Supporting Information

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SI Text

Plasmid Construction. The expression plasmids for DACH1, DACH1 DS-domain alone (DS) or DACH1 DS-domain deleted (ΔDS) (which include an N-terminal FLAG peptide), the ponasterone-regulated expression system, the full-length and point mutants of the *IL-8* promoter luciferase reporter construction, were described in refs. 1–4. The FLAG-tagged DACH1 cDNA was subcloned into the retroviral expression vector pLRT to form a tetracycline-regulated DACH1 retroviral expression system (pLRT-DACH1).

Western Blot Analysis. Western blot analysis, using antibodies to cyclin D1; ErbB2; phospho-ERK; phosphor-AKT; Myc; FLAG; and the loading control, guanine dissociation inhibitor (GDI), was performed as described in refs. 3 and 5. Whole-cell lysates (50 μg) were separated by 10% SDS (SDS/PAGE), and the proteins were transferred to a nitrocellulose membrane for Western blot analysis as described in ref. 6.

Cell Culture, Plasmid Transfection, and Luciferase Reporter Assays. Cells were cultured as recommended by ATCC and maintained in humidified atmosphere with 5% CO_2 at 37°C (6). For *in vitro* experiments with the pLRT retrovirus, Doxycycline was added to a final concentration in the media of 2 μg/ml. MCF10A-ΔRaf-ER cells were a gift from J. Downward (Cancer Research UK, London, UK) (7). MCF10A-Ras, Ras/ErbB2, ErbB2 (NeuT) and c-Myc-transformed cells, were grown as described in ref. 6. Mouse embryo fibroblasts (MEF) were prepared from *Dach1*$^{-/-}$ mice (8), a generous gift from G. Mardon (Baylor College of Medicine, Houston, TX) as described in refs. 1 and 9. PCR primers for Dach1 were 5′ACA TGC ACA TAC GCA CAC TTT and 5′AAG AGG TCA AGA CAG GAA CAT CA (265 bp) and for the knockout allele were 5′AGG CCA CTT GTG TAG CGC CAA. For 3-D culture, 1,000 cells mixed with 1:3 diluted growth factor reduced Matrigel matrix were seeded into BD Biocoated cell culture inserts (24-well) with Matrigel basement membrane matrix from BD Bioscience (catalog no. 354447) and photographed after growing for 3 to 14 days (10). Transfections were performed by using Superfect transfection reagent (Qiagen) according to the manufacturer's protocol, using 0.5 μg of the reporter, 50–150 ng of expression plasmids, or equal molar amounts of control vector. The transfection efficiency was normalized by cotransfection with 0.2 μg of the pRL-CMV plasmid (Promega) and measured with the Promega dual-luciferase reporter assay system according to the manufacturer's protocol. Statistical analyses were performed by using Student's *t* test.

Migration and Invasion Assays. Transwell migration assays were performed as described in ref. 11. Briefly, GFP-positive cells were seeded on an 8-μm-pore size Transwell filter insert (Costar) coated with 10 μg/ml fibronectin (Sigma). After 16 h of incubation at 37°C and 5% CO_2, cells adherent to the upper surface of the filter were removed by using a cotton applicator. Cells were fixed with 3.7% formaldehyde and stained with crystal violet, and the numbers of cells on the bottom were counted. Data are from at least three experiments done in triplicate (mean ± standard error).

Invasion of matrigel has conducted by using standardized conditions with BDBioCoat growth factor reduced MATRIGEL invasion chambers (PharMingen). Analysis of three-dimensional invasive activity was conducted as described in ref. 12. Cells (10^5) were embedded in 100 μl of collagen in a 96-well plate and cultured for 24 h. The collagen-cell plugs were transferred to 24 well plates, and embedded in 1 ml of collagen and cultured for 5 days. Migration from the central plug into the surrounding collagen was monitored by phase contrast microscopy. *In vivo* metastasis were assessed in nude mice from the National Cancer Institute, after tail vein injection, using 1×10^5 cells (13). Doxycycline was used to induce the DACH1 transgene expression via drinking water at a final concentration of 1 mg/ml. Staining for lung metastasis of Met-1 cells, using India ink, was conducted exactly as described in ref. 13. Anti-mouse CRCL1/KC antibody (R&D Systems) (14) was given by i.p. injection (100 μg) on days 7, 9, 11, 14, 16, 18, 21, 23, and 25 as described for IL-8 antibody in ref. 15.

