Supporting Information

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SI Experimental Procedures

Human Skin Tumor Samples. All human specimens were studied after approval by the University of Chicago Institutional Review Board. Formalin-fixed, paraffin-embedded tissue blocks were obtained from the archives in the tissue bank of the Department of Medicine, Section of Dermatology, University of Chicago. Non–sun-exposed nonlesional normal epidermis, actinic keratosis and squamous cell carcinoma tissues were used for immunohistochemical analysis of epithelial (E)-cadherin and p62 protein levels.

Cell Culture. WT, Atg5 KO mouse embryonic fibroblast (MEF) cells (obtained from Dr. Mizushima), Atg3 KO, Atg7 KO, Atg9 KO, Atg12 KO, p62 KO, control, and Atg14 cKO MEF cells, doxycycline-inducible Atg12 knockdown (KD) 4T1 cells, HaCaT (human keratinocytes, kindly provided by Nobert Fusenig), HEK-293T (human embryonic kidney cell), A431 (human squamous carcinoma cells), and A375 (human amelanotic melanoma cells) were maintained in a monolayer culture in 95% air/5% CO_2 (vol/vol) at 37 °C in DMEM supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin (Invitrogen), and 1% nonessential amino acids (Invitrogen). For the epithelial–mesenchymal transition experiment, cells were washed with PBS twice and incubated with DMEM supplemented with EGF (10 ng/mL, R&D) and TGF-β (10 ng/mL, R&D) for 48 h.

siRNA and Plasmid Transfection. MEF cells were transfected with negative control (NC) or siRNA (ON-TARGETplus SMARTpool; Dharmacon) targeting Twist1 or p62, using DharmaFECT 4 transfection reagent (Dharmacon) according to the manufacturer's instructions as described previously (1). HEK293T, and MEF cells (except for p62 KO MEF cells) were transfected with vector, Myc-Twist1, Flag-Twist1, HA-p62, HA-ubiquitin, p62, or Twist1 mutants, E-cad (108) WT-Luc, and E-cad (108) E-box Mut-Luc using X-tremeGENE 9 (Roche) as described previously (2–4). A431 and A375 cells were transfected using Amaxa Nucleofector according to the manufacturer's instructions as described previously (2, 3). Reconstitution of Atg7 and p62 and knockdown by shRNA used lentiviral vectors, with HEK293T cells used as packaging cells for the lentiviral system. pLKO.1 vectors and packaging mix (psPAX2 and pMG2) were transfected into HEK293T cells. Two days after transfection, the supernatants were collected and diluted with the same volume of fresh complete DMEM, and Polybrene was added to the infection medium at 8 μg/mL. The infection mixtures were added to cell cultures seeded on a six-well plate, incubated overnight, and then replaced with fresh complete DMEM containing 8 μg/mL puromycin. The puromycin-resistant cells were isolated and propagated for use in the experiments.

Plasmids. Myc-Twist1 pcDNA3.1 and Flag-Twist1 pcDNA3.1 were kindly provided by Tony Firuli. We cloned the Myc-Twist1 (HindIII/XbaI) from pcDNA3.1 vector to pMPB3 vector (modified piggyBac vector). HA-p62 pcDNA4 was obtained from Qing Zhong (University of California, Berkeley, Berkeley, CA) (Addgene plasmid 28027) (5). HA-Ubiquitin pcDNA3 was obtained from Edward T. H. Yeh (The University of Texas-Houston Health Science Center, Houston) (Addgene plasmid 18712) (6). PGL2 E-cad (108) WT-Luc and PGL2 E-cad (108) E-box Mut-Luc were obtained from Eric R. Fearon (University of Michigan Medical School, Ann Arbor, MI) (Addgene plasmids 19291 and 19290) (7). The transcription factor TCF/lymphoid enhancer factor luciferase reporter containing TCF binding sites TOPFLASH (TOP) and its negative control plasmid containing inactive TCF binding sites FOPFLASH (FOP) plasmids were from Tong-Chuan He. Myc-Flag-Rad23b-pCMV, Flag-Twist2, and pCMV6 vector were obtained from OriGene Technologies. pLKO.1 shp62 (human) was obtained from Sigma. pLKO.1 shp62 (mouse) and pLKO.1 shTwist1 (mouse) were obtained from Dharmacon. pENTER vector, pHAGE con, and pHAGE Atg7 (mouse) were obtained from Seungmin Hwang. pLenti CMV Dest vector was obtained from Addgene. pLKO.1 shAtg7 (human) was obtained from Dr. Kimmelman.

