Supporting Information

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SI Text

Quantitative Real-Time PCR. Oligonucleotides used for RT-PCR include: Human Twist: Forward: 5′- ggagtccgcagtcttacgag, Reverse: 5′- cctctaccaggtcctccaga; Nanog: Forward: CAGAAAAACCAGTGGTTGAAGACTAG, Reverse: GCAATGGATGCTGGGATACTC (1); Oct4: Forward: CTGTAGGGAGGGCTTCGGGCACTT, Reverse: CTGAGGGCCAGGCAGGAGCACGAG (2); Sox2: Forward: GGCAGCTACAGCATGATGCAGGAGC, Reverse: CTGGTCATGGAGTTGTACTGCAGG (2); KLF4: Forward: TGCCAGACCAGATGCAGTCAC, Reverse: GTAGTGCCTGGTCAGTTCATC (3); c-Myc: Forward: TGAGCCCCTAGTGCTGCAT, Reverse: AGCCCGACTCCGACCTCTT; 18s rRNA oligos (4).

Cell Culture, Retroviral Infection, Luciferase Reporter Assay, siRNA Transfection, and 3-D Culture. The *Twist*-specific siRNA target sequences is GGACAAGCTGAGCAAGATTCA (5, 6). The $p21^{CIP1}$ siRNA sequence is GCCTTAGTCTCAGTTTGGTGTCTT (7). *Twist* and $p21^{CIP1}$-specific siRNAs or control siRNAs (100 nM) were transfected by the Nucleofector technology using Nucleofector Kit V and program T-024 (Amaxa Biosystems). Transfection efficiency was monitored by non-silencing fluorescein-labeled siRNA from QIAGEN. For 3-D culture, 2,000 cells mixed with growth factor reduced Matrigel matrix (volume ratio 1:1) were seeded into a four-well chamber and photographed according to a previously described protocol (8, 9).

Mammosphere Formation and FACS Analysis of Stem Cell Surface Markers. Before labeling, the cells were blocked with normal mouse IgG in 1/100 dilution for 30 min and then incubated with PE labeled mouse anti-human CD24 (1/5) (clone ML5, BD PharMingen) and/or PE/Cy5 labeled rat anti human/mouse CD44 (1/200) (clone IM7, BioLegend) for 1 h. All experiments were conducted at 4 °C. Cell sorting was performed on a FACSCalibur cell sorter (BD Biosciences). The data were analyzed with FlowJo software (Tree Star, Inc.).

Western Blot Analysis, Immunofluorescence, and Immunohistochemistry (IHC). IHC was following conducted as described in ref. 10. Briefly, after slides were rehydrated in graded alcohol, retrieved for antigen using Citra (Biogenex) in a steamer, and blocked with peroxidase and goat serum, the slides were incubated with an antibody specific for E-cadherin (#610182, BD Biosciences), vimentin (M7020, Dako), or Twist (T6451, Sigma); all dilutions were 1:50. For the rabbit primary antibodies, slides were incubated with Envision System labeled polymer-HRP anti-rabbit secondary antibody (K4003, Dako) with TSA-plus Cyanine 5 system (NEL745001KT, Perkin-Elmer LAS). For the mouse primary antibodies, slides were stained using the M.O.M. basic kit (BMK-2202, Vector Laboratories). All slides were stored overnight at 4 °C. Peroxidase and protein blocking was performed again as described above. The slides were incubated with a cytokeratin primary antibody (Z0622, rabbit, Dako, 1/50), then stained with a polymer labeled secondary antibody (Alexa Flour 488 goat anti-rabbit, A11034, Invitrogen) and DAPI. Immunofluorescent images were taken using a PM2000 microscope (HistoRX; magnification, ×60). Representative images were analyzed for intensity of E-cadherin, vimentin, and Twist expression using the Image J program (NIH, Bethesda, MD), and the pixel values were averaged and graphically represented using Microsoft Excel:Mac v.x (Microsoft Corporation).

