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Transcription Elongation Regulator 1 Is a Co-integrator of the Cell Fate Determination Factor Dachshund Homolog 1*

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DACH1 (Dachshund homolog 1) is a key component of the retinal determination gene network and regulates gene expression either indirectly as a co-integrator or through direct DNA binding. The current studies were conducted to understand, at a higher level of resolution, the mechanisms governing DACH1-mediated transcriptional repression via DNA sequence-specific binding. DACH1 repressed gene transcription driven by the DACH1-responsive element (DRE). Recent genome-wide ChIP-Seq analysis demonstrated DACH1 binding sites co-localized with Forkhead protein (FOX) binding sites. Herein, DACH1 repressed, whereas FOX proteins enhanced, both DRE and FOXA-responsive element-driven gene expression. Reduced DACH1 expression using a shRNA approach enhanced FOX protein activity. As DACH1 antagonized FOX target gene expression and attenuated FOX signaling, we sought to identify limiting co-integrator proteins governing DACH1 signaling. Proteomic analysis identified transcription elongation regulator 1 (TCERG1) as the transcriptional co-regulator of DACH1 activity. The FF2 domain of TCERG1 was required for DACH1 binding, and the deletion of FF2 abolished DACH1 trans-repression function. The carboxyl terminus of DACH1 was necessary and sufficient for TCERG1 binding. Thus, DACH1 represses gene transcription through direct DNA binding to the promoter region of target genes by recruiting the transcriptional co-regulator, TCERG1.

Development of the compound eye in *Drosophila* is tightly regulated by the retinal determination gene network (RDGN),[3] which includes a number of proteins encoded by genes such as *twin of eyeless* (*toy*), *eyeless* (*ey*), *sine oculis* (*so*), *and eyes absent* (*eya*) (1). *The Dachshund* (*dac*) gene was originally cloned as a dominant inhibitor of *ellipse*. Genetic deletion of *dac* causes eye- and wing-specific defects in *Drosophila* (2). Ectopic expression of the *dac* gene, alone or together with *so* and *eya*, results in ectopic eye formation (3, 4). Vertebrate homologs of *ey* (*Pax6*), *so* (*Six*), *eya* (*Eya*), and *dac* (*DACH1*) have been identified, and the human *DACH1* (*Dach1* in mice) gene encodes a protein composed of two highly conserved domains, dachshund domain 1 (DD1, also known as Box-N) with a predicted helix-turn-helix structure, and dachshund domain 2 (DD2, also known as Box-C). Altered expression of DACH1 has been reported in a variety of human tumors (5–9). DACH1 is expressed widely in normal epithelial tissues, and reduced DACH1 expression predicts poor outcome of breast and endometrial cancer patients (6, 9). DACH1 represses TGF-β signaling, reduces DNA synthesis, and reverts the tumorigenic phenotypes induced by the oncogenes such as ErbB2, Ras, Src, and Myc in human mammary cell lines (10, 11). Reintroduction of DACH1 into breast cancer cells inhibits cellular proliferation and migration/invasion *in vitro* and tumor initiation and metastasis *in vivo* (6, 11).

Crystallization of the human DACH1 Box-N revealed that DACH1 protein forms an α/β structure resembling a DNA binding motif found in the winged helix/forkhead subgroup of transcriptional factors (12). DACH1 is capable of binding both naked DNA and the chromatin DNA template through its Box-N domain, and the DNA binding is independent of protein association with other DACH1-binding partners (13). A subsequent study using cyclic amplification and selection of targets (CAST) identified a DNA sequence that is specific for DACH1 binding (14). The DACH1 DNA binding sequence resembles a Forkhead protein binding site, and DACH1 competes with FOXM1 from being recruited to the promoter of FOXM1 target genes. The Forkhead Box (FOX) proteins are a family of evolutionarily conserved transcriptional regulators involved in diverse biological processes (15). Deregulation of FOX protein function in human tumorigenesis may occur by alteration in upstream regulators or genetic events such as

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[3] The abbreviations used are: RDGN, retinal determination gene network; C-ter, carboxyl-terminal; DACH1, Dachshund homolog 1; DBD, DNA binding domain; DD1 and DD2, dachshund domains 1 and 2, respectively; DRE, DACH1-response element; FOX, Forkhead Box; Luc, luciferase; TCERG1, transcription elongation regulator 1; WB, Western blotting.

mutations of the DNA binding domain (DBD), or translocations, which often disrupt the DBD. DACH1 inhibits FOXM1-mediated contact-independent growth, and DACH1 occupancy displaces FOXM1 in the context of local chromatin from the promoter of FOXM1-targeted genes including *CDC25B*, *SKP2*, and *CDH1* (14).

Although the role of DACH1 in tumorigenesis has been demonstrated and DACH1-specific DNA binding has been identified, the molecular mechanisms through which DACH1 conveys trans-repression function are largely unknown. The current study was designed to characterize the molecular mechanisms governing DACH1 trans-repression at its cognate DNA binding site and to identify functional and biological interactions between DACH1 and FOX protein. We demonstrate that DACH1 functionally antagonizes FOXC2-mediated cellular migration. We identify the transcriptional co-integrator, TCERG1, as rate-limiting regulator of DACH1 transcriptional activity.

EXPERIMENTAL PROCEDURES

Cell Culture—Human embryonic kidney 293T (HEK 293T), HeLa, and MCF-7 cells were maintained in DMEM containing 1% penicillin/streptomycin and supplemented with 10% fetal bovine serum (FBS). MCF-10A cells were cultured in DMEM/F12 (50:50) supplemented with 5% horse serum, 10 μg/ml insulin, 20 ng/ml EGF, 0.5 μg/ml hydrocortisone, and 100 ng/ml cholera toxin.