DNA Constructs. Twist1 deletion mutants (Myc-Twist1-WR) were generated using the following primers: N-terminal sense 5′-TACGACTCACTATAGGGAGACCC-3′ and C-terminal sense 5′-GTTCTAGACTAGCAGCTTGCCATCTTGGAGTCC-3′ from Myc-Twist1 pcDNA3.1. They were then subcloned (HindIII/XbaI) into pMPB3 vector. p62 deletion mutants (primers A–F) were generated using the primers C-terminal sense 5′-GGAATTCTATGGTGCACCCCAATGTGATCTG-3′ and N-terminal primer A 5′-GGAATTCTATGGTGCACCCCAATGTGATCTG-3′, primer B 5′-GGAATTCTATGGGTCCACCAGGAAACTGGA-3′ and primer C 5′-GGAATTCTATGGAGTCGGATAACTGTTCAGG-3′; N-terminal sense 5′-GGAATTCTATGGCGTCGCTCACCGTGAA-3′ and C-terminal primer D 5′-GAGTGCGGCCGCACGGGTCCACTTCTTTTGAAG-3′, primer E 5′-GAGTGCGGCCGCAGCTTGGCCCTTCGGATTCT-3′ and primer F 5′-GAGTGCGGCCGCGGCTTCTTTTCCCTCCGTGCT-3′ and subcloned (EcoR1/Not1) back into the HA-pcDNA4 vector. HA-p62 WT was generated using the primers N-terminal sense 5′-GTGGTACCGCTATGGCGTCGCTCACCGTGAA-3′ and C-terminal sense 5′-GAGGCGCGCCCTAAGCGTAATCTGGAACATCGT-3′ from HA-p62 pcDNA4 and subcloned (Kpn1/Asc1) into pMPB3 vector and pENTER vector or Myc-Twist1-pMPB3. Establishment of a stable transfected cell line using the piggyBac transposon system (pMPB3) was performed as described previously (8). pENTER p62 was recombined into pLenti CMV Dest vector using Gateway LR Clonase Enzyme Mix kit following the manufacturer's instructions. All constructs were confirmed by sequencing.

Site-Directed Mutagenesis. Mutations of lysine to arginine (K→R) at positions 33, 38, 73, 76, 77, 137, 146, 149, 154, and 175 of wild-type Myc-Twist1 pMPB3 plasmid were carried out using the QuikChange XL Site-Directed Mutagenesis kit following the manufacturer's instructions (Stratagene). The amino acid changes were introduced into the plasmid by using the following primers: K33R 5′-CCGGCGAGCGGCAGGCGCGGG-3′ and K33R antisense 5′-CCCGCGCCTGCCGCTCGCCGG-3′; K38R 5′-GCGGGGCTCGCAGGAGACGCAGCAG-3′ and K38R antisense 5′-CTGCTGCGTCTCCTGCGAGCCCCGC-3′; K73R 5′-CCGGCCCAGGGCAGGCGCGGC-3′ and K73R antisense 5′-GCCGCGCCTGCCCTGGGCCGG-3′; K76R 5′-GGCAAGCGCGGCAGGAAATCTGCGGGC-3′ and K76R antisense 5′-GCCCGCAGATTTCCTGCCGCGCTTGCC-3′; K77R 5′-AGCGCGGCAAGAGATCTGCGGGCGG-3′ and K77R antisense 5′-CCGCCCGCAGATCTCTTGCCGCGCT-3′; K137R 5′-CCGCCCTGCGCAGGATCATCCCCAC-3′ and K137R antisense 5′-GTGGGGATGATCCTGCGCAGGGCGG-3′; K146R 5′-CACGCTGCCCTCGGACAGGCTGAGCAAG-3′ and K146R antisense 5′-CTTGCTCAGCCTGTCCGAGGGCAGCGTG-3′;