Migration Assays. Briefly, 2.5×10^4 cells were seeded on an 8-μm-pore size Transwell filter insert (Corning Inc.) 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. Data represent at least three experiments done in triplicate (mean ± standard error).

1. Lin T, et al. (2005) p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. *Nat Cell Biol* 7:165–171.
2. Chen S, et al. (2006) Self-renewal of embryonic stem cells by a small molecule. *Proc Natl Acad Sci USA* 103:17266–17271.
3. Swamynathan SK, et al. (2007) Conditional deletion of the mouse Klf4 gene results in corneal epithelial fragility, stromal edema, and loss of conjunctival goblet cells. *Mol Cell Biol* 27:182–194.
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5. Cheng GZ, et al. (2007) Twist transcriptionally up-regulates AKT2 in breast cancer cells leading to increased migration, invasion, and resistance to paclitaxel. *Cancer Res* 67:1979–1987.
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9. Ju X, et al. (2007) Akt1 governs breast cancer progression in vivo. *Proc Natl Acad Sci USA* 104:7438–7443.
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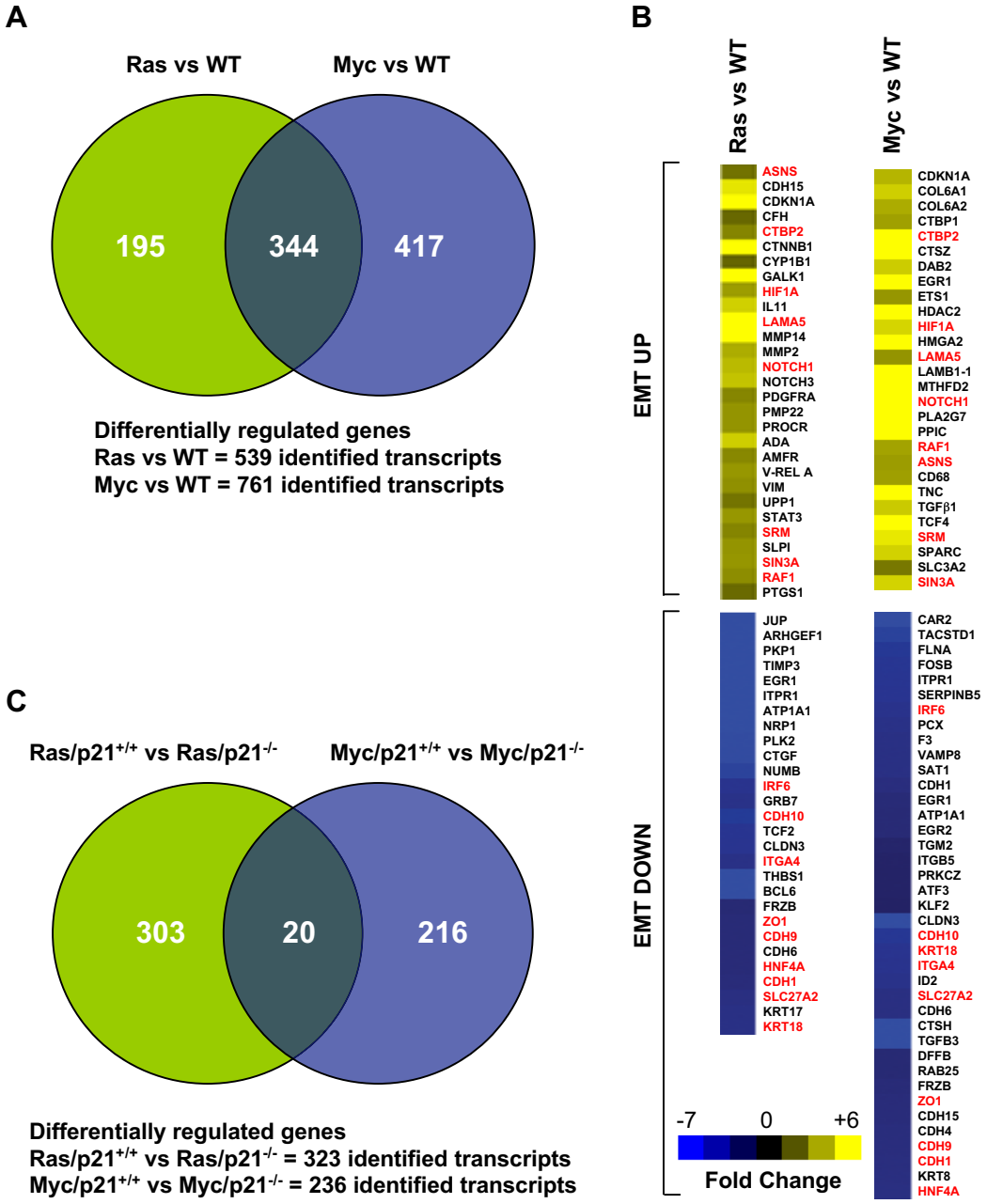