Plasmids and Small Interfering RNA—Human cDNA of DACH1 wild-type and mutants, including DNA binding domain (amino acids 183–293) deletion (ΔDBD), carboxyl-terminal (C-ter) deletion mutant (amino acids 1–565) and C-ter (amino acids 566–706), were cloned into the p3×FLAG-CMV™-10 (Sigma-Aldrich) vector. DACH1-responsive element (DRE)-Luc reporters were constructed by insertion of either single or six copies of the DACH1 binding site (DRE: TAT TTA TTT GTA TTC ATT TAT TTA ATT GTA TTG T) upstream of the distinct TATA boxes of the SV40, β-globin, and CMV-IE genes. The DRE element was assessed in each orientation by cloning the sequence into KpnI/BglII sites of pGL3 control or pGL3 basic (Promega). Expression vectors encoding TCERG1 were described previously (16). pCMV-FOXM1 expression vector and FOXA luciferase reporter vector were provided by Dr. R. Costa (17). The FOXC2 expression vector pBABE-FOXC2 was a gift from Dr. R. Weinberg (18). The plasmids encoding glutathione *S*-transferase (GST)-TCERG1 fusion proteins were gift from Dr. M. Garcia-Blanco. Myc-tagged FOXC2 was amplified by PCR and subcloned into retroviral vector MSCV-IRES-GFP. The sequence of small interfering RNA (siRNA) for human TCERG1 was described previously (19).

Transfection, Infection, and Gene Reporter Assays—DNA transfection and luciferase assays were performed as described previously (20). Briefly, cells were seeded at 50% confluence in a 24-well plate on the day prior to transfection. Cells were transiently transfected with the appropriate combination of the reporter (0.5 μg/well), expression vectors (calculated as molar concentration equal to 300 ng of control vector), and control vector (300 ng/well) via calcium phosphate precipitation for HEK 293T or Lipofectamine 2000 (Invitrogen) for remaining cell lines according to the manufacturer's instructions. 24 h after transfection, luciferase assays were performed at room temperature using an Autolumat LB 953 (EG&G Berthold) as described previously (21). The transient transfection of HEK 293T cells with siRNA targeting human TCERG1 was described previously (19).

The MSCV-IRES-GFP retrovirus vector expressing FOXC2 and the vector Psv-ψ-E-MLV that provides ecotropic packaging helper function and infection methods were described previously (22). Retroviruses were prepared by transient co-transfection of plasmid DNA using calcium phosphate precipitation. The retroviral supernatants were harvested 48 h after transfection and filtered through a 0.45-μm filter. Immortalized mammary epithelial MCF-10A cells were incubated with retroviral supernatants in the presence of 8 μg/ml hexadimethrine bromide for 24 h, cultured for a further 48 h, and subjected to fluorescence-activated cell sorting (FACS) (FACStar Plus; BD Biosciences) to select for cells expressing GFP. GFP-positive cells were used for subsequent analysis.

Cellular Migration Assay—Briefly, 2.5×10^4 cells were seeded on an 8-μm pore size Transwell filter insert (Corning Inc., Corning, NY) coated with ECM (1:7.5) (Sigma). After 6 h of incubation at 37 °C and 5% CO_2, cells adherent to the upper surface of the filter were removed using a cotton applicator. Cells were stained with 0.4% crystal violet dissolved in methanol, and the numbers of cells on the bottom were counted.

Three-dimensional Invasion Assay—100 μl of 1.67 mg/ml rat tail collagen type I (BD Biosciences) was pipetted in the top chamber of a 24-well 8-μm Transwell (Corning). The Transwell was incubated at 37 °C overnight to allow the collagen to solidify. 3×10^4 cells were then seeded on the bottom of the Transwell membrane and allowed to attach for 4 h. Serum-free growth medium was placed in the bottom chamber, whereas 5% serum was used as a chemoattractant in the growth medium of the upper chamber. The cells were then chemoattracted across the filter through the collagen above for 3 days. Cells were fixed in 4% formaldehyde and permeabilized with 0.2% Triton X-100 in PBS then stained with 40 μg/μl propidium iodide for 2 h. Fluorescence was analyzed by confocal z-sections (1 section every 4 μm) at 10× magnification from the bottom of the filter. Three-dimensional reconstructions of the propidium iodide-stained cells were done using Carl Zeiss Zen software (2007 Light Edition).

Immunoprecipitation (IP), Western Blotting (WB), and Chromatin Immunoprecipitation (ChIP) Analysis—IP and WB assays were performed in HEK 293T 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 IP and WB were: anti-T7; (Bethyl Laboratories, Montgomery, TX), anti-FLAG (M2 clone; Sigma), anti-FOXM1 (C20; Santa Cruz), and anti-DACH1 (Abcam; Cambridge, MA). ChIP analysis was performed following a protocol described previously (14).

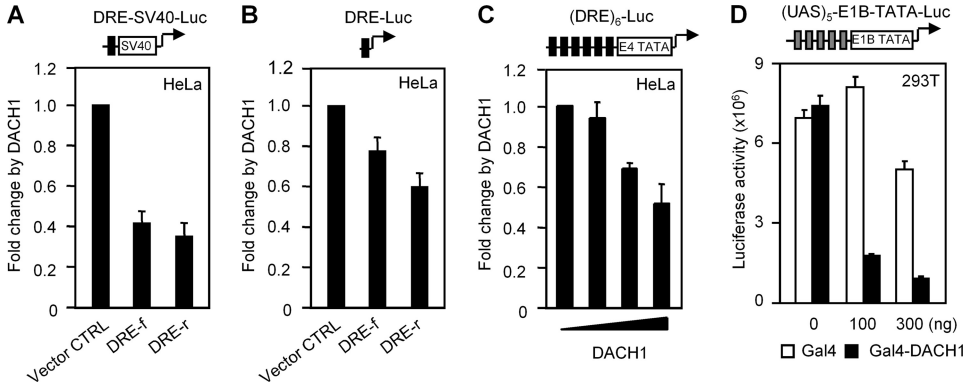

FIGURE 1. **DACH1 functions as trans-repressor through specific DNA sequence.** *A* and *B*, HeLa cells were transfected with a vector encoding the single copy of DRE in either the forward or reverse orientation upstream of either *SV40* promoter (*A*) or the promoter-less pGL3-basic reporter together with a DACH1 expression vector or control (*B*). The -fold repression by DACH1 on both reporter genes was calculated relative to vector control. The -fold change by DACH1 was further normalized to the reporter backbone (the activity from vector backbone was set as 1). *C*, cells were transfected with an expression vector encoding DACH1 and luciferase reporter construct containing six copies of DRE upstream of an E4 TATA box. *D*, DACH1 linked to the Gal4 DBD was assessed using the UAS-Gal4-DBD binding site linked to the minimal TATA box. Transfection with increasing amounts of Gal4-DACH1 repressed transcription. The data are shown as mean ± S.E. ($p < 0.05$).