K149R 5′-CTCGGACAAGCTGAGCAGGATTCAGACCCTC-AAAC-3′ and K149R antisense 5′-GTTTGAGGGTCTGAAT-CCTGCTCAGCTTGTCCGAG-3′; K154R 5′-AGCAAGATT-CAGACCCTCAGACTGGCGGCC-3′ and K154R antisense 5′-GGCCGCCAGTCTGAGGGTCTGAATCTTGCT-3′; K175R 5′-CGAGCTGGACTCCAGGATGGCAAGCTGCA-3′ and k175R antisense 5′-TGCAGCTTGCCATCCTGGAGTCCAGCTCG-3′; all mutants were confirmed by sequencing.

Immunofluorescence. Immunofluorescence was done as described previously (1, 9). Briefly, the cells attached to the coverslips were washed three times with PBS and fixed with 4% paraformaldehyde in PBS solution for 25 min. The cells were then permeabilized with cold 0.5% Triton X-100 in PBS for 20 min at 4 °C and washed with cold PBS. PBS supplemented with 5% normal goat serum (Invitrogen) was used as a blocking solution (30 min, 37 °C). After removal of the blocking solution, the cells were incubated at 4 °C overnight in primary rabbit anti–E-cadherin (1:200; Cell Signaling Biotechnology), β-catenin (1:200 BD Biosciences), HA (1:200 Cell Signaling Biotechnology), Myc (1:200 Cell Signaling Biotechnology), LC3 (1:200 Cell Signaling Biotechnology), Twist1 (1:200 Abcam), and p62 (1:200 Progene Biotechnik) monoclonal antibody. After washing with PBS, the cells were incubated at 37 °C for 1 h with Alexa Fluor 488 F (ab′) two fragments of goat anti-mouse IgG antibodies and Alexa Fluor 568 of goat anti-rabbit IgG antibodies. The cells were then fixed in Prolong Gold Antifade with DAPI (Invitrogen) to visualize the cell nuclei, and observed under a fluorescence microscope (Olympus IX71) with a peak excitation wavelength of 340 nm.

Immunoprecipitation and GST-Pulldown Assay. Immunoprecipitation was performed as described previously by using anti–HA-Tag and anti–Myc-Tag antibodies (Cell Signaling Technology), an anti-p62 antibody (Sigma), and anti-Twist1 antibody (Abcam) (1). For ubiquitin analysis, cells were treated with the proteasome inhibitor MG132 (10 μM) for 6 h before collection and *N*-ethylmaleimide (NEM, 5 mM) was added to all of the buffers. GST-P62 recombinant protein was obtained from Enzo Life Sciences, and GST (control, Con) recombinant protein was obtained from Sigma. The GST-pulldown assay was performed using Pierce GST Protein Interaction Pull-Down kit following the manufacturer's instructions. WT and Atg5 KO cells were treated with MG132 (10 μM) for 6 h before collection and NEM (5 mM) was added to all of the buffers.

Western Blotting. Western blotting was performed as described previously (10). Antibodies used were as follows: p53, Rad23b, GAPDH, and HA-Tag (mouse) (Santa Cruz), E-cadherin, LC3I/II, HA-Tag (rabbit), Myc-Tag (mouse), Myc-Tag (rabbit), Snail, Slug, Atg7, Flag-Tag, N-cadherin, ZEB1 (Cell Signaling Technology), p62 (Progene Biotechnik), and Twist1 (Abcam). Protein levels were quantified using ImageJ software and normalized so that the protein level at $t = 0$ was 100%. The $\log_{10}$ of the percentage of the protein level was plotted versus time, and the $t_{1/2}$ was calculated from the $\log_{10}$ of 50%.

