Attenuation of Forkhead signaling by the retinal determination factor DACH1

Jie Zhou[a,b], Chenguang Wang[a,b,c,1], Zhibin Wang[d], Will Dampier[e], Kongming Wu[a,b], Mathew C. Casimiro[a,b], Iouri Chepelev[d], Vladimir M. Popov[a,b], Andrew Quong[a,b], Aydin Tozeren[e], Keji Zhao[d], Michael P. Lisanti[a,b,c], and Richard G. Pestell[a,b,1]

Departments of [a]Cancer Biology and [c]Stem Cell Biology and Regenerative Medicine, [b]Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA 19107; [d]Laboratory of Molecular Immunology, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD 20892; and [e]Center for Integrated Bioinformatics, School of Biomedical Engineering, Science and Health Systems, Drexel University, Philadelphia, PA 19104

Communicated by Hilary Koprowski, Thomas Jefferson University–Jefferson Medical College, Philadelphia, PA, March 3, 2010 (received for review December 29, 2009)

The *Drosophila* Dachshund (*Dac*) gene, cloned as a dominant inhibitor of the hyperactive growth factor mutant *ellipse*, encodes a key component of the retinal determination gene network that governs cell fate. Herein, cyclic amplification and selection of targets identified a DACH1 DNA-binding sequence that resembles the FOX (Forkhead box–containing protein) binding site. Genome-wide *in silico* promoter analysis of DACH1 binding sites identified gene clusters populating cellular pathways associated with the cell cycle and growth factor signaling. ChIP coupled with high-throughput sequencing mapped DACH1 binding sites to corresponding gene clusters predicted *in silico* and identified as weight matrix resembling the cyclic amplification and selection of targets–defined sequence. DACH1 antagonized FOXM1 target gene expression, promoter occupancy in the context of local chromatin, and contact-independent growth. Attenuation of FOX function by the cell fate determination pathway has broad implications given the diverse role of FOX proteins in cellular biology and tumorigenesis.

cell fate determination | Forkhead protein | transcription factor | tumor suppressor | dachshund homolog 1

The *Dachshund* gene (*dac*) was originally cloned in *Drosophila* as a dominant inhibitor of *ellip*se and encodes a key component of the retinal determination gene network (RDGN). In *Drosophila* retinal determination, dac interacts with EGFR, Decapentaplegia, and Wingless. The human *DACH1* gene encodes a protein with a predicted helix-turn-helix family structure. Altered expression or function of mammalian RDGN components have been described in human tumors (1–5). DACH1 is expressed widely in normal adult tissues, and loss of DACH1 expression in human breast and endometrial cancers predicts poor outcome (2, 6). DACH1 blocks c-Jun–induced DNA synthesis, reverts the H-Ras and c-Myc–induced oncogenic phenotype in the immortalized human breast epithelial cell line, and inhibits breast tumor metastasis in mice (2, 7). Although DACH1 is thought to regulate gene expression by complexing with DNA-binding transcription factors including Six, Jun, and Smad4 (2, 8–10), no sequence specific DNA binding of DACH1 has been identified so far to our knowledge.

Crystallization of the human DACH1 Box-N revealed that the DACH1 protein forms an α/β structure containing a DNA-binding motif similar to that found in the winged helix/Forkhead subgroup of DNA-binding proteins (11). We sought to determine whether *DACH1* encoded a protein with sequence-specific DNA binding activity. Cyclic amplification and selection of targets (CAST) identified a DNA sequence that was specific for DACH1 binding. Genome-wide identification of DACH1 binding sites with sequential functional pathway analysis identified DACH1 sites within genes populating the cell cycle and the glioma pathways associated with tumorigenesis. The DACH1 DNA-binding sequence resembled the FOXM1 binding site. DACH1 selectively inhibited FOXM1-mediated contact-independent growth and occupancy in the context of local chromatin. DACH1 displaced FOXM1 from the known target gene promoters including *Cdc25B, Survivin*, *CENPA*, and *CENPB*. The cell fate determination factor DACH1 competes as a DNA binding–specific transcription factor to attenuate signaling by the Forkhead family of transcription factors.

Results

Identification of a DNA-Specific Sequence for DACH1 Binding. CAST analysis was conducted to determine whether *DACH1* encodes a DNA sequence–specific binding protein. The DACH1 consensus-binding sequence was deduced from 33 sequenced colonies (Table S1). Electrophoretic mobility shift assay (EMSA) was used to further characterize the DNA/protein interaction. The DNA probe was incubated with nuclear extracts prepared from HEK 293T cells transfected with an expression vector encoding FLAG-tagged DACH1. The protein/DNA complex detected (Fig. 1*A*, lane 1) was competed by 100-fold molar excess cold probe cognate competitor (Fig. 1*A*, lane 2), indicating the DNA specificity to form the complex. Preincubation of nuclear extracts with anti-FLAG antibody delayed migration of the DNA/DACH1 complex (Fig. 1*A*, lane 4 vs. lane 3). FLAG-DACH1 was expressed in HEK 293T cells and purified to homogeneity using an anti-FLAG–agarose affinity column. DACH1 purified in this manner was capable of complexing with the DACH1 DNA probe (Fig. 1*B*, lane 1). The complex was dissociated by the competitor (Fig. 1*B*, lane 2) and was supershifted by the anti-FLAG antibody (Fig. 1*B*, lane 4). In vitro–translated DACH1 produced using TNT T7 Coupled Reticulocyte Lysate System (Promega) also formed a protein/DNA complex that was specific to both DNA probe and DACH1 protein (Fig. 1*C*, lane 4 vs. lane 3 and lane 6 vs. lane 5). DACH Box-N is predicted to form an α/β structure containing a DNA binding domain found in the winged helix/Forkhead subgroup of the helix-turn-helix family (11). We therefore determined whether deletion of this domain abrogated DNA binding. The deletion mutant of this domain (ΔDBD) was unable to bind the DNA probe (Fig. 1*A*, lanes 5–7; and Fig. 1*C*, lane 7). Thus, the DNA sequences selected by CAST serve as a DNA target site for both in vivo and in vitro synthesized DACH1 protein and DNA binding requires the DACH1 Box-N domain, designated as DNA binding domain (DBD).