Fig. S1. Microarray analysis of MMTV-Ha-Ras- and MMTV-c-Myc-induced tumors. (*A*) Venn diagram of genes differentially regulated between MMTV-Ras and MMTV-c-Myc compared to WT mammary gland. (*B*) Genes differentially regulated by MMTV-Ras and MMTV-c-Myc compared to WT mammary gland that overlap with genes associated with EMT (13, 17). Genes upregulated in yellow and downregulated in blue relative to WT mammary gland ($P < 0.05$). Genes represented as gray were not differentially expressed at a significance of $P < 0.05$. (*C*) Venn diagram of genes differentially regulated by $p21^{CIP1}$ in MMTV-Ras- and MMTV-c-Myc induced tumors. (*D*) Analysis of sample set enrichment scores (ASSESS) was used to classify the pathways differentially regulated by $p21^{CIP1}$ in MMTV-Ras and (*E*) MMTV-c-Myc. Sample set enrichment scores concordant across each sample were used in a hierarchical cluster with complete linkage. Red denotes positive enrichment and upregulation, blue denotes negative enrichment and down regulation. Pathways indicated in red illustrate upregulation of EMT and LTHSC in $p21^{CIP1-/-}$ tumors.

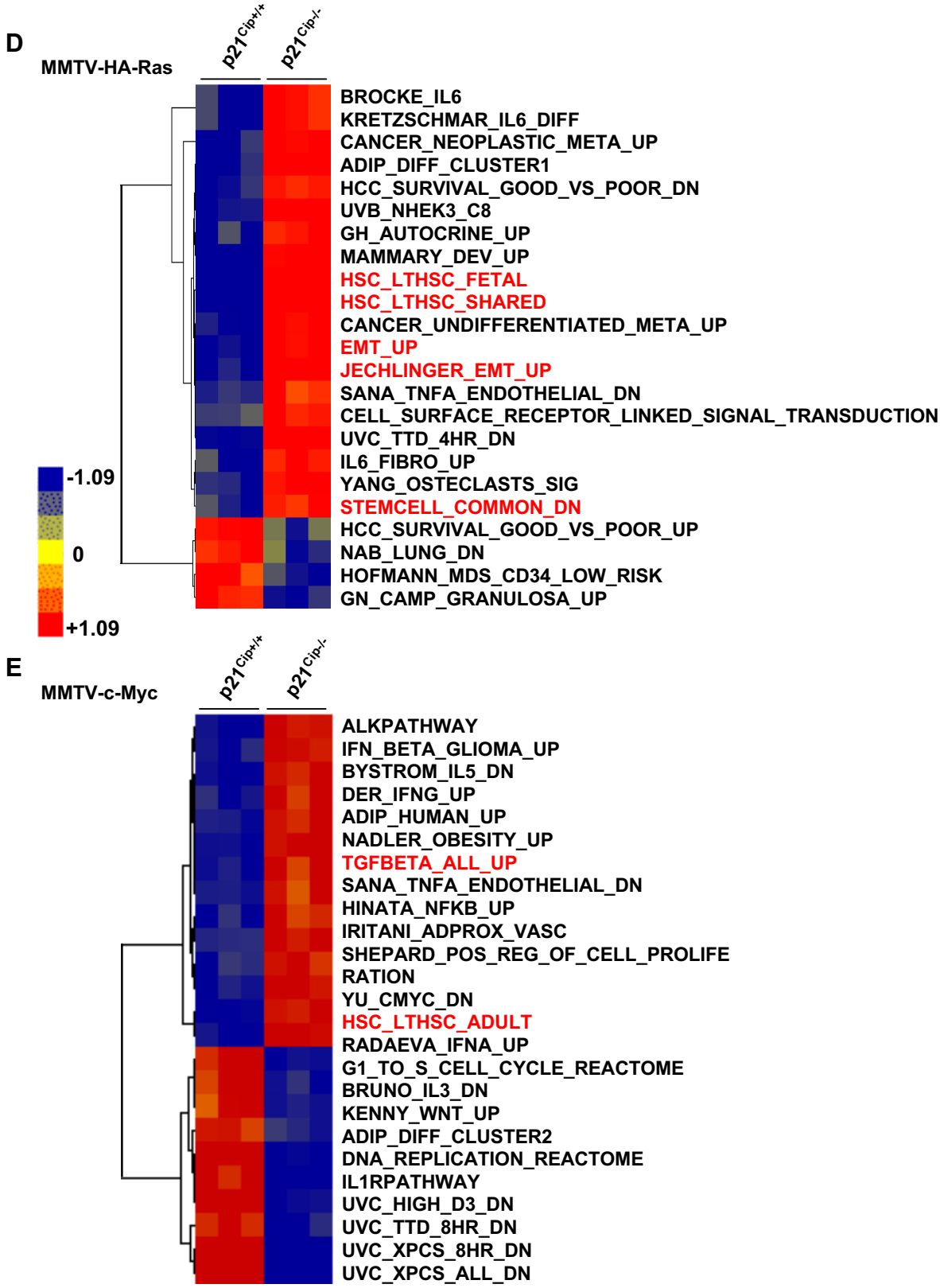

Fig. S1. Continued.