Luciferase Reporter Assays. PGL2 E-cad WT-Luc, PGL2 E-cad E-box Mut-Luc (obtained from Eric R. Fearon, Addgene plasmids 19291 and 19290), and 0.025 μg of pRL-TK (Promega, used as a transfection efficiency control) were transfected with X-treme 9 Transfection Reagent (Roche) or Amaxa Nucleofector according to the manufacturer's protocols. The luciferase activity was measured as described previously (2).

Immunohistochemical Analysis. E-cadherin and Ki67 levels were determined using immunohistochemical analysis by the Immunohistochemistry core facility (University of Chicago). E-cadherin (Cell Signaling) and Ki67 (Cell Signaling) antibodies were used, with the protein levels visualized with the diaminobenzidine (DAB) method (brown color). To exclude the contribution of endogenous brown pigmentation due to melanin, we also performed hematoxylin and eosin (H&E) staining.

Real-Time PCR. Quantitative real-time PCR assays were performed using ABI7300 (Applied Biosystems) in 96-well plates with the SYBR Green PCR Master Mix (Applied Biosystems) as in our previous studies (11). The threshold cycle number (CT) for each sample was determined in triplicate. The CT for values for E-cadherin and Twist1 were normalized against GAPDH as described previously (2, 3, 11). Amplification primers were 5′-CAGGTCTCCTCATGGCTTTGC-3′ (forward), 5′-CTTCCG-AAAAGAAGGCTGTCC-3′ (reverse) for the mouse E-cadherin gene; 5′-AGGTCGGTGTGAACGGATTTG-3′ (forward) and 5′-TGTAGACCATGTAGTTGAGGTCA-3′ (reverse) for GAPDH; 5′-CAGAGAAGCCCATGGACAG-3′ (forward) and 5′-AGCT-GCCTTGTACCCACATC-3′ (reverse) for the human p62 gene; 5′-CGGGAGTCCGCAGTCTTA-3′ (forward) and 5′-GCTTG-AGGGTCTGAATCTTG-3′ (reverse) for the human Twist1 gene; and 5′-GCAGGACGTGTCCAGCTC-3′ (forward) and 5′-CTG-GCTCTTCCTCGCTGTT-3′ (reverse) for the mouse Twist1 gene. The CT for each sample was determined in triplicate. The CT values for E-cadherin and Twist1 were normalized against GAPDH as described previously (2, 3, 11).

Animal Treatments. All animal procedures have been approved by the University of Chicago Institutional Animal Care and Use Committee. K14-Cre mice were obtained from The Jackson Laboratories and $Atg7^{flox/flox}$ mice were a gift from Masaaki Komatsu (Tokyo Metropolitan Institute of Medical Science, Tokyo).

Analysis of Tumor Growth and Metastasis in Nude Mice. All animal procedures have been approved by the University of Chicago Institutional Animal Care and Use Committee. Athymic nude mice were obtained from Harlan Sprague–Dawley. A total of 1×10^6 A431-Con, A431-p62, A431-Twist1 and A431-Twist1-p62, and A375-Con and A375-p62 cells were injected s.c. into the right flank of 6-wk-old female nude mice. Tumors were monitored weekly and tumor volume (TV) was calculated using the following formula: TV (mm^3) = $d^2 \times D/2$, where d and D are the shortest and the longest diameters, respectively. For metastasis analysis, cells were injected via tail vein as described previously (12). Lungs and other tissues with metastasis were harvested for metastasis analysis. Tumors were fixed in formalin for histological analysis or immunohistochemical analysis for E-cadherin and Ki67-positive cells (Immunohistochemistry core facility).