Genome-Wide Screening of Putative DACH1 Targets. To determine candidate DACH1 target genes at the whole-genome level, the

Author contributions: J.Z., C.W., Z.W., K.W., V.M.P., and K.Z. performed research; J.Z., C.W., W.D., M.C.C., I.C., A.Q., A.T., and M.P.L. analyzed data; C.W. and R.G.P. designed research; and C.W. and R.G.P. wrote the paper.

The authors declare no conflict of interest.

[1]To whom correspondence may be addressed. E-mail: chenguang.wang@jefferson.edu or richard.pestell@jefferson.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/1002746107/DCSupplemental.

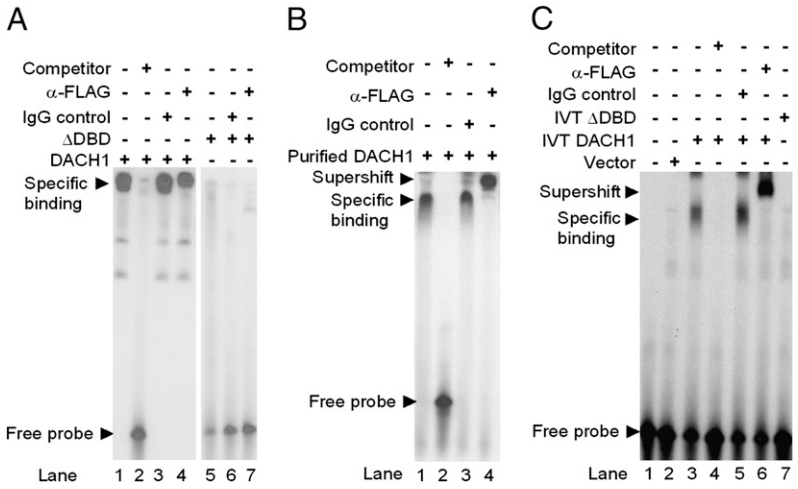
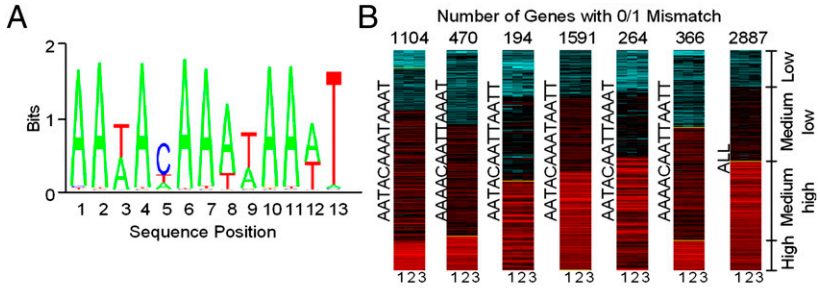


Fig. 1. Identification of DACH1-response element. (*A*) The ^{32}P-labeled oligonucleotide of DACH1 binding sequence (DACH1 probe) was used in EMSA. The probe migration was retarded by DACH1 binding (lane 1). The protein/DNA complex was competed by 100-fold cognate cold competitor (lane 2). The addition of anti-FLAG antibody delayed mobility of the DNA–protein complex (lane 4). The DACH1 DBD deletion (ΔDBD) failed to bind the DNA probe (lanes 5–7). (*B*) Immunopurified DACH1 was incubated with DACH1 probe. The DNA–protein complex was competed by cold probe (lane 2) and the mobility of the complex was delayed by anti-FLAG antibody (lane 4 vs. 3). (*C*) In vitro–translated DACH1 or ΔDBD were incubated with DACH1 probe. No addition of DNA (lane 1) or empty vector DNA (lane 2) was included as a negative control. The DACH1/DNA complex (lane 3) was supershifted by anti-FLAG antibody (lane 6) compared with IgG control (lane 5). Deletion of DBD abolished DNA binding (lane 7).

2,000 bp upstream of the transcriptional start site in Promoter Analysis and Interaction Network generation Tool were scrutinized using the DACH1 DNA binding templates (Table S1). A linear sequence search revealed a total of 52 exact matches for the six DNA templates and 2,887 unique genes with exact or single-nucleotide (0/1) mismatches (Datasets S1 and S2), presenting a sequence logo for the DACH1 binding site (Fig. 2*A*). The KEGG cell cycle pathway and the glioma pathway are populated with genes that have DACH1 binding sequences (Figs. S1 and S2), providing a context for the possible roles of these genes in the development and progression of cancer. The GO terms that are statistically enriched ($P < 0.05$) for the sum of the 0/1 mismatch gene lists are shown in Table S2. Proteins encoded by genes with DACH1 binding sites preferentially populate biological processes essential for signaling such as protein modification, phosphorylation, and translation, as well as metabolic processes associated with nucleosides, RNA, and serine family amino acids.