Modeling Studies of DACH1 and FOXM1 Binding with DNA—The crystal structure of the DBD of human FOXM1 complexed with DNA duplex (21 nucleotides long) is available in the Protein Data Bank (code 3G73). Fig. 2*A* (*right*) shows the ribbon representation of the x-ray crystal structure of the complex of the DBD FOXM1 with DNA. The sequence of the human DACH1 (NCBI sequence ID NP_542937) was searched against the NCBI Protein Data Bank structure data base, we found that this sequence has 100% homology with that of the x-ray crystal structure of the retinal determination protein Dachshund (Protein Data Bank code 1L8R) (5). The three-dimensional structure of DACH1 (amino acids 3–101) was used to dock into the major groove of a segment of DNA double helical structure. The whole complex was energy-minimized using the program AMBERB8 Molecular Stimulation and Modeling software to refine the interactions between the human DACH1 protein and DNA.

Identification of DACH1-associated Protein by Mass Spectrometry—HEK 293T cells were transiently transfected with expression vector encoding FLAG-tagged DACH1. 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 anti-FLAG antibody affinity column and washing buffer (Technical Bulletin no. MB-925, Sigma-Aldrich). The DACH1 complex was eluted from the column with TBS buffer containing 3×FLAG peptide and concentrated with P10 (10-kDa cutoff column; Millipore) and loaded onto 8% SDS-polyacrylamide gel. The silver-stained bands were excised from SDS-polyacrylamide gels and completely destained with 200 mM ammonium bicarbonate. These gel pieces were treated with 10 mM DTT in 0.1 M ammonium bicarbonate for protein reduction. Free cysteine residues were alkylated with freshly made 55 mM iodoacetamide in 0.1 M ammonium bicarbonate. Proteins were digested by the addition of 25 ng/μl sequence-grade modified trypsin (Promega) in ammonium bicarbonate buffer for 16 h at 30 °C with agitation. The digestion products were cleaned and concentrated using micro-C18 ZipTip (Millipore) mixed with 0.5 μl of 10 mg/ml α-cyano-4-hydroxysuccinamic acid in 50% acetonitrile, 0.1% (v/v) trifluoroacetic acid, and applied onto a MALDI plate.

RESULTS

DACH1 Encodes a Heterologous Transcriptional Repressor—A DACH1-specific DNA binding sequence was recently identified (14). To characterize the biological significance of DACH1 DNA binding further, we cloned a 13-bp DACH1 binding sequence, designated as DRE, into pGL3 luciferase reporter vectors pGL3-SV40 and pGL3-basic (Promega). Transfection of DRE-Luc in HeLa cells demonstrated that DACH1 conveys transcriptional repression through the DNA binding sequence, independent of sequence orientation (Fig. 1, *A* and *B*). A multimeric DRE was cloned upstream of either the E4 TATA minimal or β-globin promoter to determine whether the transcriptional repression by DACH1 was promoter-selective. DACH1 repressed DRE-Luc transcriptional activity when linked to the E4 TATA box or the β-globin TATA box (Fig. 1*C* and data not shown). To determine whether DACH1 conveys heterologous transcriptional repression function, DACH1 was expressed as a Gal4 fusion protein (Gal4-DACH1) and analyzed using the upstream activator sequence linked to the minimal TATA box ($(UAS)_5$-E1B-TATA-Luc). DACH1 expression conveyed trans-repression function independently of its DNA binding sequence (Fig. 1*D*). Thus, DACH1 is capable of repressing transcriptional activity in a DNA sequence-specific manner.

Modeling Studies of DACH1 and FOX Protein Binding with DNA—Given the evidence that a subset of genes regulated by DACH1 in MDA-MB-231 cells was also enriched in their promoter region for the Forkhead family of transcription factors, we proposed a competition model of DACH1 and Forkhead proteins in regulating gene expression. Comparisons of the CAST-deduced DACH1 binding sequence identified homology with the consensus Forkhead family protein binding site, in particular the oncoprotein FOXM1 binding site (Fig. 2*B*), raising the possibility that DACH1 may block Forkhead protein function through direct competition for promoter DNA