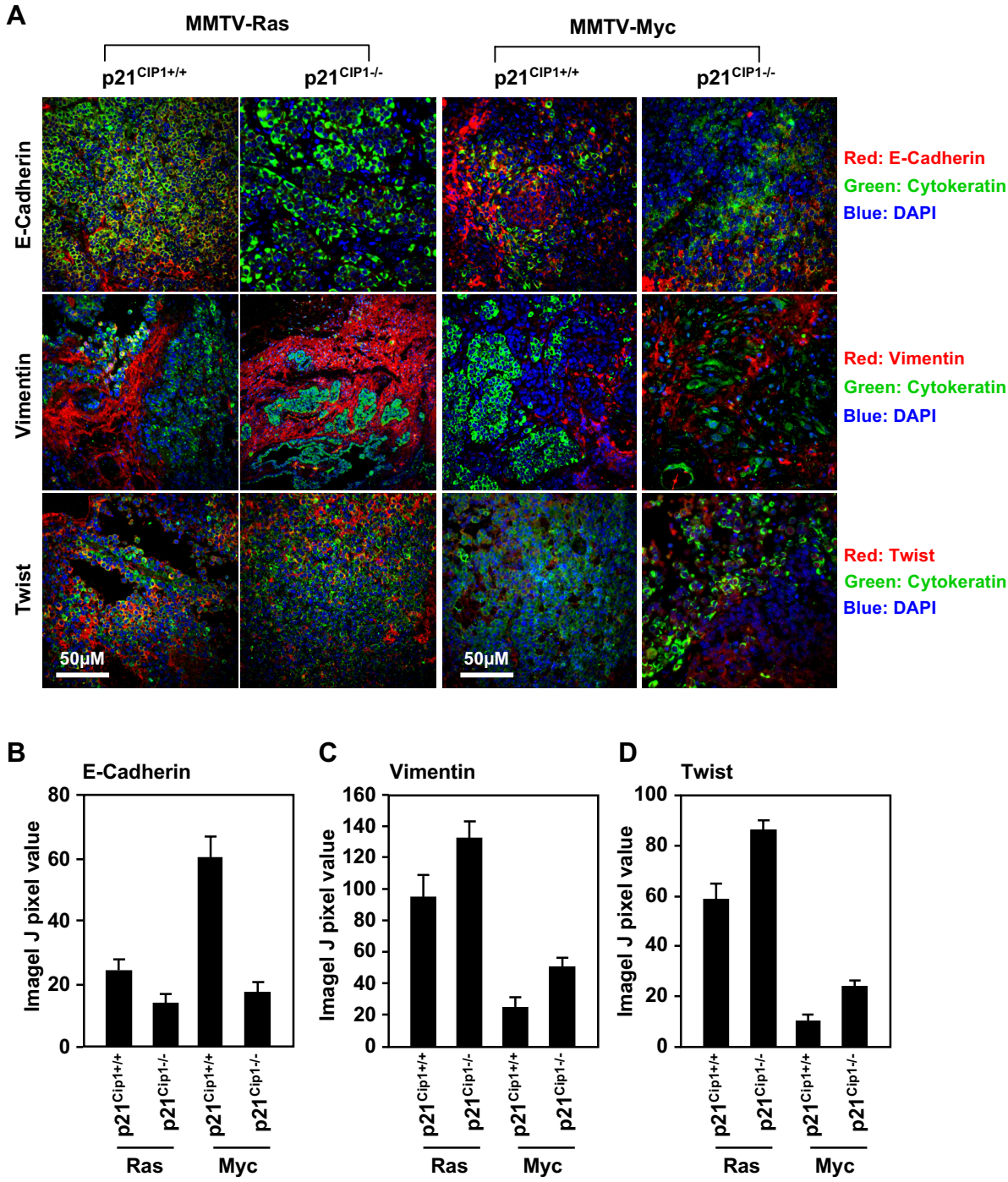

Fig. S2. p21^{CIP1} suppresses EMT in MMTV-Ha-Ras and MMTV-c-Myc mice. (*A*) Representive immunohistochemical staining for E-cadherin (top panel), vimentin (middle panel), and Twist (bottom panel) in mammary tumors of MMTV-Ras *p21*$^{CIP1+/+}$, MMTV-Ras/*p21*$^{CIP1-/-}$, MMTV-c-Myc/*p21*$^{CIP1+/+}$ and MMTV-c-Myc/*p21*$^{CIP1-/-}$ mice. (*B–D*) Relative intensity of E-cadherin (*B*), vimentin (*C*), and Twist (*D*) protein abundance in murine mammary tissue showed by pixel value.