To identify potential transcription factor partners of DACH1, we compared the gene sets with upstream DACH1 binding sites to the basal gene expression profile generated in MDA-MB-231 cells (9). Specifically, we used hierarchal clustering to cluster genes with DACH1 binding sites as well as their sum into four groups: low, medium low, medium high, and high expression (Fig. 2*B*). The choice of four groups was optimal as they partitioned the set of genes on the array into subgroups of approximately equal size. The median expression values for the four low to high clusters as log2 of the signal intensity are as follows: 1.2016, 2.2016, 4.2901, and 8.0081. Fig. 2*C* shows the ranked list of transcription factors that has a binding site(s) in genes that also possess potential DACH1 binding site(s) ($P < 0.05$). The medium-high and high sets were of particular interest because these represent genes that are robustly expressed based on their signal intensity. These sets contained a number of Forkhead family transcription factors such as FOXD3, XFD-1, FOXJ2, HFH-3, and XFD2. These results implicate Forkhead family



C

Medium Low			Medium High			High Expression		
TF	Homology	p-value	TF	Homology	p-value	TF	Homology	p-value
Nkx2-5	72.96%	0.0046	Pax-4	85.42%	0.0004	Pax-4	85.63%	0.0000
FOXD3	**53.86%**	**0.0000**	Nkx2-5	74.41%	0.0000	Nkx2-5	73.57%	0.0000
RFX1	7.73%	0.0068	**FOXD3**	**52.80%**	**0.0000**	**FOXD3**	**47.42%**	**0.0000**
XFD-1	**4.08%**	**0.0012**	**FOXJ2**	**31.09%**	**0.0000**	Pax-6	43.65%	0.0001
NRF-2	3.00%	0.0103	**HFH-3**	**6.01%**	**0.0143**	**FOXJ2**	**28.59%**	**0.0003**
			Arnt	4.79%	0.0499	v-Myb	17.85%	0.0168
			XFD-2	**4.38%**	**0.0011**	NF-Y	10.32%	0.0000
			XFD-1	**2.65%**	**0.0389**	**HFH-3**	**5.65%**	**0.0193**
			MEIS1A	1.94%	0.0021	E2F	4.67%	0.0209
			MEIS1B	1.83%	0.0014	SREBP-1	4.67%	0.0169
			RREB-1	1.63%	0.0122	GATA-2	3.35%	0.0000
			Sox-5	1.43%	0.0101	E2	1.88%	0.0286
			Ik-3	1.33%	0.0454	MEIS1A	1.46%	0.0280
			MEF-2	1.12%	0.0417	MEIS1B	1.12%	0.0160
			Clox	1.02%	0.0463	HEN1	0.84%	0.0111

Fig. 2. Genome-wide *in silico* promoter analysis. (*A*) Sequence logo depicts sequence conservation of the template sequence for all single-mismatch hits. (*B*) The clustered gene expression data from MDA-MB-231 breast cancer cells for the exact and single-mismatch genes with minimal expression in cyan and high expression in red. The yellow bars indicate the cutoffs between minimal, low, medium, and high expression subsets. (*C*) Transcription factors that are enriched for the medium low, medium high, and high expression subsets of the genes in MDA-MB-231 cells with exact match or single-mismatch DACH1 binding site templates. Homology refers to the percentage of genes within the subset that possess the element transcription factor binding site. The *P* values were calculated using the hypergeometric test ($P < 0.05$).

CELL BIOLOGY

transcriptional factors as potential coregulator in DACH1-associated gene regulation.

DACH1 Represses FOXM1 Target Gene Expression. Through literature search, we found that DACH1 shares the DNA binding sequence at a high similarity with FOX proteins. Together with the *in silico* analysis suggesting that DACH1 may interact with FOX family members, we speculated that DACH1 regulates FOX-dependent gene expression via competition with cognate DNA-binding sites. FOXM1-targeted genes, which have been previously reported, including *Aurora A* (*STK6*), *Aurora B* (*AURKB*), *CDC25A*, *CDC25C*, *E-cadherin* (*CDH1*), and *CENPB* (12) have DACH1 binding sequences within their 2-kb promoter region (Dataset S2). We therefore determined whether DACH1 regulated these FOXM1-activated genes. Induction of DACH1 expression by Ponasterone A in MDA-MB-231 cells (2) repressed expression of FOXM1 target genes (Fig. 3*A*). To further determine whether DACH1 regulation of these gene expression through direct DACH1 binding to the targeted gene promoters, ChIP assays were conducted of this inducible line to determine DACH1 occupancy at FOXM1 DNA-binding sites in the context of local chromatin using an anti-FLAG antibody directed to the FLAG tag sequence of DACH1 or control IgG. Induction of DACH1 expression by Ponasterone A was associated with increased occupancy of DACH1 at the promoters of the known FOXM1 targeted genes (Fig. 3*B*). DACH1 expression repressed activity of luciferase reporters driven by the *Cdc25B*, *Skp2*, and *E-cadherin* gene promoters (Fig. 3*C*). Deletion of the putative DACH1 DNA-binding domain abrogated repression of reporter gene activity (Fig. 3*C*), suggesting that direct DNA binding is required for transcriptional repression.

DACH1 Competes for Occupancy of FOXM1 Regulatory Genes in the Context of Local Chromatin. DACH1 repression of FOXM1-targeted gene expression promoted us to further explore the functional interactions between DACH1 and FOXM1. We used an osteosarcoma cell line (U2OS C3) expressing doxycycline (Dox)–inducible FOXM1, which has been well characterized in a prior publication (13). FOXM1 induces, and is required for, the transcription of *Skp2* and *CENPB* genes (Fig. 4*A*), consistent with the previous observations made in this cell line (12). The U2OS-FOXM1 cell line was transduced with either a retroviral expression vector encoding DACH1 or a control retrovirus. The induction of FOXM1 by Dox in the U2OS-FOXM1 cells increased Skp2 and CENPB abundance, and overexpression of DACH1 reduced both the basal and the induced Skp2 and CENPB expression (Fig. 4*A*). Next, we investigated the possibility that DACH1 may compete with FOXM1 in the context of local chromatin. ChIP assays conducted of FOXM1 target gene promoters with anti-FOXM1 antibodies demonstrated FOXM1 occupancy at the promoters (Fig. 4*B*). The induction of DACH1 expression reduced FOXM1 occupancy at the promoters of the *Cdc25B*, *CENPA*, *CENPB*, *Aurora B kinase*, *Skp2*, and *E-cadherin* genes, associated with increased occupancy of DACH1 (Fig. 4*B*). DACH1 repressed FOXM1 activation of the *Cdc25B* promoter (Fig. 4*C*).

