

Supplemental Information

A Switch in the Expression of Embryonic EMT-Inducers Drives the Development of Malignant Melanoma

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Inventory of Supplemental Information

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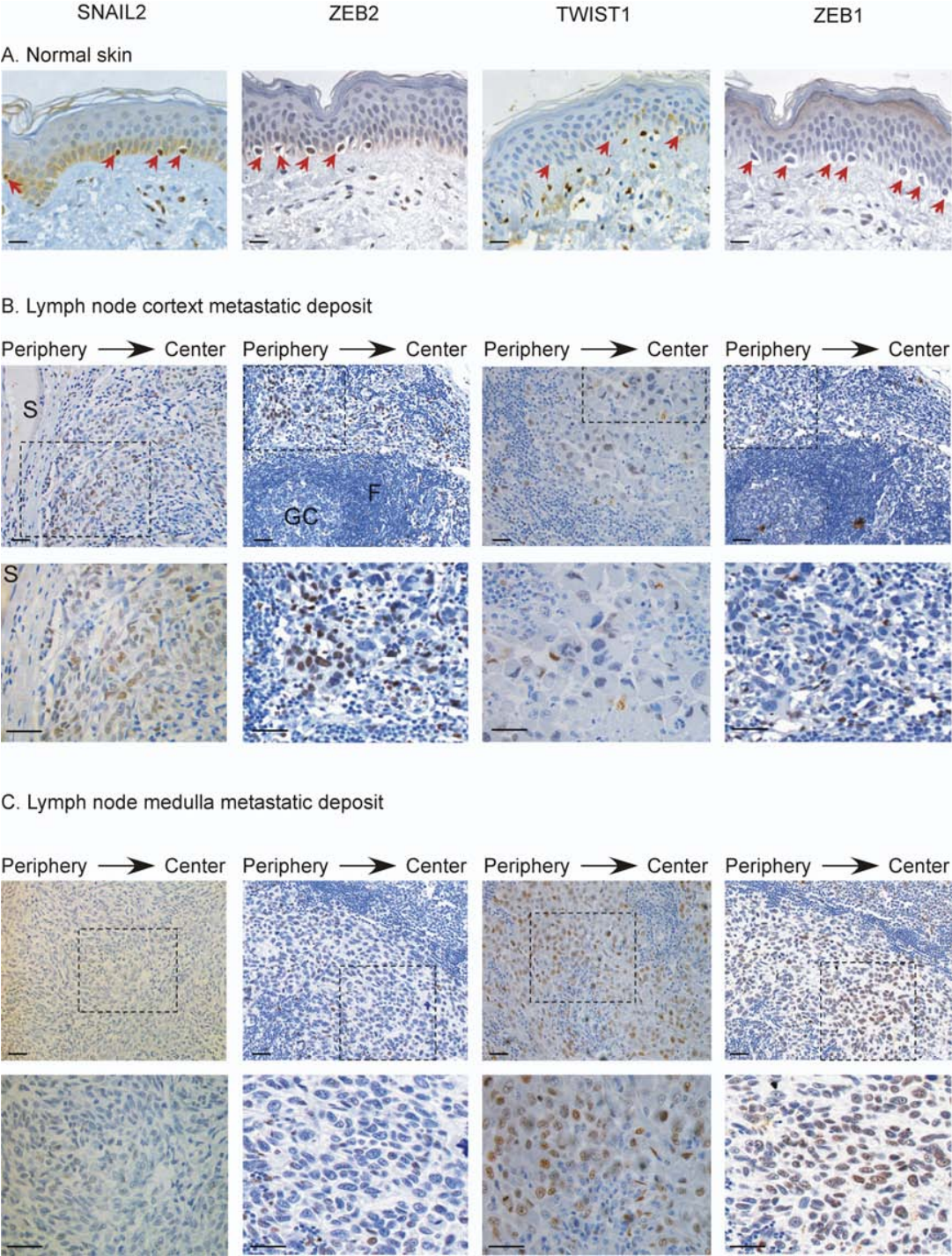
Figure S5, related to Figure 7