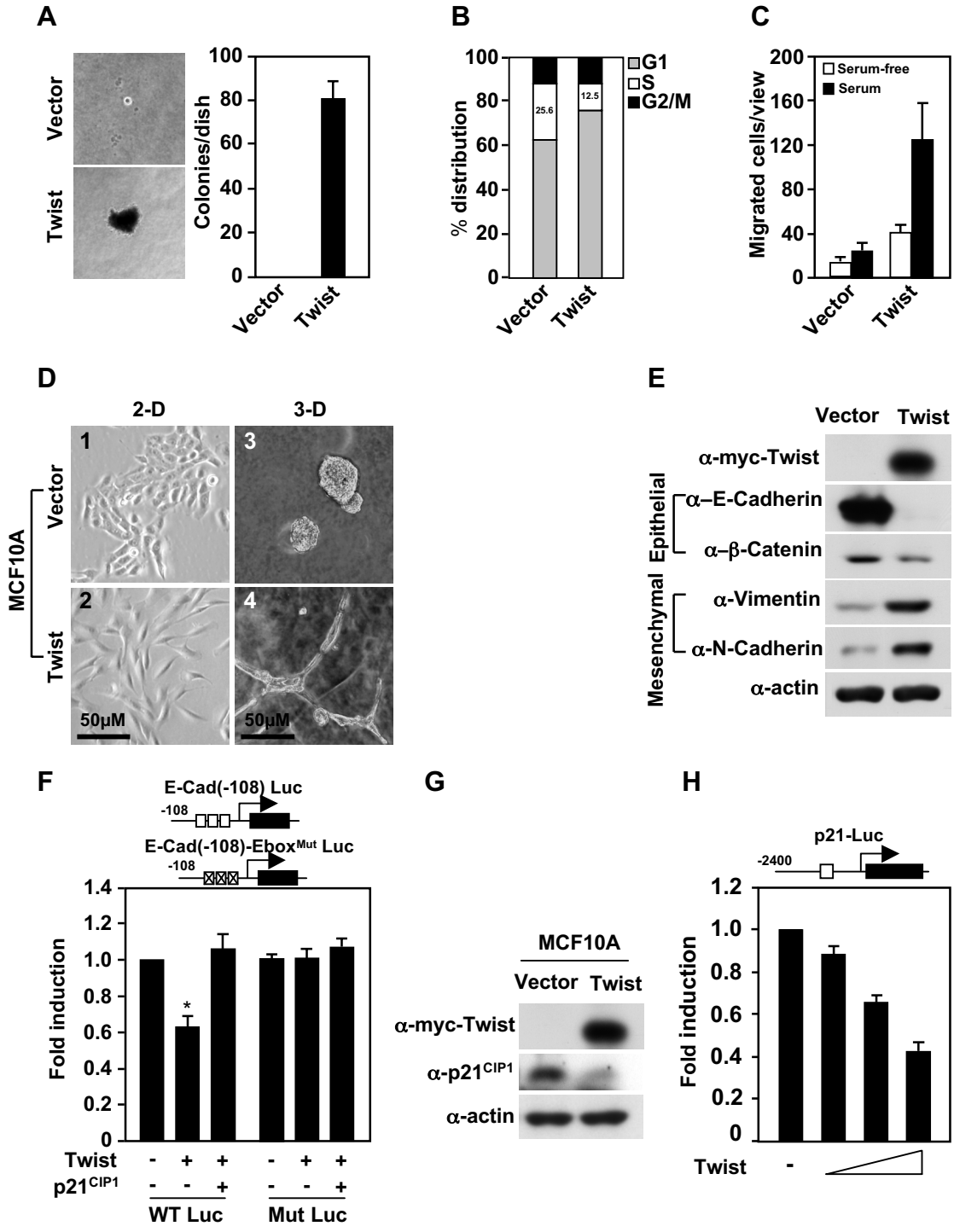

Fig. S3. Twist induces epithelial to mesenchymal transition in human MCF10A mammary epithelial cells. (*A*) Twist induces colony formation of MCF10A in soft agar. (*B*) Twist reduces S-phase in MCF10A cells determined by FACS assay. (*C*) Twist enhances migratory capability of MCF10A cell in a Transwell assay. (*D*) Twist induces mesenchymal morphologies in 2D culture (panels 1 and 2) and induces collagen matrix invasion in 3D culture (panels 3 and 4). (*E*) Expression of the epithelial markers E-cadherin, β-catenin, and mesenchymal markers vimentin and N-cadherin were examined by immunoblotting in MCF10A-Twist and control cells. (*F*) $p21^{CIP1}$ antagonizes Twist-mediated repression of E-cadherin promoter activity. (*G* and *H*) $p21^{CIP1}$ expression level (*G*) and promoter activity (*H*) was inhibited by Twist.