DACH1 Inhibits FOXM1 Induction of Contact-Independent Growth. Conditional expression of FOXM1 protein enhances anchorage-independent growth of U2OS cells (13). The U2OS C3 clone was transduced with a retroviral vector expressing DACH1 and soft agar assays were performed. Expression of DACH1 decreased anchorage-independent growth as evidenced by a reduction in both the size and number of FOXM1-induced colonies (Fig. 4*D*). Thus, DACH1 inhibits FOXM1-induced contact-independent growth.

Identification of DACH1 DNA Binding in the Human Genome. To characterize genome-wide DACH1 DNA binding in vivo, ChIP coupled with high-throughput sequencing (ChIP-Seq) was conducted of MDA-MB-231 cells (14). Using an FDR threshold of 10^{-3} we identified a total of 1,845 binding sites (1,608 unique genes) for DACH1 (Dataset S3). The genome-wide contribution of the DACH1 binding sites relative to RefSeq is shown in Fig. 5*A*. We examined the Tag density landscape and deduced the frequency for each nucleotide for target genes. The motif deduced by ChIP-Seq shared 11 of 13 nucleotides with the motif identified *in silico*. The genes with putative DACH1 binding in their promoter sequence by *in silico* analysis were enriched by ChIP-Seq (P = 0.0013; Fig. 5*B*). DACH1-dependent tag density at FOXM1 target gene promoters that contain peaks defined by TIRDE algorithms are shown in Fig. 5*C*. In addition, we validated a subset of genes whose promoters contain the putative DACH1 binding sequence using quantitative RT-PCR, showing increased expression in MEFs derived from *DACH1* gene knockout mice compared with littermate controls (Fig. 5*D*). These genes identified by ChIP-Seq correspond to the genes identified by ChIP analysis and promoter studies as regulated by DACH1 and FOX proteins.

Discussion

In this study, we provide evidence that the RDGN and Forkhead pathways integrate to control the expression of genes that associate with contact-independent growth. The key RDGN protein, DACH1, directly binds DNA and competes in the context of local chromatin with FOXM1, thereby attenuating key Forkhead regulatory gene

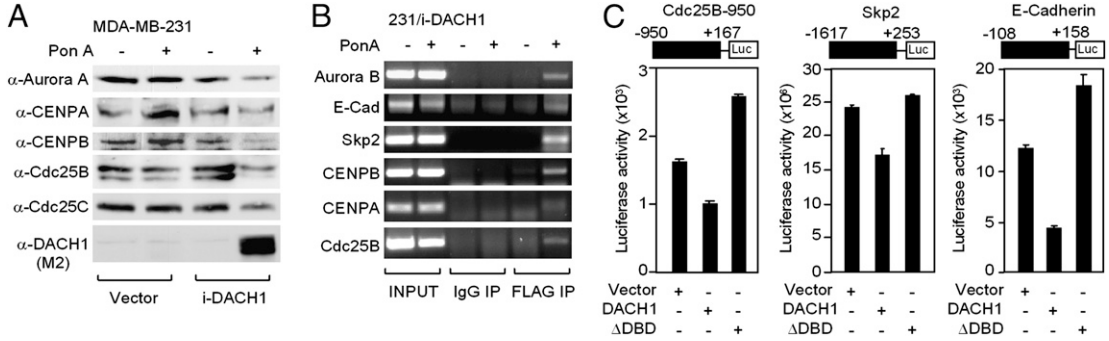


Fig. 3. DACH1 inhibits expression of FOXM1 targeted genes. (*A*) Western blot analysis of DACH1 protein abundance in breast cancer cell lines. (*B*) ChIP assay was conducted with Ponasterone A–inducible DACH1 MDA-MB-231 cells with either the antibody against FLAG epitope (FLAG tag in the aminoterminus of DACH1) or control IgG. DACH1 occupies the FOXM1-binding sites of G_2/M regulatory proteins included in ChIP assays. (*C*) DACH1 inhibits the transcriptional activity of G_2/M regulatory genes. The promoter sequences of the *Cdc25B*-950, *Skp2*, *and E-cadherin* genes were linked to the luciferase reporter. The reporter was transfected together with vectors expressing WT DACH1 and DBD-deletion mutant (ΔDBD). The data are shown as mean ± SD.