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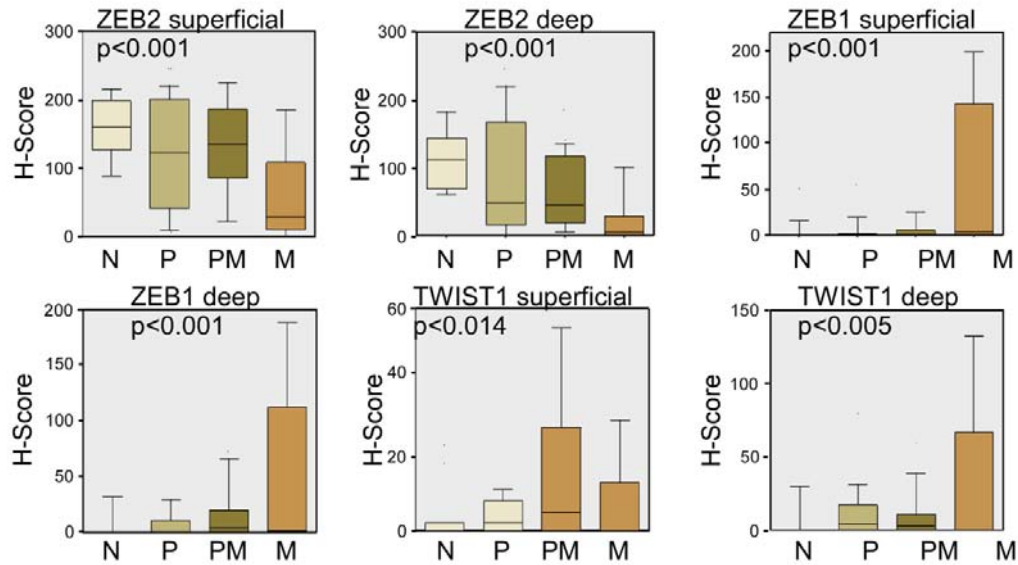
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SUPPLEMENTAL DATA



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E

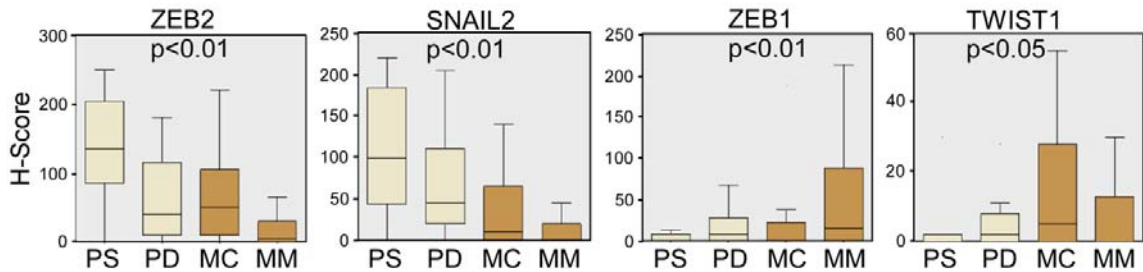


Figure S1, related to Figure 1.

Opposing trends of EMT-TF expression in melanocytic lesions.

(A) IHC analysis of EMT-TF expression in the skin.

Melanocytes embedded in the basal layer of the epidermis are indicated by red arrows. Note that melanocytes are positive for ZEB2 and SNAIL2, but negative for ZEB1 and TWIST1. Keratinocytes do not express EMT-TFs. Scale bar = 40 μ m.

(B, C) Examples of IHC analyses of EMT-TF expression in lymph nodal metastases in patients with advanced MM. Cortical (B) and medullar (C) regions of affected lymph nodes are shown. Images within boxed areas represent predominantly tumor cells and are shown at 40x magnification in adjacent photomicrographs. F, follicles; GC, germinal center; S, subcapsular sinus. Scale bar = 40 μ m.

(D, E) Box and whisker plots of EMT-TF IHC staining (H-Score) for MM independent progression series (D) or matched primary tumor/metastatic lesions series (E). Staining was measured at superficial and deep sites for each melanocytic lesion. Error bars represent the 10th and 90th percentiles. p values represent the significance of a trend test using Jonckheere-Terpstra trend test for the independent series (D) and the Page L trend test for the matched series (E). N, nevi; P, primary melanoma; PM, metastatic primary melanoma; M, metastases. PS, superficial areas in primary melanomas; PD, deep areas in primary melanomas; MC, cortical metastatic deposits in lymph nodes; MM, metastatic deposits in medullary areas. Note opposing gradients of ZEB2 and SNAIL2 versus ZEB1 and TWIST1 expression within the lesions.

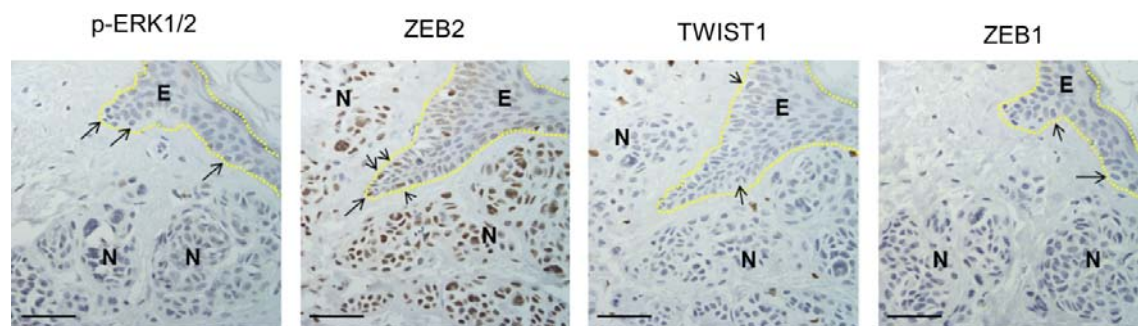
Table S1, related to Figure 1.