Wound Healing. Cells were grown to confluence on 12-well plates, and the monolayers were wounded with a P10 micropipette tip (16). DMEM with 10% FBS and Hepes was changed immediately after scoring. The wound-healing videos were taken at 15-min intervals using a Nikon Eclipse TE-300 inverted microscope system. The cell movement velocity was determined by tracing single cells at different time points, using MetaMorph software (Molecular Devices).

Time-Lapse Video. For time-lapse observation of cell movement, cells on 12-well plates were maintained in DMEM with 10% FCS (FCS) and Hepes. Cells were placed in a temperature and CO_2 controlled incubator to maintain the temperature at 37°C and CO_2 at 5%. Cell movements were monitored by using a Zeiss inverted microscope. Video images were collected with a charge-coupled-device camera (model 2400) at planned intervals, digitized, and stored as images, using Metamorph software, Version 3.5 (11). Images were converted to QuickTime movies, and the positions of nuclei were tracked to quantify cell motility. Cellular velocity was calculated in micrometers, using Metamorph software. The persistence for migratory directionality was determined as a relative D/T ratio representing the ratio of the direct distance (D) from start point to end point divided by the total track distance (T) (11). Net displacements were measured every 15 min from start point to end point. Data from at least 100 cells were collected for each set or treatment.

Cytokine Array Analysis. Human cytokine arrays spotted on nitrocellulose membranes were obtained from Raybiotech (10). Conditioned medium from MDA-MB-231 cells expressing ponasterone-regulated DACH1 was prepared by culturing cells in serum free DMEM for 24–48 h. Membranes were then processed according to the manufacturers instructions for assessment of secreted cytokines and growth factors present in conditioned medium.

Real-Time PCR for Assessment of Expression of IL-8 Transcripts and Chromatin Immune Precipitation (ChIP) Assays. RNA was extracted by using standard GITC method from MDA-MB-231 stable cells and MCF10A-Ras cells transfected with DACH1. All RNA samples were subjected to DNase I (RQ1 DNase; Promega) treatment to remove contaminating DNA from RNA preparations followed by their repurification, using Qiagen RNAEasy RNA columns. Equal amounts of purified RNA samples were then reverse transcribed by using Iscript reverse transcriptase kit (Bio-Rad) to form c-DNA, which was then subjected to SYBR Green based Real-Time PCR relative quantification method for

amplification of IL-8 transcripts, using standard hot-start reaction mixes and conditions (12) in an ABI Prism 7900HT (Applied Biosystems). The primers used for amplification of IL-8 transcripts were: forward, 5′-TTT TGC CAA GGA GTG CTA AAG A-3′, and reverse, 5′-ACC CTC TGC ACC CAG TTT TC-3′. Amplification of the 18s rRNA housekeeping gene transcript [primer (12)] was performed in every sample and the obtained Ct values for each sample were used for normalization of data for IL-8 expression, using the ABI Prism SDS 2.3 software. To calculate the fold change in *IL-8* gene expression between various treatments, Ct values obtained from amplification of IL-8 transcripts in MCF10A-Ras cells were used as the calibrator.

Analysis of the endogenous *IL-8* promoter was conducted in ChIP assays, using antibodies directed to either the FLAG epitope of DACH1 under described conditions (12). The primers for IL-8 prompter were: forward, 5′-AGTGTGATGACTCAGGTTTGCCCT-3′, and reverse, 5′-TGGTTTCTTCCTGGCTCTTGTCCT-3′. The primers for negative site were: forward, 5′-CCTTGTTCCACTGTGCCTTGGTTT-3′, and reverse 5′-ACCAACACAGCTGGCAATGACAAG-3.