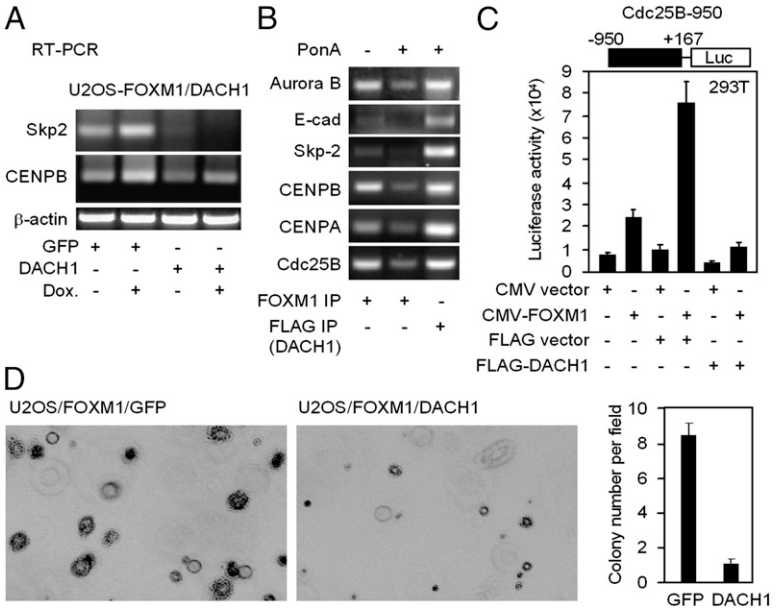


Fig. 4. DACH1 competes with FOXM1 for DNA binding and inhibits FOXM1-mediated contact-independent growth. (*A*) The Dox-inducible U2OS/FOXM1 cells were transduced with a DACH1 retroviral vector. Cells were treated with either Dox or vehicle control. The transcript abundance of G_2/M regulatory genes including *Skp2* and *CENPB* were measured with RT-PCR. β-Actin served as an internal loading control. (*B*) A Ponasterone A–inducible MDA-MB-231 cell line was induced to express DACH1. ChIP analysis was conducted for the G_2/M regulatory genes promoters using antibody to FOXM1 or DACH1 (anti-FLAG) as indicated. FOXM1 occupancy at the promoters of G_2/M regulatory genes was reduced following DACH1 recruitment. (*C*) HEK 293T cells were cotransfected with the indicated luciferase reporter genes together with expression vectors FOXM1, DACH1, or control vector. DACH1 repressed FOXM1-induced *Cdc25B* promoter activity. (*D*) U2OS cells stably expressing FOXM1 were transduced with MSCV-DACH1-IRES-GFP and vector control. Colony growth was measured by size and number. DACH1 expression reduced the FOXM1-induced colony formation.

networks. The cell fate determination factor, DACH1, is thus a DNA sequence–specific inhibitor of Forkhead signaling.

The FOX proteins are a family of evolutionarily conserved transcription regulators involved in diverse biological processes (15). FOX protein function can either promote or inhibit tumorigenesis, and deregulation of FOX protein function in human tumorigenesis may occur by alteration in upstream regulators or genetic events such as mutations of the DBD, or translocations, which often disrupt the DBD (16). FOXM1 is overexpressed in human tumors (13, 17–22) and promotes tumor growth (18, 23, 24). Inhibition of FOXM1 expression reduces growth of murine tumors in response to carcinogens, and diminishes DNA replication and mitosis of tumor cells (12, 13, 17, 18). FOXC2, associated with aggressive basal-like breast cancer, enhances tumor metastasis and invasion (25).

Herein, DACH1 inhibited FOXM1-mediated contact-independent growth of U2OS cells, antagonized FOXM1-mediated gene expression, and reduced occupancy of FOXM1 at target genes known to regulate the G_2/M phase progression. Through competitive inhibition of FOXM1 occupancy at target genes, DACH1 could be essential for transcription of F-Box protein S-phase kinase–associated protein 2 (Skp2) and the Cdk subunit 1 (Cks1), which are substrate-specific

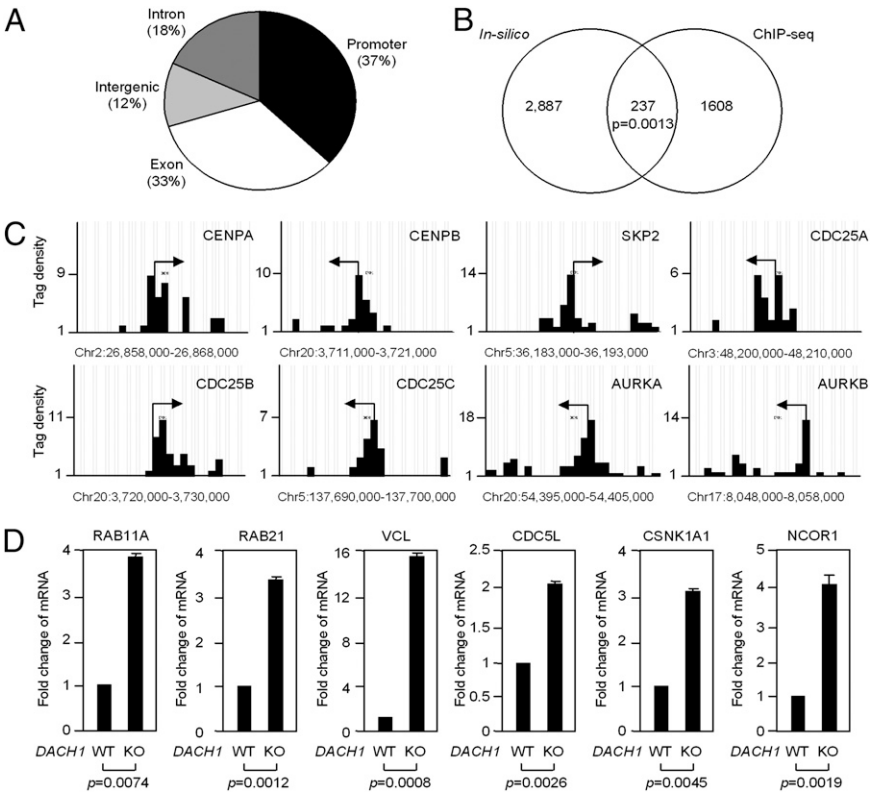


Fig. 5. Genome-wide identification of DACH1 DNA binding by ChIP-Seq. (*A*) Distribution of DACH1 binding sites in the resulting nonredundant and nonoverlapping classes of regions by ChIP-Seq analysis within a promoter of 2 kb upstream of transcription start site. (*B*) Overlap between *in silico* and ChIP-Seq analysis of target genes. (*C*) DACH1-dependent tag density at selected gene promoter that contains DACH1 binding sites defined by the ChIP-Seq. Arrow indicates the start and direction of transcription. (*D*) Validation of a subset of genes whose promoters contain DACH1 binding site(s).