Univariate and multivariate Cox proportional hazard analysis for clinical prognostic factors, age, gender, tumor Breslow depth and ulceration with EMT-TF and phospho-ERK immunostaining.

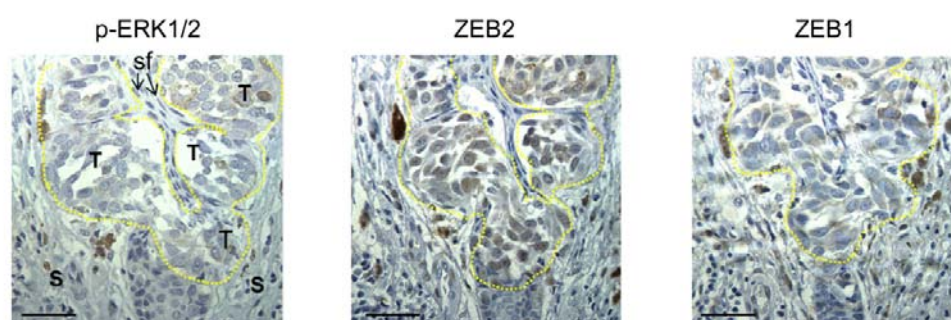
			Univariate Cox Proportional hazard analysis of EMT-TF and p-ERK IHC with metastasis free survival			Multivariate Cox Proportional hazard analysis of EMT-TF or p-ERK IHC and clinical prognostic factors with metastasis free survival		
Variable	Evaluation	N#	HR (95% confidence interval)		P value	HR (95% confidence interval)		P value
Age	<60	46	1			1		
	>60	52	1.64	0.91-2.97	0.01*	0.781	0.40-1.51	0.46
Gender	Female	57	1			1		
	Male	41	1.99	1.12-3.55	0.02*	1.98	1.09-3.62	0.081
Breslow	<1.5 mm	29	1			1		
	>1.5 mm < 4.0mm	43	2.67	1.16-6.17	0.02*	3.09	1.18-8.08	0.022*
	>4.0mm	26	5.10	2.14-12.17	< 0.001***	5.35	1.89-15.12	0.002**
Ulceration	No ulceration	56	1			1		
	ulceration	39	1.81	1.01-3.24	0.049*	1.178	0.58-2.374	0.65
ZEB1 Superficial	H Score (100)	98	6.22	2.19-17.71	<0.001***	5.413	1.74-16.86	0.004**
ZEB2 Superficial	H Score (100)	98	0.89	0.61-1.30	0.557	1.061	0.69-1.63	0.79
TWIST1 Superficial	H Score (100)	92	4.04	1.23-13.29	0.022*	6.452	1.94-21.45	0.002**
p-ERK1/2 Superficial	H Score (100)	63	1.83	1.052-3.19	0.032*	1.40	0.74-2.657	0.302
ZEB1 Deep	H Score (100)	98	4.16	2.02-8.54	<0.001***	3.42	1.53-7.59	0.003**
ZEB2 Deep	H Score (100)	98	0.931	0.35-0.93	0.025*	0.75	0.45-1.25	0.27
TWIST1 Deep	H Score (100)	92	1.73	0.497-6.04	0.389	6.022	1.58-22.94	0.008**
p-ERK1/2 Deep	H Score(100)	63	1.98	1.28-3.07	0.002**	1.72	1.04-2.83	0.034*

Cases were excluded where time-to-event was zero (i.e. 4 cases with pre-existing metastases) P * <0.05 **<0.01 *** < 0.001