Migration and Invasion Assay. For 2D migration assays, cells were plated onto Radius 24-Well Cell Migration Assay plates according to the manufacturer's instructions (Cell Biolabs). To analyze the invasion activity of WT, Atg3 KO, Atg5 KO, Atg9 KO, and Atg12 KO MEF cells, the upper surface of the Transwell filters (8-μm pore size Millipore MultiScreen plates) was coated with Matrigel as described previously (13). For 2D migration assays, cells were plated into a Radius 24-Well Cell Migration Assay plate (Cell Biolabs). Each well contains a circular 680-μm diameter "gel spot" to which cells do not attach. Before the experiment, cells were confluent and quiescent. At the start of the experiment, the gel spot was removed within 5 min, and cells then populated the circular void space. Digital images of the gap closure were taken with an inverted microscope (Olympus IX71) with a 10× phase lens. Images were analyzed with CellProfiler cell image analysis software. To analyze the invasion activity of WT, Atg3 KO, Atg5 KO, Atg9 KO, and Atg12 KO MEF cells, the upper surface of the Transwell filters

(8-μm pore size Millipore MultiScreen-MIC plates) was coated with Matrigel. Cells suspended in 60 μL serum-free media (5×10^4) were then added to the chamber, and the chamber was placed in a 96-well plate containing complete medium. After a 24-h incubation at 37 °C, the filters were gently taken out and Matrigel on the upper surface of the filters was removed by cotton swabs. Cells on the undersides of Transwell filters were fixed with 4% paraformaldehyde for 30 min, stained with 0.1% crystal violet for 10 min, and then photographed. The extent of cell migration was quantified as the number of migrated cells per field as described (13).

In Vitro Cell Proliferation Assay. Cell proliferation of MEF cells was analyzed using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (MTS) (Promega) according to the manufacturer's instructions as in our recent studies (4).

Statistical Analyses. Statistical analyses were performed using Prism 5 (GraphPad). Data were expressed as the mean of at least three independent experiments and analyzed by Student *t* test. Error bars indicate the SDs of the means. $P < 0.05$ was considered statistically significant.

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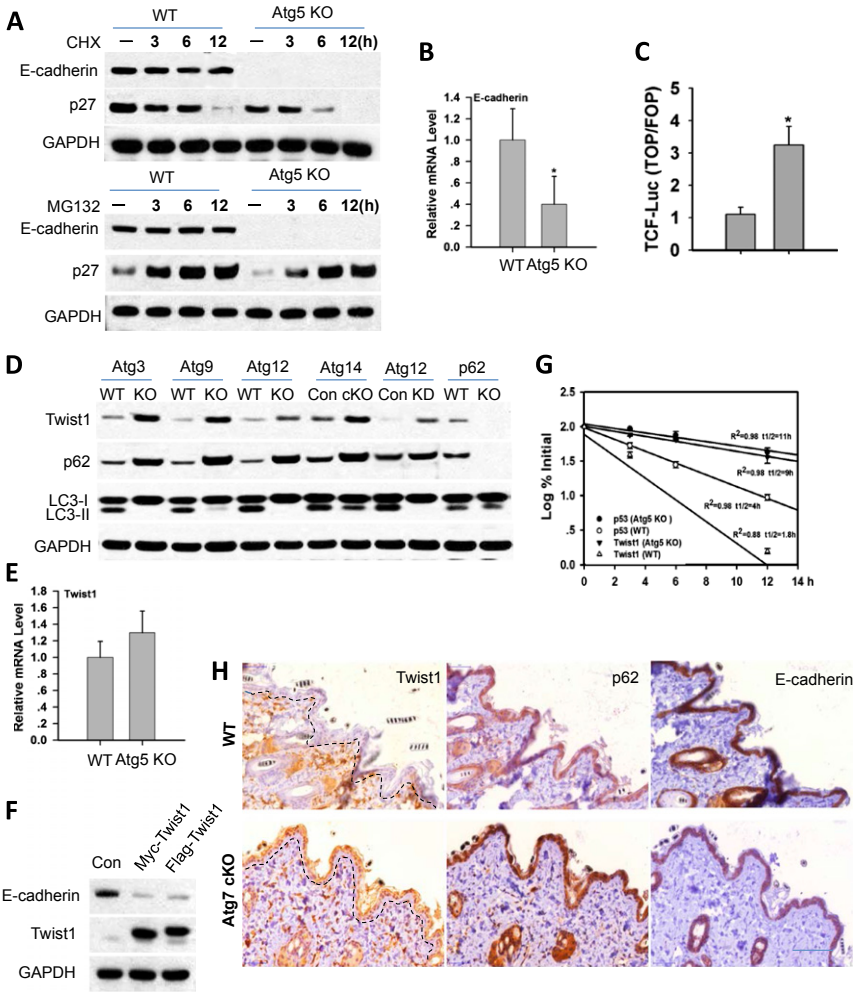