subunits of the Skp1–Cullin-F-box protein (SCF) ubiquitin–ligase complex, that regulate $p21^{CIP1}$ and $p27^{KIP1}$. FOXM1 protein activates gene transcription and DACH1 competes with FOXM1 in the context of local chromatin to repress gene expression. Loss of DACH1 expression and increased oncogenic FOX protein expression could lead to deregulation of a subset of genes required for tumorigenesis. We had previously shown that loss of DACH1 expression in human breast and endometrial cancers predicts poor outcome (2, 6). Future studies will address whether loss of DACH1 correlates with increased FOXM1 expression during the progression of breast and endometrial cancers as well as other type of cancers.

Identification of DACH1 DNA binding sequences by CAST, together with genome-wide *in silico* screening for putative target genes and ChIP-Seq for genes whose promoters were engaged by DACH1 through direct DNA binding, provided us with an alternative approach to establish the regulatory networks or individual genes that are governed by DACH1. *In silico* screening identified 2,887 genes whose promoter regions contain a potential DACH1 binding site. Many FOXM1-targeted genes including *Aurora A* (*STK6*), *Aurora B* (*AURKB*), *CDC25A*, *CDC25C*, *E-cadherin* (*CDH1*), and *CENPB* have DACH1 binding sequences within their 2-kb promoter region. A subset of these genes, chosen based on gene expression change as a result of DACH1 induction into MDA-MB-231 cells, were also enriched in their promoter region for the Forkhead family of transcription factors, supporting the competition/cooperation model of DACH1 and Forkhead transcription factors in gene regulation. Genes with DACH1 binding sites in their promoters populate cellular pathways associated with cancers such as the cell cycle pathway and the glioma pathway. Taken together, these results provide a rationale for further investigations into DACH1-mediated gene regulation in tumorigenesis. Given the diverse roles of Forkhead family proteins in cellular differentiation, survival, and DNA repair, the finding that the RDGN network protein DACH1 intersects FOX signaling may have broad implications for the understanding of cellular biology and tumorigenesis.

Materials and Methods

Cell Culture. Human embryonic kidney 293T (HEK 293T), HeLa, and breast cancer cell lines MDA-MB-231 were maintained in DMEM containing 1% penicillin/streptomycin and supplemented with 10% FBS. Doxycycline-inducible U2OS C3 stable cell line was previously described (12).

Plasmids. Human cDNA of DACH1 WT and mutants including DS-domain deletion (ΔDBD), carboxyl-terminal (C-ter) deletion, and C-ter were cloned into p3×FLAG-CMV-10 (Sigma-Aldrich) vector. pCMV-FOXM1 expression vector and FOXA luciferase reporter vector were provided by R. Costa (University of Chicago, Chicago, IL) (26). Cdc25B promoter reporter was a gift from Rolf Müller (Philipps-University Marburg, Marburg, Germany) (27). Skp2 and E-cadherin promoter reporter were previously described (28, 29).

CAST (30) was conducted as previously described using the random sequence 75-mer [5′-GC GTC GAC AAG CTT TCT AGA $(N)_{35}$ GAA TTC GGA TCC CTC GAG CG-3′], which begins with 20 nucleotides of forward primer sequence, followed by 35 random bases, and ends with a reverse primer sequence of 20 nucleotides. This double-stranded DNA was made by synthesis with the reverse primer (5-CGC TCG AGG GAT CCG AAT C-3). FLAG tagged-DACH1 proteins, either transiently expressed in 293T cells or in vitro–translated, were incubated with anti-FLAG M2 antibody beads (Sigma-Aldrich). Mouse IgG conjugated to protein G agarose beads (Santa Cruz Biotechnology) was included as control. For the initial reaction, dsDNA was added and the mixture was incubated for 20 min at room temperature in PBS solution supplemented with 0.1% BSA and 0.1% Nonidet P-40. Protein–DNA complexes were recovered by centrifugation and washed three times with modified PBS buffer. Enriched PCR products by DACH1 from the initial reaction were subjected to a second round of CAST. The immunoprecipitated DNA was amplified using forward primer (5-GC GTC GAC AAG CTT TCT AGA-3) and the reverse primer (5-CGC TCG AGG GAT CCG AAT C-3). Purified PCR products were subjected to additional rounds of binding site selection. After six rounds of CAST, the purified products were digested with restriction enzymes and cloned into pGEM-3Zf(+) (Promega) and sequenced.

Transfections and Gene Reporter Assays. DNA transfection and luciferase assays were performed as previously described (31). Briefly, cells were seeded at 50% confluence in a 24-well plate the day before transfection. Cells were transiently transfected with the appropriate combination of the reporter (0.5 μg per well), expression vectors (calculated as molar concentration equal to 300 ng of control vector), and control vector (300 ng per well) via calcium phosphate precipitation for HEK 293T or Lipofectamine 2000 (Invitrogen) for remaining cell lines according to the manufacturer's instructions. Twenty-four hours after transfection, luciferase assays were performed at room temperature using an Autolumat LB 953 (EG&G Berthold) as previously described (32).