A. Expression in nevi



B. Expression in cortical area



C. Expression in medullary area

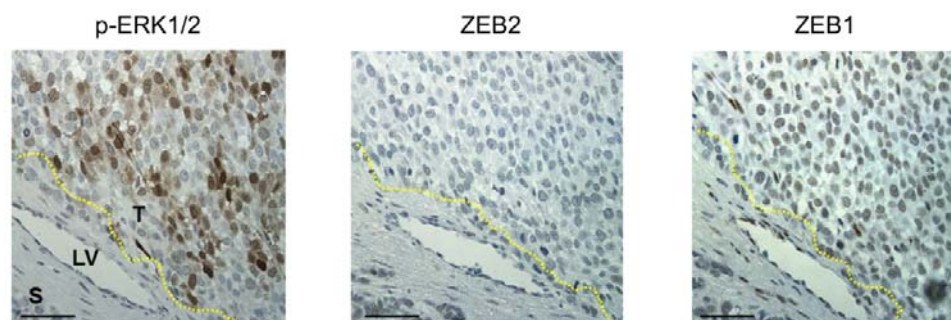
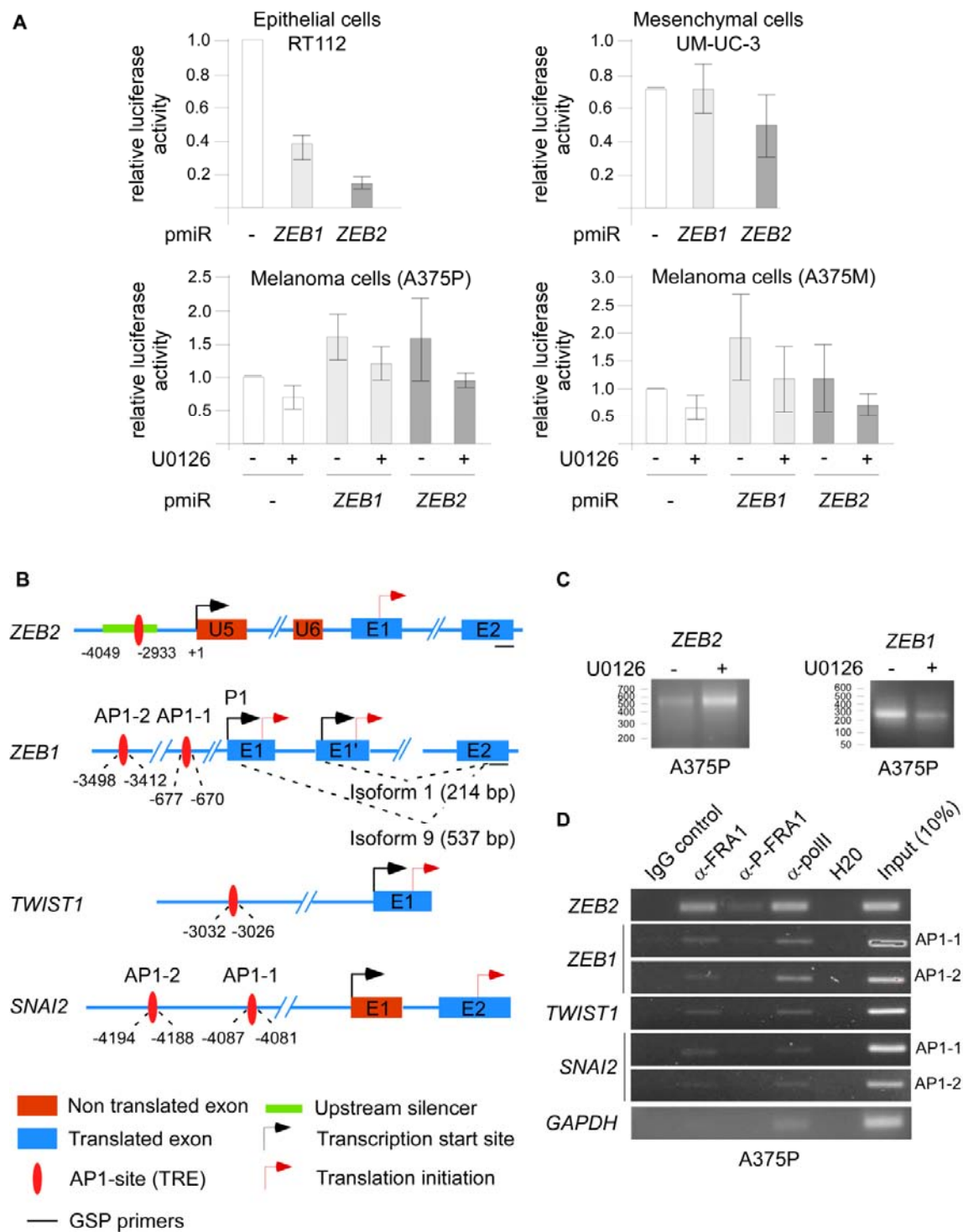


Figure S2, related to Figure 3.

EMT-TF switch correlates with ERK activity. Examples of IHC analyses of phospho-ERK (p-ERK1/2) and EMT-TF expression in a nevus (A) and lymph nodal metastasis (B, C). E, epidermis; N, nevus, melanocytes are indicated by arrows. T, tumor; S, stroma; sf, stromal fibroblasts; LV, lymphatic vessel. The micrographs demonstrate a correlation between EMT-TF reprogramming and phospho-ERK immunopositivity. Scale bar = 40 μ m.