Fig. S1. (Related to Fig. 2.) Autophagy regulates Twist1 protein degradation involving both autophagosome and proteasome. (*A*) Immunoblot analysis of E-cadherin and GAPDH in WT and Atg5 KO MEF cells treated with cycloheximide (CHX, 100 μg/mL) or MG132 (10 μM) for the indicated times as in Fig. 2 *E* and *F*. (*B*) Real-time RT-PCR analysis of E-cadherin mRNA levels in WT and Atg5 KO MEF cells. (*C*) Luciferase reporter assay of the TCF/lymphoid enhancer factor luciferase reporter (TOP) and its negative control (FOP) in WT and Atg5 KO MEF cells. The results were obtained from three independent experiments [mean ± SD (error bars), $n = 3$; $^{*}P < 0.05$; $^{**}P < 0.01$, compared with the Con group). (*D*) Immunoblot analysis of Twist1, p62, LC3-I/II, and GAPDH in WT and Atg3, Atg9, Atg12, and p62 KO MEF cells; Con and Atg14 cKO MEF cells; and Con and Dox-inducible Atg12 knockdown (KD) 4T1 cells. (*E*) Real-time RT-PCR analysis of Twist1 in WT and Atg5 KO MEF cells. (*F*) Immunoblot analysis of E-cadherin, Twist1, and GAPDH in WT MEF cells transfected with vector control (Con), Flag-Twist1, or Myc-Twist1. (*G*) Quantification of the Twist1 levels in Fig. 2*E* using ImageJ. H, Representative immunohistochemical analysis of Twist1, p62 and E-cadherin protein (brown) in the skin of WT and Atg7 cKO mice. Scale bar, 50 μm. The results were obtained from three independent experiments (mean ± SD, $n = 3$; $^{*}P < 0.05$ compared with the WT MEF; $^{\#}P < 0.05$ compared with the WT E-cadherin promoter).