Immunoprecipitation and Western Blot. Immunoprecipitation and Western blot analysis were performed in HEK 293T, MDA-MB-231, MCF-7, and U2OS 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 protease inhibitor mixture (Roche Diagnostics). Antibodies used for immunoprecipitation and Western blot are as follows: anti-FLAG (M2) and CENPA from Sigma-Aldrich; anti-DACH1 from Abcam; Cyclin D1 (Clone Ab-3) from NeoMarker; anti-FOXM1 (H300), anti–Aurora A (H-130), anti-Cdc25B (H-85), and anti-Cdc25C (C-20) from Santa Cruz Biotechnology.

EMSA. Complementary oligodeoxyribonucleotide strands of the DACH1 binding site (CAA GCT TTC TAG ATG TTT ATT TGT ATT CAT TTA TTT AAT TGT ATT GTG AAT TCG GAT CCA) were used for EMSA as previously described (31).

Purification of DACH1 Protein. HEK 293T cells were transiently transfected with expression vectors encoding either FLAG-tagged DACH1 or DACH1 ΔDBD. Total protein extracts were prepared using lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) supplemented with protease inhibitor mixture (Roche Diagnostics). The manufacturer's instructions were followed to prepare the column and washing buffer (technical bulletin MB-925; Sigma-Aldrich).

ChIP Assay. ChIP analysis was performed following a protocol described previously (33) using a breast cancer cell line MDA-MB-231 expressing DACH1 upon Ponasterone A treatment. Cell lysates were prepared using a ChIP assay kit (Upstate Chemicon) following the manufacturer's guidelines. Chromatin solutions were precipitated overnight with agitation at 4 °C using 30 μL of agarose preconjugated with anti-FLAG antibody (Sigma-Aldrich). For a negative control, mouse IgG was immunoprecipitated by incubating the supernatant fraction for 1 h at 4 °C with rotation. Precipitated DNAs were analyzed by PCR. The primers used for PCR of the Cdc25B, *AuroraB kinase*, *E-Cadherin*, *Skp2*, *CENPA*, and *CENPB* promoters were previously described (12).

RT-PCR and Quantitative PCR. Total RNA was prepared using TriZol reagent (Invitrogen) following manufacturer's instructions. Five micrograms of total RNA was subjected to reverse transcription to synthesize cDNA using the SuperScript II Reverse Transcriptase Kit (Invitrogen). A 25 μL volume reaction consisted of 1 μL reverse transcription product and 100 nM of each primer. The primers used for RT-PCR of the *Cdc25B*, *AuroraB kinase*, *E-Cadherin*, *Skp2*, *CENPA*, and *CENPB* gene products were sense and antisense primer sequences and annealing temperatures were previously described (12).

ChIP-Seq. ChIP-Seq was conducted using a breast cancer cell line MDA-MB-231 stably expressing DACH1. Chromatin template preparation, ChIP, and ChIP-Seq were previously described (14, 34). 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 is meaningful to directly compare window read counts of the two samples and look for DACH1 enriched regions. We identified DACH1 binding sites from ChIP-Seq

data using SISSRs software as the transition points between sense and antisense reads density profiles in the genome. A background model for genome-wide reads distribution was built using IgG control data. Using a *P* value cutoff of 0.01, we detected 6530 DACH1 binding sites.

Gene and exon location information was obtained from the Ensembl database (www.ensembl.org) using the BioMart tool. Based on this location information, we defined exonic, intronic, promoter, and intergenic regions as follows. A promoter is 2 kb/10 kb upstream of transcription start site. To avoid double counting at the promoter overlaps, we merged coordinates of all promoters to define a nonredundant set of promoter regions. Similarly we merged other classes of regions to avoid double counting. Pieces of genes, exons, and introns that overlap promoters are assumed to belong to promoter region. The intergenic region is the complement of promoter and genic regions. We then counted the number of DACH1 binding sites in the resulting nonredundant and nonoverlapping classes of regions and created pie charts (Fig. 5*A*).

ACKNOWLEDGMENTS. This work was supported in part by Award BCTR0504227 from the Susan Komen Breast Cancer Foundation (to C.W.) and National Institutes of Health Grants R01CA70896, R01CA75503, and R01CA86072 (to R.G.P.), R01CA080250, R01CA098779, R01CA120876, and R01AR055660 (to M.P.L.), and 232240 (to A.T.). Work conducted at the Kimmel Cancer Center was supported by National Institutes of Health Cancer Center Core Grant P30CA56036 (to R.G.P.). This project was funded in part by a grant from the Margaret Q. Landenberger Research Foundation (to K.W.), the Pennsylvania Department of Health (to C.W., M.P.L., and R.G.P.), and by the Intramural Research Program of the National Institutes of Health, National Heart, Lung, and Blood Institute (K.Z.).