(A) Activity of the firefly luciferase reporters linked to the 3'UTRs of *ZEB1* or *ZEB2* was measured after transient transfection in epithelial (RT112), mesenchymal carcinoma (UM-UC-3) or MM (A375P and A375M) cell lines. U0126 was added to the transfected cells as indicated. The reporter activity was normalized to that of the pMIR-REPORT vector containing no UTRs (-). Data represent mean \pm SD of three independent experiments.

(B) A scheme depicts localization of AP-1 binding sites relative to the *ZEB2*, *ZEB1*, *TWIST1* and *SNAI2* gene structures.

(C) Results of 5'RACE experiments mapping *ZEB2* and *ZEB1* transcription start sites. Note the transcriptional activation of *ZEB2* and repression of *ZEB1* by U0126 in A375P cells.

(D) ChIP using anti-FRA1 and anti-phospho-FRA1 (P-FRA1) antibodies revealed FRA1 interactions with DNA fragments containing AP-1 binding sites upstream of transcription start sites within *ZEB2*, *ZEB1*, *TWIST1* and *SNAI2* genes. ChIP with an anti-PolIII antibody and negative IgG was used as positive and negative controls. Input shows amplification of DNA that was sheared but not subjected to immunoprecipitation.

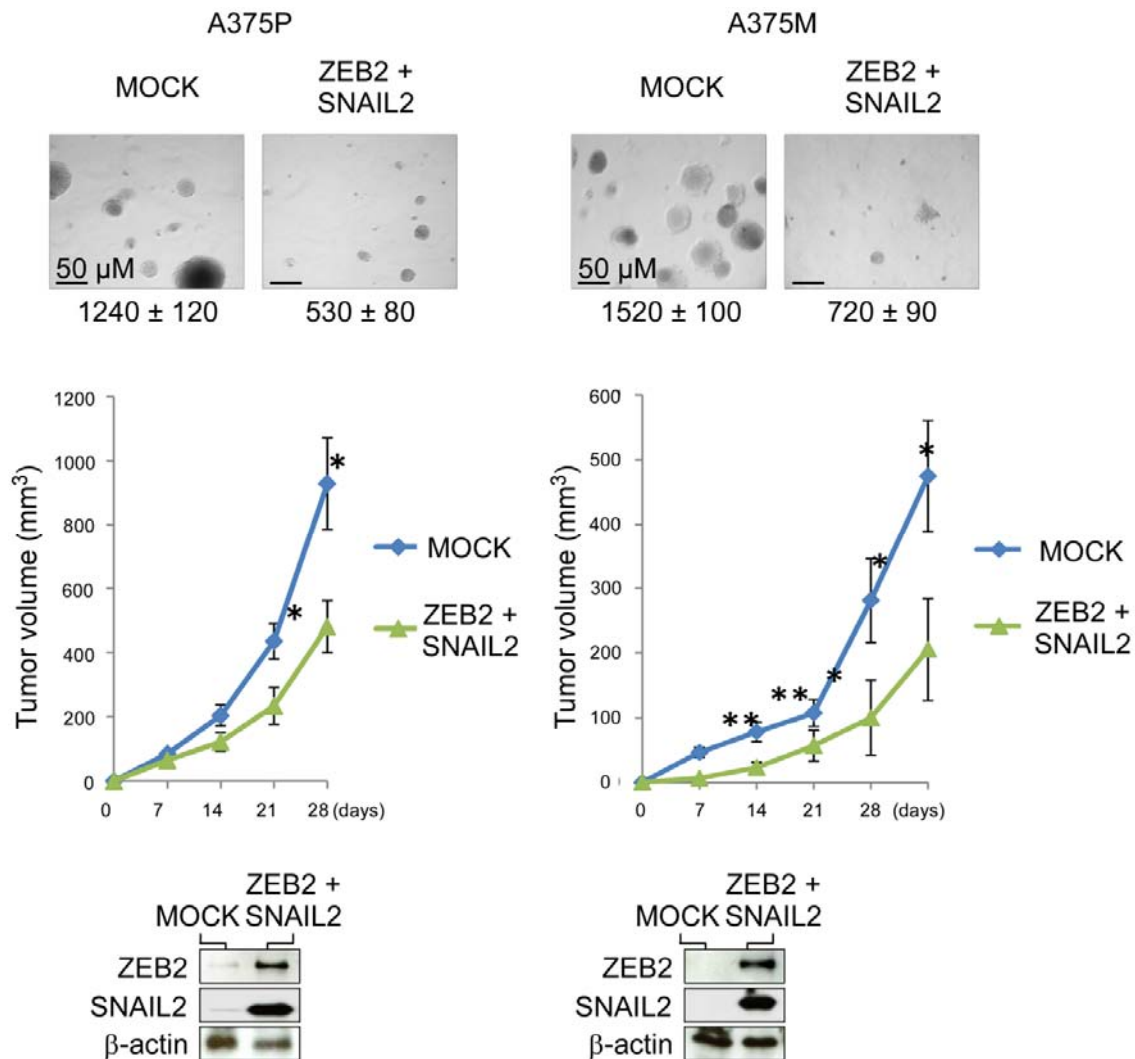
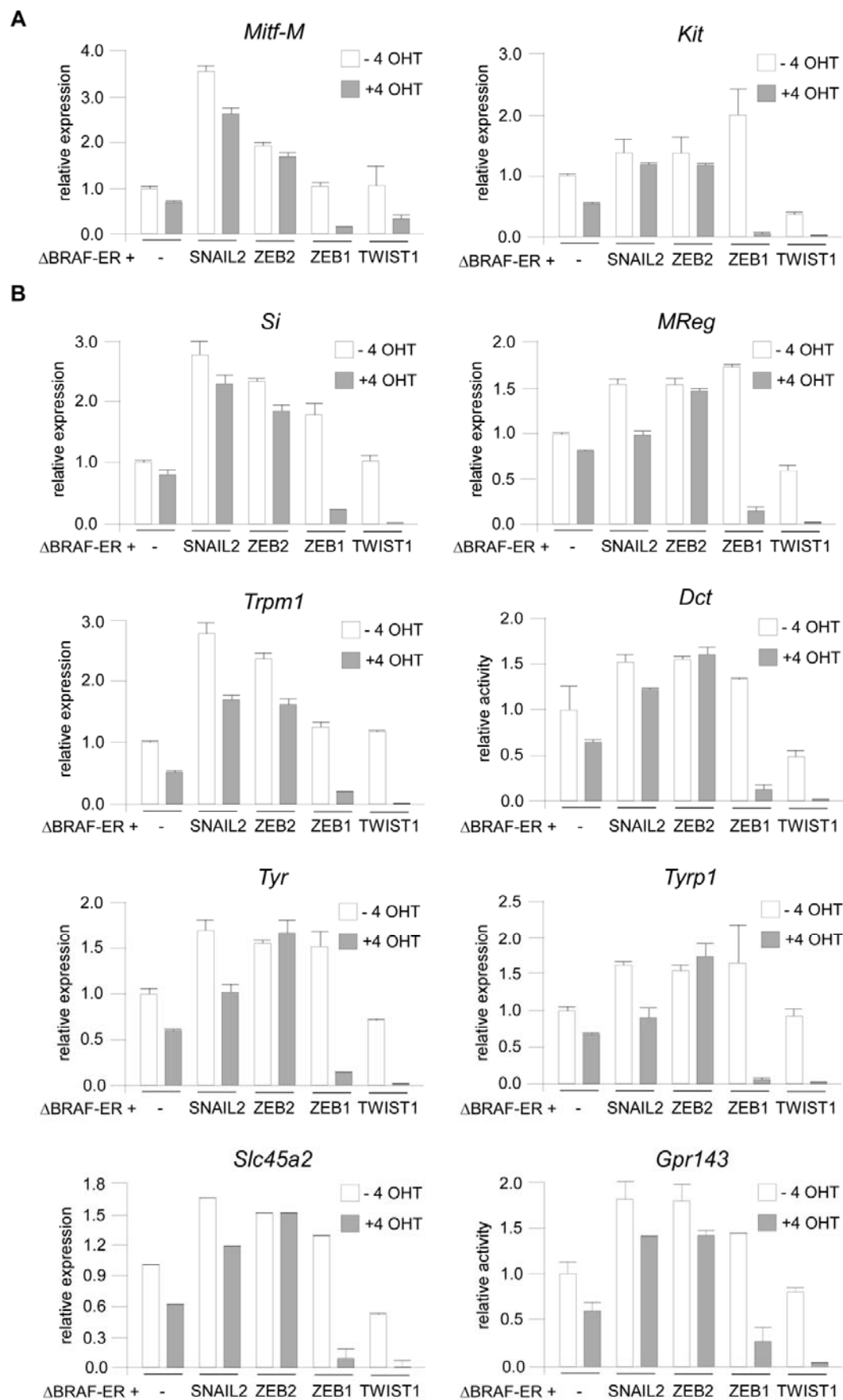


Figure S4, related to Figure 6.

EMT-TF reprogramming impacts on tumorigenicity of melanoma cells.