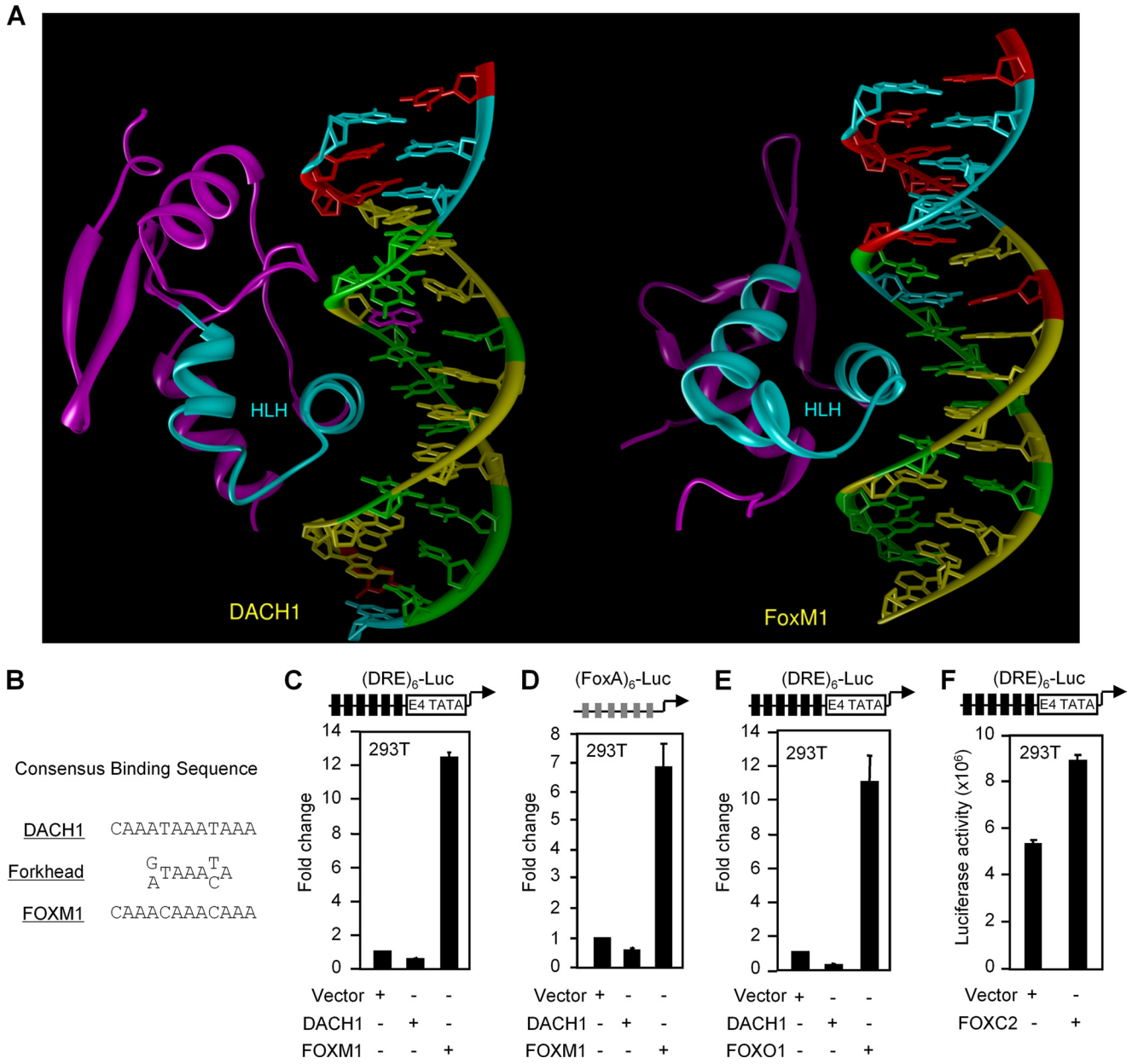

FIGURE 2. **DACH1 inhibits transcription through the Forkhead protein response element.** *A*, *ribbon model* representation of the FOXM1 protein with a DNA binding site is shown. The helix loop-helix (*HLH*) domain of DACH1 resembles the FOXM1 DBD. The DNA is shown as a *stick model*. The *color coding* for bases are: A, *yellow*; T, *green*; G, *cyan*; and C, *red*. The x-ray structure of the DNA binding motif of Dachshund is shown in *ribbon* representation. For both proteins, the HLH segments are shown by *cyan-colored ribbon*, and the rest of the *ribbon* structures are *colored magenta*. The picture was generated by the UCSF Chimera visualization program (23). *B*, comparison of DACH1 binding nucleotides to the consensus Forkhead binding site and FOXM1 binding site. *C–E*, $(DRE)_6$-Luc or $(FoxA)_6$-Luc transfected into HEK 293T cells, showing that DACH1 repressed reporter activity, whereas FOXM1 and FOXO induced the reporter activity. *F*, 293T cells transfected with $(DRE)_6$-E4-TATA-Luc, showing that FOXC2 induced the reporter activity. The data throughout are shown as mean ± S.E. (*error bars*) from at least two separate experiments with triplicate samples each ($p < 0.01$).

binding. A prediction model of the DBD of DACH1 was generated (Fig. 2*A*). For DACH1 and FOXM1, the HLH motifs are shown by *cyan ribbon spirals*, and the rest of the ribbon structures for both complexes are shown in *magenta*. Despite these differences in secondary structures shown on the *top* and the *left side* of the proteins; the DNA interaction helix-loop-helix motifs (*cyan ribbon spirals*) are similar in orientation between DACH1 and FOXM1.

DACH1 Attenuates Forkhead-dependent Transcriptional Activity—To investigate the possibility that DACH1 may regulate Forkhead trans-activation, luciferase reporter assays were conducted with several Forkhead family proteins, including FOXM1, FOXO1 (FKHR), and FOXC2. FOXM1 induced the DRE_6-Luc activity ~12-fold (Fig. 2*C*). DACH1 repressed activity of a reporter containing six copies of the FOXM1 binding consensus sequence $(FoxA)_6$-Luc by ~60% (Fig. 2*D*). Expression of the FOX protein, FOXO1, activated the $(DRE)_6$-Luc ~11-fold (Fig. 2*E*). FOXC2 induction of $(DRE)_6$-Luc was also observed (Fig. 2*F*).

These studies suggested that DACH1 may function to repress FOX protein-dependent gene expression via competition with cognate DNA binding sites. Prior to conducting