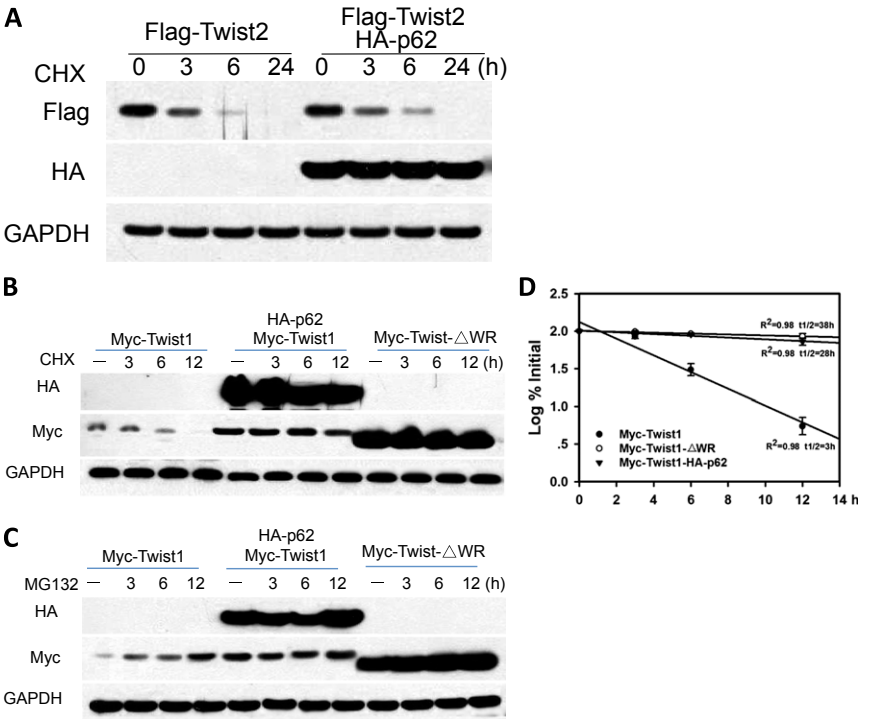

Fig. S2. (Related to Fig. 4.) p62 binds to Twist1 through its ubiquitin-associated domain. (*A*) Immunoblot analysis of HA, Flag, and GAPDH in 293T cells transfected with Flag-Twist2 alone or in combination with HA-p62 for 48 h, and then treated with CHX (100 μg/mL) for the indicated times. The results were obtained from three independent experiments. (*B* and *C*) Immunoblot analysis of HA and Myc in 293T cells transfected with Myc-Twist1, a combination of Myc-Twist1 and HA-p62, or Myc-Twist1-ΔWR for 48 h, and then treated with CHX (100 μg/mL) (*B*) or MG132 (10 μM) (*C*) for the indicated times. (*D*) Quantification of *B* using ImageJ.