ZEB2 and SNAIL2 were ectopically expressed in A375P and A375M melanoma cell lines. Tumorigenicity was examined using a soft agar colony assay (top panels) or by xenografting cells in immunocompromised mice. The graphs illustrate the mean tumor size in each group of mice (n=5). Error bars represent standard error of the mean. Significance was analyzed using one tail t test. * p<0.05; ** p<0.01. Note that simultaneous expression of exogenous ZEB2 and SNAIL2 reduces colony formation in soft agar and alleviates tumor growth in xenografted mice.



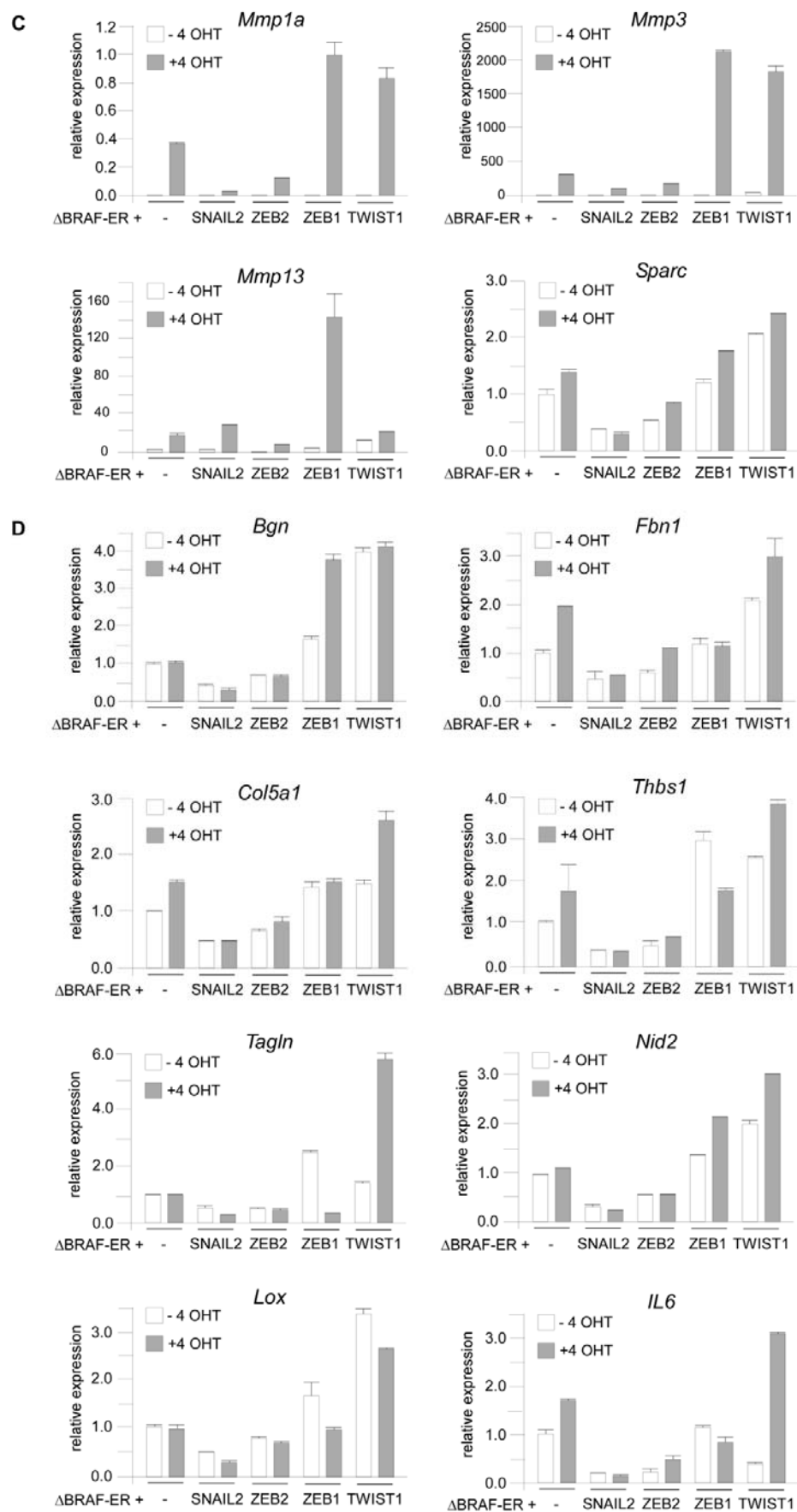


Figure S5, related to Figure 7.

Validation of the array data.

Expression of indicated genes from the following clusters was validated by qPCR.

(A), differentiation; (B), target genes of MITF; (C), invasion; (D), TGF- β pathway.

Data show mean \pm SD of triplicate experiments.