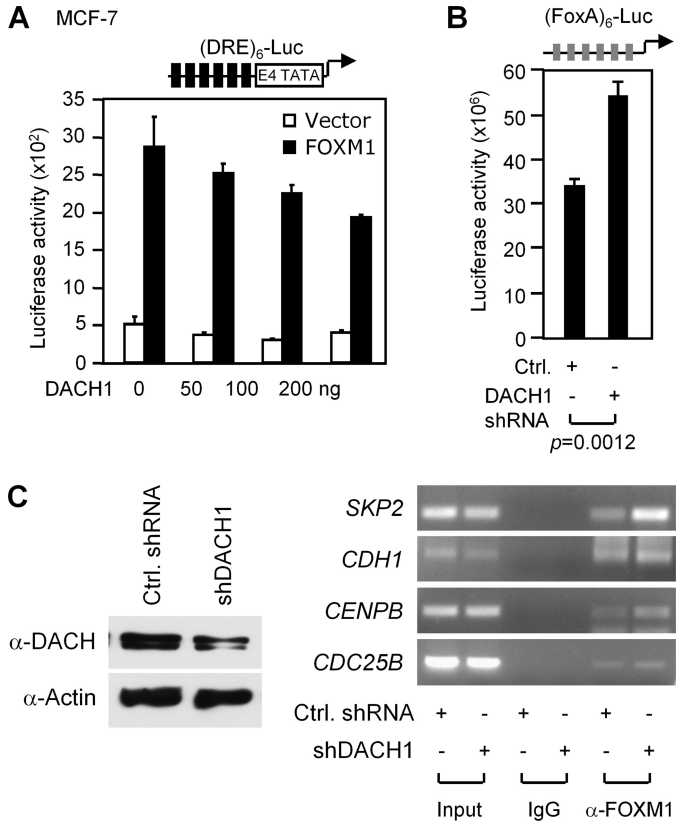

FIGURE 3. **DACH1 inhibits FOXM1 transactivation.** *A*, FOXM1 expression vector was co-transfected with increasing amounts of DACH1 expression vector together with the $(DRE)_6$-Luc reporter into MCF-7 breast cancer cells. *B*, luciferase reporter assay was conducted in HEK 293T cells transduced with either shRNA for DACH1 or control. Data are mean ± S.E. (*error bars*). *C*, ChIP assays were conducted using anti-FOXM1 antibody in cells transfected with DACH1 shRNA or control.

promoter occupancy studies to prove this, we examined the relative abundance of DACH1 and FOXM1 in cell lines. DACH1 was readily detected in HEK 293T and HeLa cells by WB (data not shown). FOXM1 was detectable in each cell line. DACH1 abundance was regulated by serum addition with a nadir at 4–6 h (~8-fold variation in abundance) (data not shown). FOXM1 induced activity of the consensus DRE in MCF-7 cells, and this activity was repressed by DACH1 in a dose-dependent manner (Fig. 3*A*). Luciferase reporter assay was conducted in HEK 293T cells transduced with either shRNA for DACH1 or control, showing enhanced FOX transactivation (Fig. 3*B*). We have previously shown that overexpression of DACH1 competes with FOXM1 for DNA binding (14). We further confirmed this observation by reducing the endogenous levels of DACH1 in HEK 293T. The amount of FOXM1 recruited to the promoter of its target genes was increased upon reduction of DACH1 expression (Fig. 3*C*).

DACH1 Inhibits FOXC2-induced Cellular Migration—It has been shown previously that FOXC2 is overexpressed in breast cancers and high levels of FOXC2 correlate with aggressiveness of ductal breast carcinoma (18). MCF-10A cells overexpressing FOXC2 enhanced Transwell migration in a Boyden chamber ~9-fold (Fig. 4*A*). Retroviral transduction of FOXC2-expressing MCF-10A cells increased cell invasion in a three-dimensional invasion assay. Introduction of DACH1 into these cells abolished the FOXC2-induced cellular migration and invasion (Fig. 4*B*), suggesting a functional repression of FOXC2 by DACH1.

TCERG1 Is a DACH1 Transcriptional Co-integrator—To determine, at a high level of resolution, the molecular mechanisms by which DACH1 conducts transcriptional repression, a proteomic approach was used to identify can-

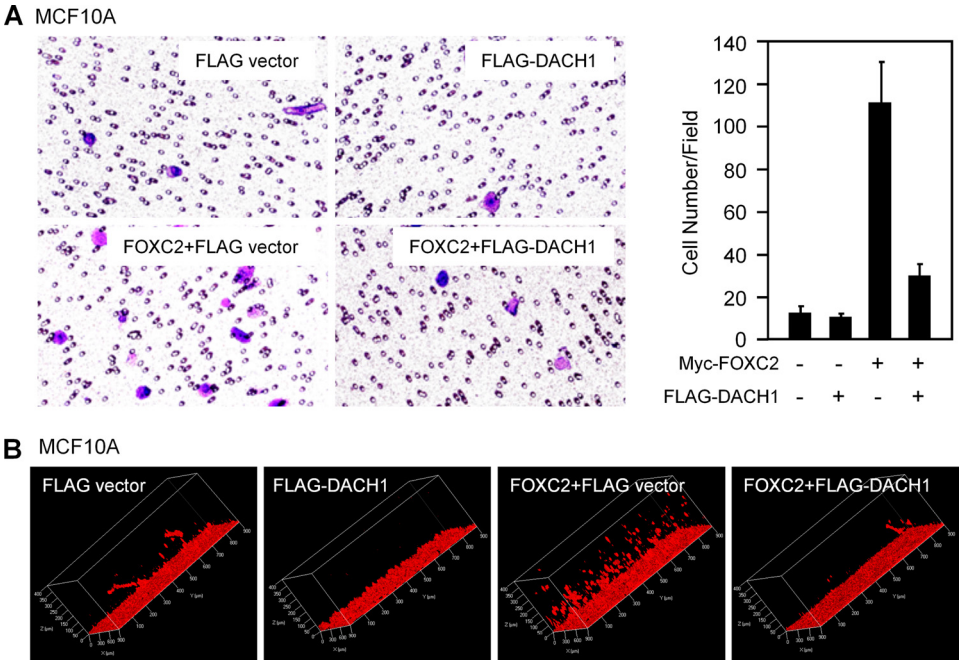

FIGURE 4. **DACH1 represses FOXC2-dependent cellular migration.** MCF-10A cells were transduced with retroviral expression vectors encoding FOXC2 and subjected to GFP-FACS with subsequent transient transfection with vector encoding FLAG-tagged DACH1. Cells were then analyzed for cellular migration by Transwell assay (*A*) and for invasion by three-dimensional invasiveness assay (*B*). Crystal violet dye staining of cells that migrated in the Transwell assays is shown. The data are shown as mean ± S.E. (*error bars*) of the number of cells migrated in three separate experiments.