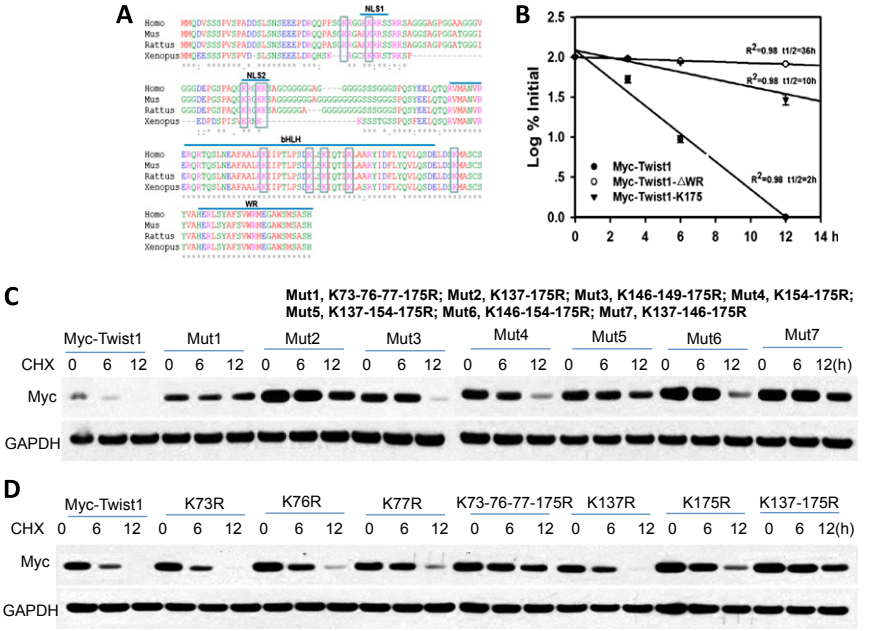

Fig. S3. (Related to Fig. 5.) Twist1 lysine 175 is critical for Twist1 ubiquitination, degradation, and binding with p62. (*A*) Schematic for comparison of amino acid sequences among human (*Homo*), mouse (*Mus*), rat (*Rattus*), and *Xenopus*. Lysine sites are marked by rectangles. "*" indicates the identical residues in all sequences in the alignment; ":" indicates conserved substitutions; "." indicates semiconserved substitutions; and "-" indicates gaps in the alignment. (*B*) Quantification of Fig. 5*B* using ImageJ. (*C*) Immunoblot analysis of Myc and GAPDH in 293T cells transfected with Myc-Twist1 (K→R) mutation constructs (Mut1, K73-76-77-175R; Mut2, K137-175R; Mut3, K146-149-175R; Mut4, K154-175R; Mut5, K137-154-175R; Mut6, K146-154-175R; and Mut7, K137-146-175R), and then treated with CHX (100 μg/mL) for the indicated times. K73-76-77-175R, Twist1 mutant with K→R mutations for all the K73, K76, K77, and K175 sites. We used a similar format to denote other Twist1 mutants with K-R mutations for more than one site. (*D*) Immunoblot analysis of Myc and GAPDH in 293T cells transfected with Myc-Twist1 or constructs with mutations of K73R, K76R, K77R, K73-76-77-175R, K137R, K175R, or K137-175R for 48 h, and then treated with CHX (100 μg/mL) for the indicated times. The results were obtained from three independent experiments.

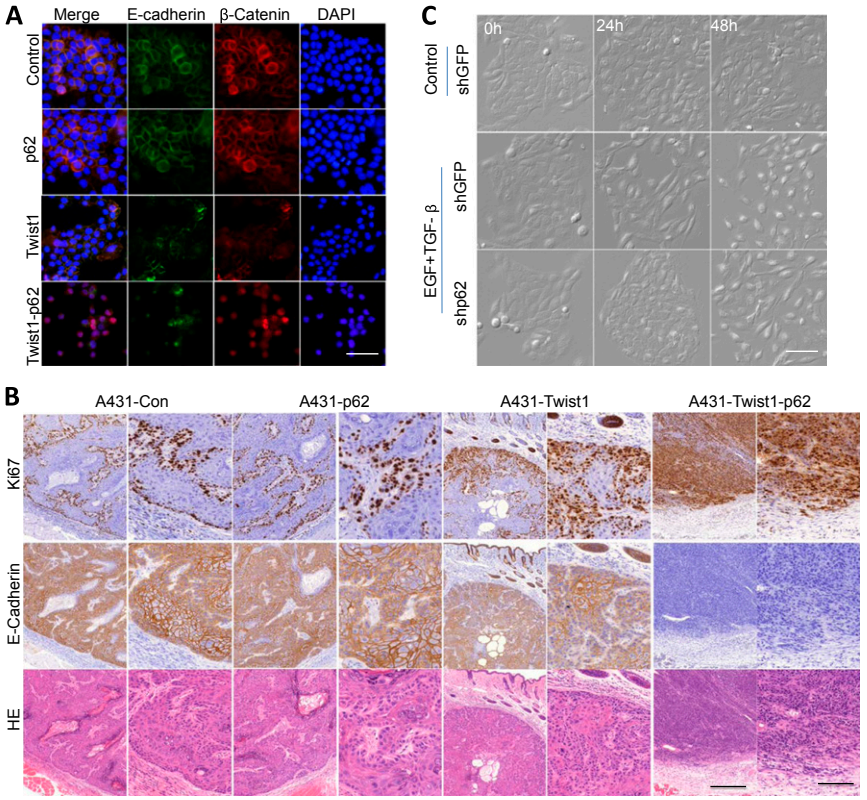

Fig. S4. (Related to Fig. 6.) p62 promotes tumor cell growth and metastasis in vivo through regulating Twist1. (*A*) Immunofluorescence assay of β-catenin in WT and Atg5 KO MEF cells. (Scale bar, 50 μm.) (*B*) Histological H&E staining and immunohistochemical analysis of E-cadherin and Ki67 (brown) in A431-Con, A431-p62, A431-Twist1, and A431-Twist1-p62 tumors. (Scale bar, 200 μm and 50 μm for *Left* and *Right*, respectively.) (*C*) Morphology of HaCaT human epithelial cells transfected with vector or shRNA target p62 (shp62), following treatment with control vehicle or the combination of EGF (10 ng/mL) and TGF-β (10 ng/mL) for 48 h. (Scale bar, 50 μm.)