collaborates with FoxG1 in generating self-renewing neural precursor cells (29). DACH1 binds the SOX2 gene promoter in ChIP-Seq and inhibits SOX2 expression in breast cancer cells (18). DACH1 binds FOXO binding sites, competing with endogenous FOXO proteins in ChIP assays and antagonized FOXO signaling (12). Thus, DACH1 inhibits 2 key pathways promoting stem cell renewal. DACH1 and SOX2 expression were inversely correlated in NSCLC but not SCLC and DACH1 repressed SOX2 via the promoter of SOX2. The DACH1-mediated repression of SOX2 was p53-dependent. SOX2 contributes to the expansion of several stem cell types. It has been proposed that a cancer stem or progenitor cell contributes to the onset of NSCLC (30). The repression of SOX2 by DACH1 may contribute a mechanism to inhibit NSCLC.

The loss of normal stem cells is largely due to p53-p21^{CIP1} mediated proliferative arrest, senescence, and apoptotic elimination (31, 32). The diminished stem cell function observed in certain strains of mice encoding germ line alleles of mutant p53 (the gene encoding p53, also known as Trp53; refs. 33, 34), and the diminished stem cell pool in Dach1^-/- mice (27), is consistent with the possibility that DACH1 and p53 may also cooperate in stem cell function. It is known that p53 deletion improves stem cell function in mice, in particular with animals with short dysfunctional telomeres (35, 36). p53 may reduce stem cell number through regulating the polarity of cell division, with recent studies showing that loss of p53 favors symmetric division of cancer stem cells (37). DACH1 has been shown to regulate the polarity of mammary epithelial cells in 3D Matrigel (15). It is possible that DACH1 and p53 may regulate stem cell signaling modules although common gene pathways, as ChIP-Seq analysis of p53 binding sites in IMR90 cells (37) compared with DACH1 binding sites, indicated approximately 15% of p53 binding sites also bound DACH1.

The functional significance of p53 in DACH1-dependent regulation of cell polarity remains to be determined, but may provide an additional mechanism for coordinating progenitor cell expansion.

Disclosure of Potential Conflicts of Interest

R.G. Pestell holds minor (<$10,000) ownership interests in and serves as the CSO/Founder of the biopharmaceutical companies ProstaGene, LLC and AAA Phoenix, Inc. In addition, R.G. Pestell holds ownership interests (value unknown) for several submitted patent applications. 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.

Authors’ Contributions

Conception and design: K. Chen, K. Wu, M.P. Lisanti, R.G. Pestell
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Chen, S. Cai, J. Wang, H. Rui, S.B. McMahon
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Chen, K. Wu, A. Ertel, H. Rui, A. Quong, A. Tozeren, C. Tanes, S. Addya, M. Gormley, C. Wang, R.G. Pestell
Writing, review, and/or revision of the manuscript: K. Chen, A. Ertel, H. Rui, A. Quong, M.P. Lisanti, A. Tozeren, C. Tanes, M. Gormley, R.G. Pestell
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Chen, W. Zhang, J. Zhou, Z. Li
Study supervision: K. Chen, K. Wu, Z. Li, R.G. Pestell

Acknowledgments

The authors thank Jeannine Nicole Moore for preparation of this manuscript.

Grant Support

This work was supported in part by grants no. R01CA70896, R01CA132115, R01CA75503, R01CA86072, (R.G. Pestell), the Kimmel Cancer Center NIH Cancer Center grant P30CA56036 (R.G. Pestell), a generous grant from the Dr. Ralph and Marian C. Falk Medical Research Trust (R.G. Pestell), a grant from the Pennsylvania Department of Health (R.G. Pestell), Margaret Q. Landenberger Research Foundation, and the Department of Defense Concept Award W81XWH-11-1-0033 (K. Wu). M.P. Lisanti and his laboratory were supported via the resources of Thomas Jefferson University.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 10, 2012; revised February 1, 2013; accepted February 1, 2013.

Published OnlineFirst March 14, 2013.

References


www.aacajournals.org Cancer Res; 73(11) June 1, 2013 3273

Downloaded from cancerres.aacrjournals.org on April 14, 2017. © 2013 American Association for Cancer Research.


Dachshund Binds p53 to Block the Growth of Lung Adenocarcinoma Cells

Ke Chen, Kongming Wu, Shaoxin Cai, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-12-3191

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2013/03/14/0008-5472.CAN-12-3191.DC1

Cited articles
This article cites 37 articles, 16 of which you can access for free at:
http://cancerres.aacrjournals.org/content/73/11/3262.full.html#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
/content/73/11/3262.full.html#related-urls