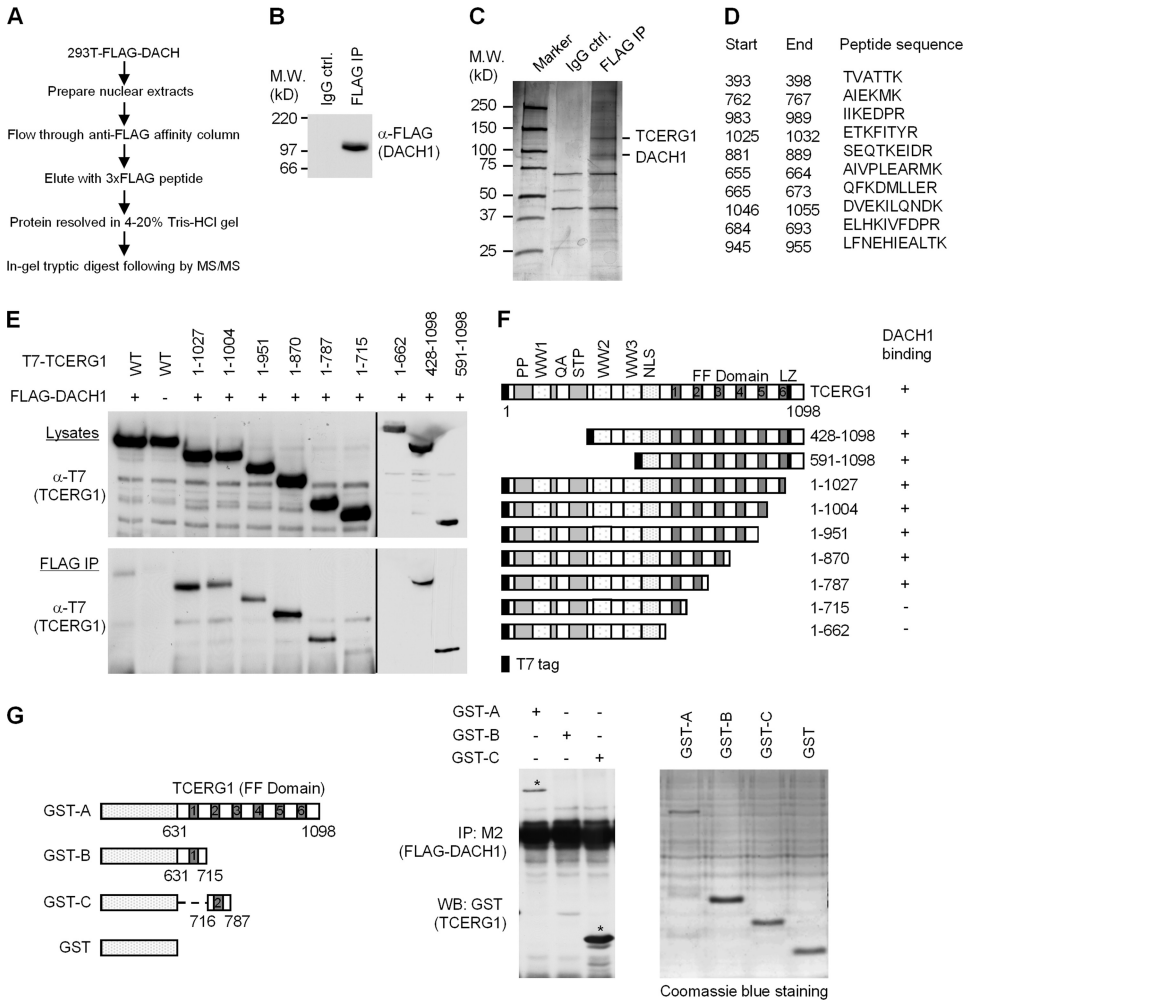

FIGURE 5. **DACH1 binds the co-integrator TCERG1.** *A*, experimental approach used for identification of DACH1-binding proteins. *B*, HEK 293T cells transiently transfected with FLAG-DACH1-expressing vector. 50 mg of whole cell lysates were subjected to an immune-affinity column preloaded with a 1-ml slurry of M2 agarose beads (Sigma). The proteins associated with agarose beads were eluted with buffer containing 100 μM FLAG peptide. Western blotting was conducted of the eluted DACH1 using the anti-FLAG antibody (Sigma). *C*, eluted proteins separated on 4–20% gradient SDS-PAGE using a silver-stained gel. *D*, peptide sequence aligning to TCERG1. *E*, IP-Western blotting to determine DACH1 and TCERG1 binding. Expression vector for DACH1 (FLAG-tagged) and a series of TCERG1 deletion mutants (T7-tagged) were used to transfect HEK 293T cells. IP was conducted with anti-FLAG antibody for DACH1 and Western blotting with T7 antibody for TCERG1. *F*, schematic representation of the TCERG1 expression vectors and observed DACH1 binding ability. *G*, co-immunoprecipitation assays performed by incubating GST fusion proteins of TCERG1 with FLAG-DACH1 protein expressed in HEK 293T cells. IP was conducted using anti-FLAG (M2) antibody followed by Western blotting using GST antibody to detect mutants of TCERG1.

didate DACH1-binding co-regulator proteins (Fig. 5*A*). DACH1 protein complexes were prepared from HEK 293T cells transfected with a FLAG-DACH1 expression vector (Fig. 5*B*). DACH1-associated proteins were resolved on a 4–10% Tris-HCl gel and silver-stained (Fig. 5*C*). The proteins recovered from the gel were subjected to in-gel tryptic digestion and sequential MS/MS. One of the excised bands corresponding to 145 kDa was identified as TCERG1 (Fig. 5*D*).

To determine the domain of physical interaction between DACH1 and TCERG1, FLAG-DACH1 was co-expressed with T7-tagged wild-type and a series of deletion mutants of TCERG1. IP was conducted with anti-FLAG with sequential WB for the T7 tag of TCERG1 (Fig. 5*E*). The *upper panel* shows the WB of the cell extracts, and the *lower panel* shows the IP-WB. IP-WB analysis demonstrated the co-precipitation of TCERG1 with DACH1. The WW domain binds to the SF1 splicing factor (24). Deletion of all three WW domains did not affect DACH1 binding (Fig. 5, *E* and *F*); however, deletion of the carboxyl terminus (TCERG1, amino acids 1–662) abrogated DACH1 binding (Fig. 5, *E* and F). Sequential carboxyl-terminal deletion of the FF domain abrogated DACH1 binding upon deletion of FF2 (construct 1–787 *versus* 1–715). Thus, DACH1 binding to the TCERG1 co-integrator protein requires the FF2 domain. Next, we determined whether the FF2 domain of TCERG1 is sufficient for binding to DACH1. We performed immunoprecipitation assays by incubating cell extracts prepared from HEK 293T cells transfected with FLAG-DACH1 plasmid with the FF2 domain expressed in bacteria as a GST fusion protein (25). The FF2 domain that was required for DACH1 binding was also sufficient for DACH1 binding (Fig. 5*G*).