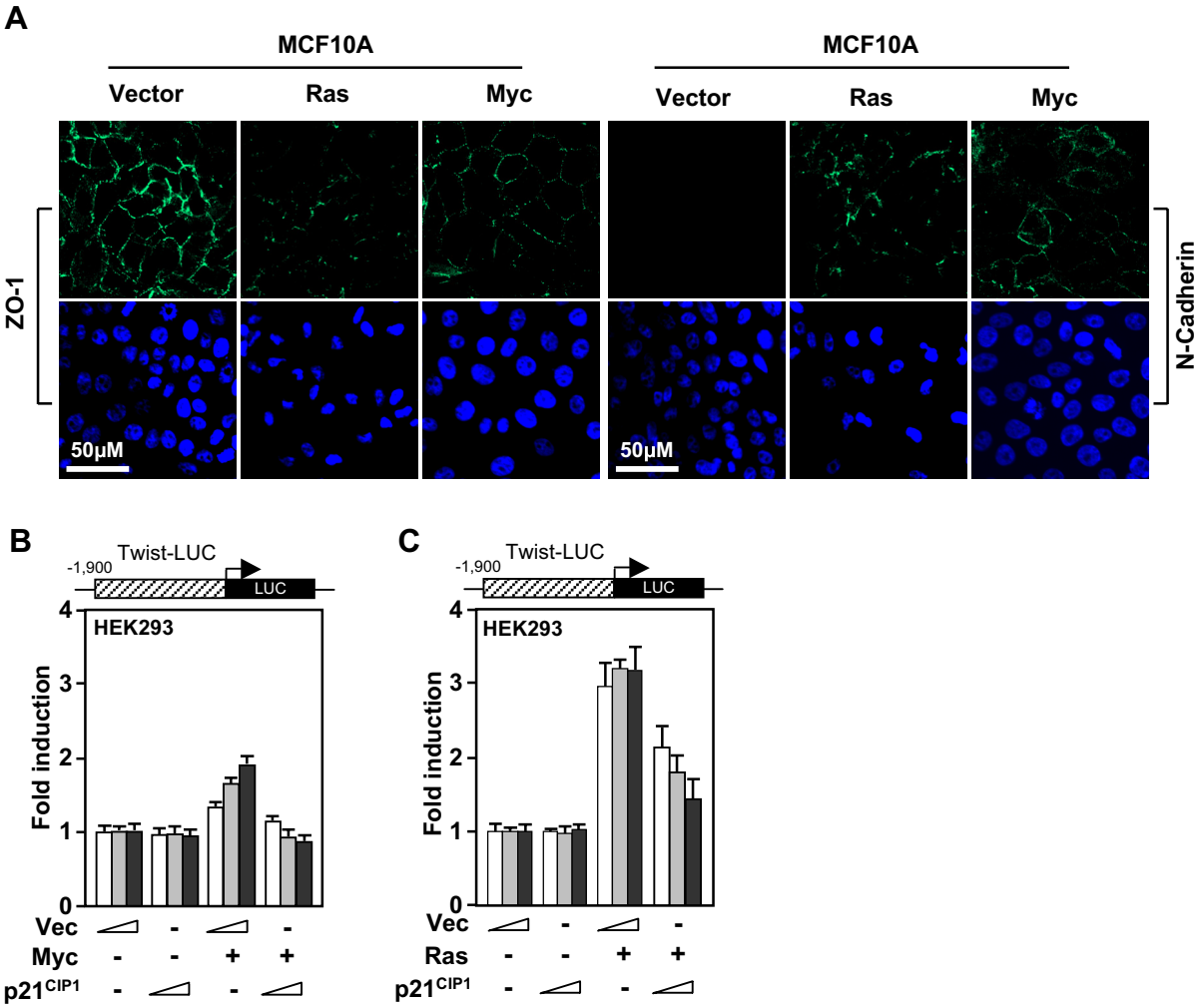

Fig. S4. p21^{CIP1} inhibits Ha-Ras-mediated induction of Twist. (*A*) Immunofluorescence staining of epithelial marker (ZO-1) and mesenchymal marker (N-Cadherin) in MCF10A-Ras and MCF10A-c-Myc. (*B* and *C*) p21^{CIP1} inhibits *Twist* promoter activity induced by Ras and c-Myc. All luciferase reporter assays were normalized to Renilla Luciferase and represent ± SEM ($n \geq 3$).