1. Albanese C, *et al*. (2005) Sustained mammary gland-directed, ponasterone A-inducible expression in transgenic mice. *FASEB J* 14:877-884.
2. Albanese C, *et al*. (2003) IKKalpha Regulates Mitogenic Signaling through Transcriptional Induction of Cyclin D1 via Tcf. *Mol Biol Cell* 14:585-599.
3. Wu K, *et al*. (2003) DACH1 inhibits TGF-beta signaling through binding Smad4. *J Biol Chem* 278: 51673-51684.
4. Casola A, *et al*. (2002) Interleukin-8 gene regulation in intestinal epithelial cells infected with rotavirus: role of viral-induced IkappaB kinase activation. *Virology* 298:8-19.
5. Lee RJ, *et al*. (2000) Cyclin D1 is required for transformation by activated Neu and is induced through an E2F-dependent signaling pathway. *Mol Cell Biol* 20:672-683.
6. 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
7. Schulze A, Lehmann K, Jefferies HB, McMahon M, Downward J (2001) Analysis of the transcriptional program induced by Raf in epithelial cells. *Genes Dev* 15:981-994.
8. Davis RJ, Pesah YI, Harding M, Paylor R, Mardon G (2006) Mouse Dach2 mutants do not exhibit gross defects in eye development or brain function. *Genesis* 44:84-92.
9. Albanese C, *et al*. (1999) Activation of the *cyclin D1* gene by the E1A-associated protein p300 through AP-1 inhibits cellular apoptosis. *J. Biol Chem.* 274:34186-34195.
10. Ju X, *et al*. (2007) Akt1 governs breast cancer progression *in vivo*. *Proc Natl Acad Sci USA* 104:7438-7443.
11. Li Z, *et al*. (2006) Cyclin D1 regulates cellular migration through the inhibition of thrombospondin 1 and ROCK signaling. *Mol Cell Biol* 26:4240-4256.
12. Katiyar S, Jiao X, Wagner E, Lisanti M.P, Pestell RG. (2007) Somatic excision demonstrates c-Jun induces cellular migration and invasion through induction of stem cell factor. *Mol Cell Biol* 27:1356-1369.
13. Williams TM, *et al*. (2004) Caveolin-1 gene disruption promotes mammary tumorigenesis and dramatically enhances lung metastasis *in vivo*. Role of Cav-1 in cell invasiveness and matrix metalloproteinase (MMP-2/9) secretion. *J Biol Chem* 279:51630-51646.
14. Witowski J, *et al*. (2000) IL-17 stimulates intraperitoneal neutrophil infiltration through the release of GRO alpha chemokine from mesothelial cells. *J Immunol* 165:5814-5821.
15. Mizukami Y, *et al*. (2005) Induction of interleukin-8 preserves the angiogenic response in HIF-1alpha-deficient colon cancer cells. *Nat Med* 11:992-997.
16. Neumeister P, *et al*. (2003) Cyclin D1 governs adhesion and motility of macrophages. *Mol Biol Cell* 14:2005-2015.

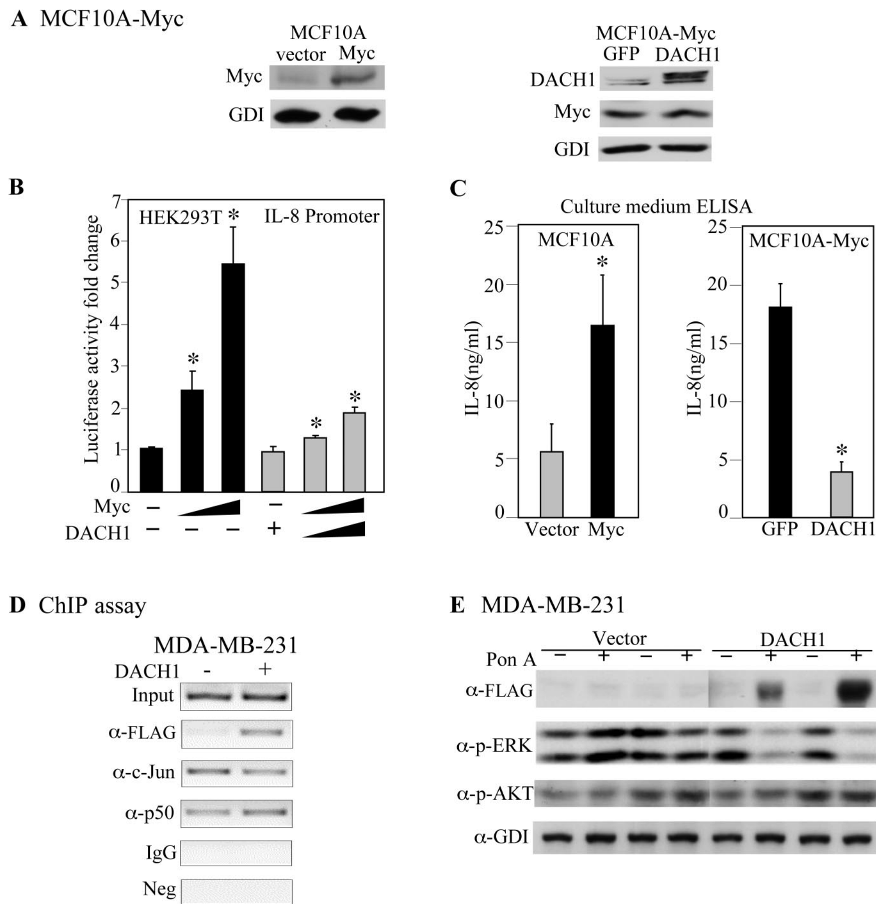
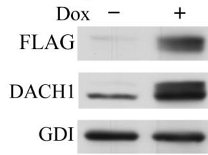
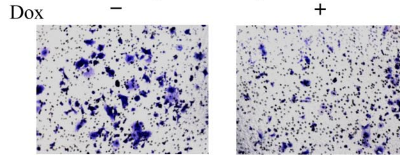