1. Winchester C, Robertson S, MacLeod T, Johnson K, Thomas M (2000) Expression of a homeobox gene (SIX5) in borderline ovarian tumours. *J Clin Pathol* 53:212–217.
2. Wu K, et al. (2006) DACH1 is a cell fate determination factor that inhibits cyclin D1 and breast tumor growth. *Mol Cell Biol* 26:7116–7129.
3. Laflamme C, et al. (2003) The homeotic protein Six3 is a coactivator of the nuclear receptor NOR-1 and a corepressor of the fusion protein EWS/NOR-1 in human extraskeletal myxoid chondrosarcomas. *Cancer Res* 63:449–454.
4. Li CM, et al. (2002) Gene expression in Wilms' tumor mimics the earliest committed stage in the metanephric mesenchymal-epithelial transition. *Am J Pathol* 160: 2181–2190.
5. Coletta RD, et al. (2004) The Six1 homeoprotein stimulates tumorigenesis by reactivation of cyclin A1. *Proc Natl Acad Sci USA* 101:6478–6483.
6. Nan F, et al. (2009) Altered expression of DACH1 and cyclin D1 in endometrial cancer. *Cancer Biol Ther* 8:1534–1539.
7. Wu K, et al. (2008) Dachshund inhibits oncogene-induced breast cancer cellular migration and invasion through suppression of interleukin-8. *Proc Natl Acad Sci USA* 105:6924–6929.
8. Wu K, et al. (2007) Cell fate determination factor DACH1 inhibits c-Jun-induced contact-independent growth. *Mol Biol Cell* 18:755–767.
9. Wu K, et al. (2003) DACH1 inhibits transforming growth factor-beta signaling through binding Smad4. *J Biol Chem* 278:51673–51684.
10. Li X, Perissi V, Liu F, Rose DW, Rosenfeld MG (2002) Tissue-specific regulation of retinal and pituitary precursor cell proliferation. *Science* 297:1180–1183.
11. Kim SS, et al. (2002) Structure of the retinal determination protein Dachshund reveals a DNA binding motif. *Structure* 10:787–795.
12. Wang IC, et al. (2005) Forkhead box M1 regulates the transcriptional network of genes essential for mitotic progression and genes encoding the SCF (Skp2-Cks1) ubiquitin ligase. *Mol Cell Biol* 25:10875–10894.
13. Kalinichenko VV, et al. (2004) Foxm1b transcription factor is essential for development of hepatocellular carcinomas and is negatively regulated by the p19ARF tumor suppressor. *Genes Dev* 18:830–850.
14. Wang Z, et al. (2009) Genome-wide mapping of hats and hdacs reveals distinct functions in active and inactive genes. *Cell* 138:1019–1031.
15. Myatt SS, Lam EW (2007) The emerging roles of forkhead box (Fox) proteins in cancer. *Nat Rev Cancer* 7:847–859.
16. Arden KC (2007) FoxOs in tumor suppression and stem cell maintenance. *Cell* 128: 235–237.
17. Kalin TV, et al. (2006) Increased levels of the FoxM1 transcription factor accelerate development and progression of prostate carcinomas in both TRAMP and LADY transgenic mice. *Cancer Res* 66:1712–1720.
18. Kim IM, et al. (2006) The Forkhead Box m1 transcription factor stimulates the proliferation of tumor cells during development of lung cancer. *Cancer Res* 66: 2153–2161.
19. Liu M, et al. (2006) FoxM1B is overexpressed in human glioblastomas and critically regulates the tumorigenicity of glioma cells. *Cancer Res* 66:3593–3602.
20. van den Boom J, et al. (2003) Characterization of gene expression profiles associated with glioma progression using oligonucleotide-based microarray analysis and real-time reverse transcription-polymerase chain reaction. *Am J Pathol* 163:1033–1043.
21. Yoshida Y, Wang IC, Yoder HM, Davidson NO, Costa RH (2007) The forkhead box M1 transcription factor contributes to the development and growth of mouse colorectal cancer. *Gastroenterology* 132:1420–1431.
22. Wang IC, et al. (2008) Transgenic expression of the Forkhead box M1 transcription factor induces formation of lung tumors. *Oncogene* 27:4137–4149.
23. Teh MT, et al. (2002) FOXM1 is a downstream target of Gli1 in basal cell carcinomas. *Cancer Res* 62:4773–4780.
24. Madureira PA, et al. (2006) The Forkhead box M1 protein regulates the transcription of the estrogen receptor alpha in breast cancer cells. *J Biol Chem* 281:25167–25176.
25. Mani SA, et al. (2007) Mesenchyme Forkhead 1 (FOXC2) plays a key role in metastasis and is associated with aggressive basal-like breast cancers. *Proc Natl Acad Sci USA* 104: 10069–10074.
26. Al-Kuraya K, et al. (2004) Prognostic relevance of gene amplifications and coamplifications in breast cancer. *Cancer Res* 64:8534–8540.
27. Körner K, Jerôme V, Schmidt T, Müller R (2001) Cell cycle regulation of the murine cdc25B promoter: Essential role for nuclear factor-Y and a proximal repressor element. *J Biol Chem* 276:9662–9669.
28. Li Z, et al. (2006) Cyclin D1 induction of cellular migration requires $p27^{(KIP1)}$. *Cancer Res* 66:9986–9994.
29. Hajra KM, Chen DY, Fearon ER (2002) The SLUG zinc-finger protein represses E-cadherin in breast cancer. *Cancer Res* 62:1613–1618.
30. Hewetson A, Hendrix EC, Mansharamani M, Lee VH, Chilton BS (2002) Identification of the RUSH consensus-binding site by cyclic amplification and selection of targets: Demonstration that RUSH mediates the ability of prolactin to augment progesterone-dependent gene expression. *Mol Endocrinol* 16:2101–2112.
31. Wang C, et al. (2001) Inhibition of cellular proliferation through IkappaB kinase-independent and peroxisome proliferator-activated receptor gamma-dependent repression of cyclin D1. *Mol Cell Biol* 21:3057–3070.
32. Wang C, et al. (2003) Cyclin D1 repression of peroxisome proliferator-activated receptor gamma expression and transactivation. *Mol Cell Biol* 23:6159–6173.
33. Wang C, et al. (2005) Cyclin D1 antagonizes BRCA1 repression of estrogen receptor alpha activity. *Cancer Res* 65:6557–6567.
34. Wang Z, et al. (2008) Combinatorial patterns of histone acetylations and methylations in the human genome. *Nat Genet* 40:897–903.