TCERG1 is a promoter-specific transcriptional co-regulator of human immunodeficiency virus (HIV) *Tat* gene (26, 27). We therefore sought to determine whether TCERG1 functions as a co-regulator of DACH1. HEK 293T cells were

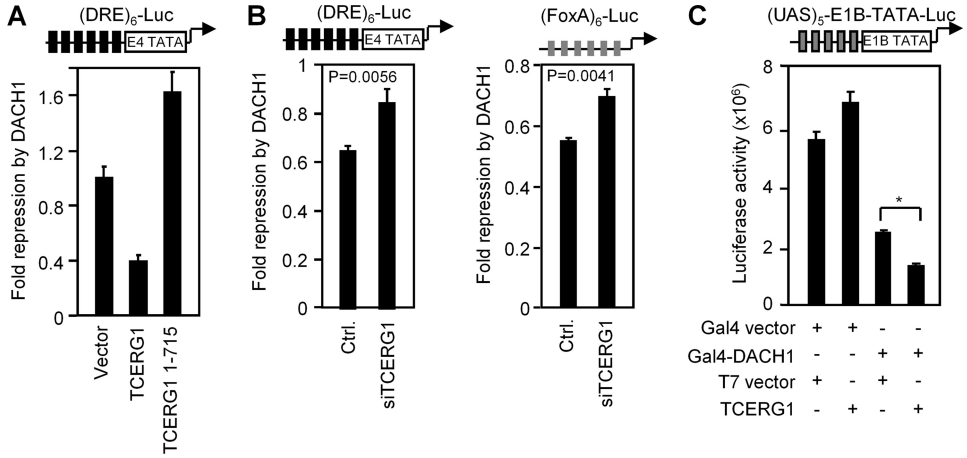

FIGURE 6. **TCERG1 is required for DACH1 trans-repression.** *A*, HEK 293T cells transfected with DACH1 and TCERG1 mutants together with $(DRE)_6$-Luc. The carboxyl-terminal deletion of TCERG1, which does not bind DACH1, is defective for repression. *B*, HEK 293T cells transfected with TCERG1 siRNA and control for 48 h followed by transfection with indicated luciferase reporter plasmids. *C*, HEK 293T cells transfected with TCERG1 and Gal4-DACH1 or control vector together with Gal4 reporter, showing that TCERG1 enhanced DACH1-mediated transcriptional repression.

transfected with $(DRE)_6$-Luc reporter and a DACH1 expression vector. TCERG1 expression enhanced DACH1 transcriptional repression (Fig. 6*A*). We next determined the domain of TCERG1 that is required to enhance DACH1 trans-repression. The carboxyl-terminal deletion mutant (amino acids 1–715), which does not bind to DACH1, was defective in enhancing DACH1-mediated trans-repression function (Fig. 6*A*). TCERG1 enhancement of DACH1 repression was further confirmed by knockdown of TCERG1 with siRNA, showing that reduced TCERG1 expression reversed DACH1 repression in the reporter assay (Fig. 6*B*). The synergistic repression of TCERG1 and Gal4-DACH1 on $(UAS)_5$-E1B-TATA-Luc reporter suggested that TCERG1 enhances DACH1-mediated trans-repression independent of its DNA binding (Fig. 6*C*).

Carboxyl Terminus of DACH1 Exhibits Trans-repression through Binding TCERG1—To identify the domain of DACH1 required for TCERG1 binding, IP-WB was conducted. DACH1 mutants were assessed using anti-FLAG antibody with sequential blotting to the T7 tag of TCERG1 (Fig. 7*A*). Deletion of the DACH1 carboxyl-terminal (amino acids 566–706) residues abrogated binding to TCERG1 (Fig. 7*B*). Deletion of the DNA binding domain (ΔDBD) did not affect DACH1 binding to TCERG1 (data not shown).

The ΔDBD mutant of DACH1 was capable of binding TCERG1 and showed a dominant negative effect on DACH1 repression (Fig. 7*C*). The dominant negative effect may be due to competition with wild-type DACH1 through its carboxyl terminus for a limited TCERG1 pool in the cell. Consistent with this model, the C-ter of DACH1, which is sufficient for TCERG1 binding by itself, exhibited a dominant negative effect on the DRE reporter gene activity (Fig. 7*C*). Therefore, we conclude that the carboxyl terminus of DACH1 conveys a trans-repression function through binding TCERG1. Consistent with the importance of the DACH1 binding to TCERG1 via its C terminus, the deletion of the DACH1 carboxyl terminus (ΔC) impaired DACH1 trans-repression function and ΔC exhibited strong dominant negative effect on full-length DACH1 in gene reporter assays (Fig. 7*D*).

DISCUSSION

This study provides the first mechanistic evidence that DACH1 is a transcription factor (14) that represses gene transcription through binding transcription elongation regulator 1 (TCERG1, also known as CA150 or TAF2S). TCERG1 is a nuclear protein associated with the RNA polymerase II holoenzyme. FOX proteins activate gene transcription, and DACH1 competes with FOX protein in the context of local chromatin to repress gene expression. Decreased DACH1 expression and overexpression of oncogenic FOX protein could lead to deregulation of a subset of genes required for tumorigenesis.