Fig. S1. DACH1 inhibits ERK activity and Myc-induced IL-8. (*A*) Western blot of MCF10A, MCF10A-Myc cells transduced with MSCV-DACH1-IRES-GRP. (*B*) IL-8 promoter luciferase activity in cell cotransfected with expression vectors encoding c-Myc and DACH1. (*C*) IL-8 abundance by ELISA in MCF10A or MCF10A-c-Myc cells transduced with MSCV-DACH1 or MSCV-GFP control vector. (*D*) ChIP analysis of the endogenous IL-8 promoter in MDA-MB-231 cells. DACH1 expression and recruitment to the IL-8 promoter is identified by αFlag ChIP. (*E*) Induction of DACH1 expression inhibits ERK phosphorylation (Tyr-204) but does not effect Akt phosphorylation (Ser-473). GDI is a protein loading control. *, $P < 0.01$.





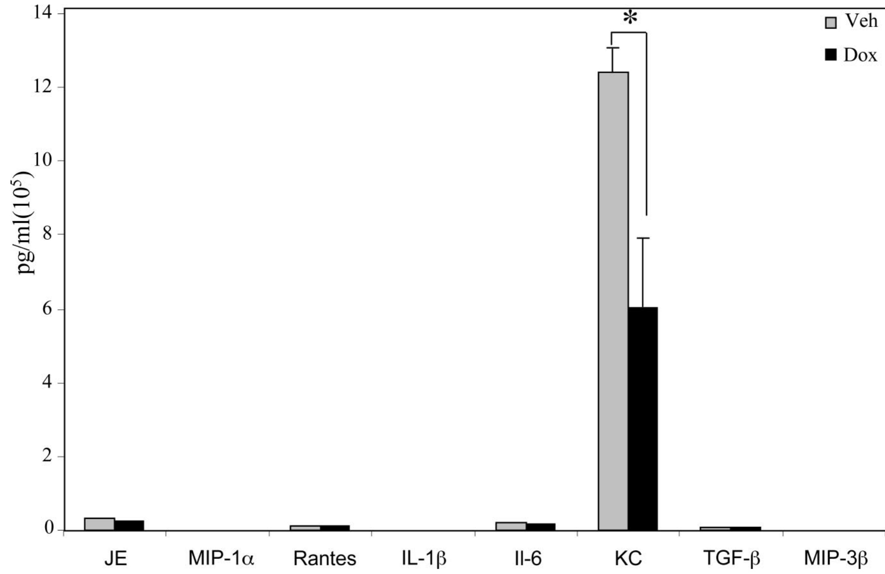


Fig. S2. DACH1 inhibits IL-8 production and migration of Met-1 cells. (*A* and *B*) The Met-1 cell line expressing inducible DACH1 (pLRT-DACH1) was assessed for DACH1 expression by Western blot (*A*), and transwell migration in Boyden chamber assays (*B*). (*C*) Quantitation of secreted cytokines was conducted by using the Searchlight kit ($n = 3$). *, $P < 0.01$.