DACH1 regulates gene expression by complexing with DNA-binding transcription factors, including Six, Jun, and Smad4 (6, 10, 28, 29). Analysis of the components of the RDGN has implicated *dac* in cell fate specification. *Six6*$^{-/-}$ mutant mice present hypoplastic pituitary glands with a variable penetrance and retinal hypoplasia with decreased ganglion cell layer cell number. Mammalian 2-hybrid experiments suggest that Six6 interacts with DACH1, whereas molecular mapping studies reveal co-precipitation of DACH1 with NCoR, HDAC3, and Sin3a/b (28). These molecular interactions are consistent with studies by Ikeda *et al.*, which suggest that Eya interacts with Six6 in mammalian two-hybrid, but does not interact with DACH1 (13). DACH1, however, is capable of transactivating in the presence of an Eya fusion protein, suggesting that the CREB-binding protein, CBP, mediates the interaction between Eya and DACH1. The functional significance of CBP *versus* TCERG1 in DACH1-mediated function remains to be determined. DACH1 is also known to inhibit TGF-β signaling in ovarian cancer. Future studies will address whether loss of DACH1 in cancers correlates with increased expression of oncogenes, whose promoter have DACH1 binding site(s).

DACH1 conveys transcriptional repression when linked to a heterologous DNA binding domain. Proteomic analysis identified the co-integrator protein TCERG1 as a candidate mediator of DACH1 transcriptional repression. TCERG1 is a

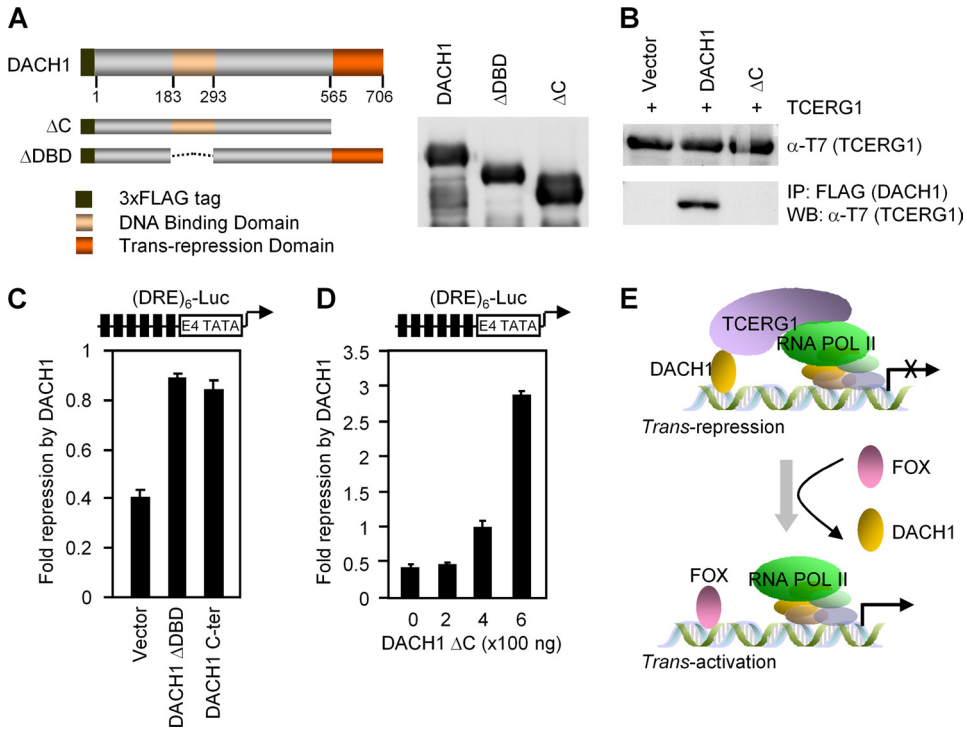

FIGURE 7. **C terminus of DACH1 conveys trans-repression function.** *A*, schematic representation of DACH1 expression vectors used for IP-Western blotting to determine the minimal region of DACH1 required for TCERG1 association. *B*, HEK 293T cells co-transfected with T7-tagged TCERG1 and FLAG-tagged full-length DACH1 or carboxyl-terminal truncated form (ΔC). IP was conducted with anti-FLAG antibody, followed by Western blotting with anti-T7 antibody. *C*, effect of ΔDBD and carboxyl terminus (*C-ter*) on wild-type DACH1 measured by reporter assay using $(DRE)_6$-Luc. *D*, HEK 293T cells co-transfected with increased amount of carboxyl-terminal deletion mutant (ΔC) plasmid DNA and vector expressing full-length DACH1 together with $(DRE)_6$-Luc reporter. The dominant effect is dose-dependent. -Fold repression was calculated as the ratio of activities measured from DACH1-transfected cells to those in vector control-transfected cells. The data are shown as mean ± S.E. (*error bars*) from at least two separate experiments with triplicate samples each. *E*, schematic representation of proposed model by which DACH1 and FOXM1 compete to regulate gene expression.

promoter-specific transcription co-regulator of HIV *Tat* gene (26, 27). TCERG1 expression enhanced DACH1-mediated trans-repression. It has been reported previously that the carboxyl-terminal and amino-terminal halves of TCERG1, containing FF repeats and WW domains, respectively, are required for efficient repression of the α4-integrin gene expression (24). We included two mutants of TCERG1 representing the FF domain (FF2–6) deletion and amino-terminal deletion (591–1098) in DACH1 trans-repression assays. The carboxyl-terminal deletion of TCERG1 (1–715) defective for DACH1 binding failed to enhance DACH1 repression, although the WW domain deletion has no effect on TCERG1 function as a co-repressor of DACH1. Through multiple WW domains in its amino terminus, TCERG1 associates with splicing factor 1 (SF1) (24), a protein that functions in the assembly of a pre-RNA splicing complex. We failed to observe synergistic repression of DACH1 and SF1 binding domain of TCERG1 (data not shown). However, this does not rule out the possibility that pre-RNA splicing mediated by the TCERG1-SF1 complex is a possible mechanism that DACH1 utilizes to repress gene expression because the reporter systems we used here do not contain introns.

Herein DACH1 inhibited Forkhead signaling and blocked FOXC2-mediated cellular migration and invasion. These findings are consistent with recent studies that DACH1 displaces FOXM1 in the context of local chromatin from the promoter of FOXM1-targeted genes (14). The current findings provide further evidence that the RDGN pathway protein, DACH1, modulates Forkhead signaling and function (Fig. 7*E*). In this study, we identified TCERG1 as a transcriptional co-integrator of the RDGN factor, raising the intriguing possibility that TCERG1 may functions as the limiting co-integrator of the RDGN and Forkhead signaling pathways.

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