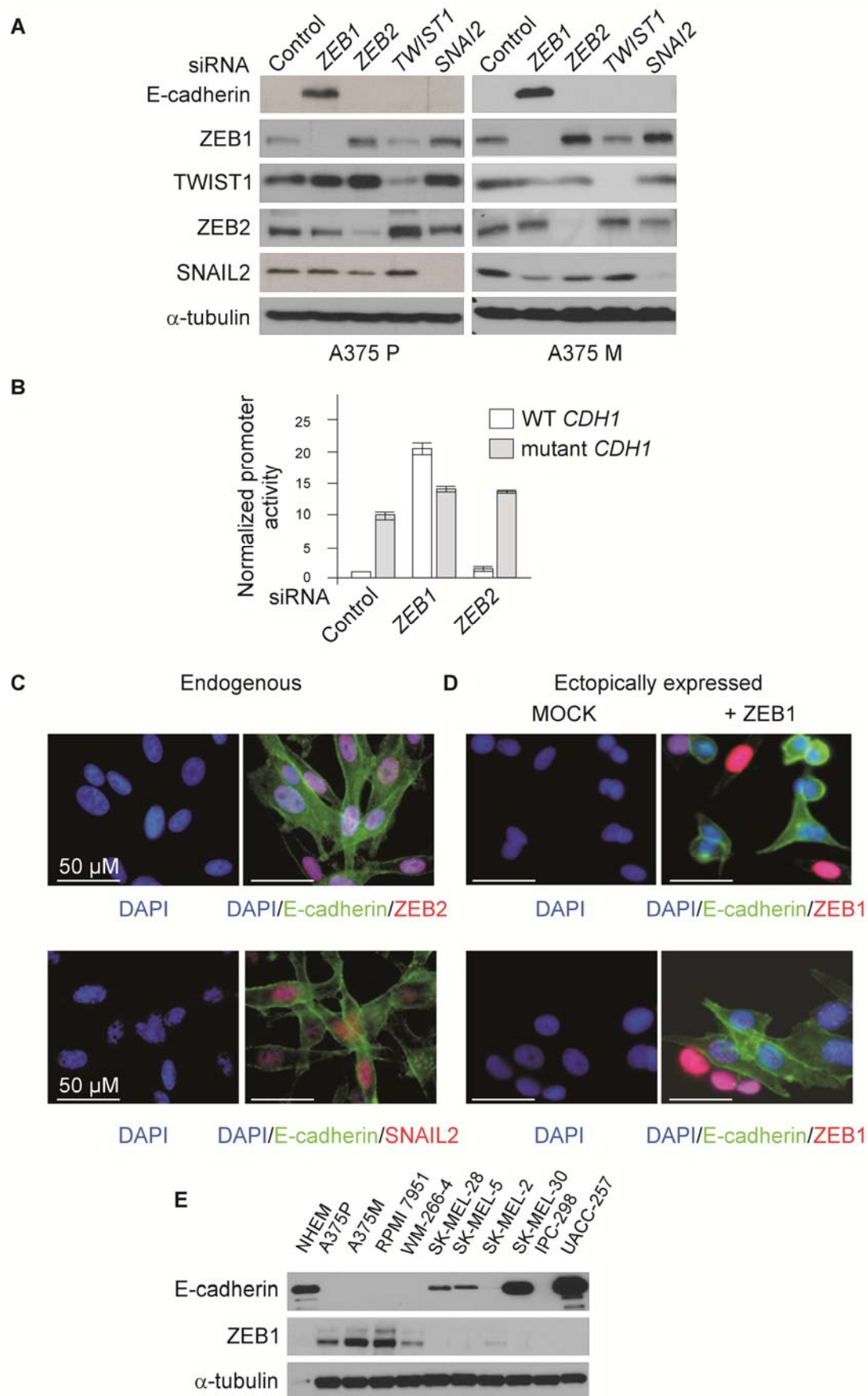


Figure S6, related to Figure 8.

ZEB1 represses *CDH1* expression in melanoma cells.

(A) Analysis of the effect of EMT-TF depletion on the expression of E-cadherin and other EMT-TFs in A375P or A375M cells as assessed by immunoblotting.

(B) Impact of ZEB1 or ZEB2 depletion on *CDH1* promoter activity. Luciferase reporters containing either wild type or mutant E boxes (Bolos et al., 2003) were transiently expressed in A375M cells. Luciferase activity was determined in three independent experiments performed in duplicate with similar results. Results are mean \pm SD of a representative experiment.

(C) E-cadherin is co-expressed with ZEB2 and SNAIL2 in UACC-257 cells as demonstrated by immunofluorescent microscopy.

(D) Ectopic expression of ZEB1 down-regulates E-cadherin in UACC-257 melanoma cells.

(E) Expression levels of E-cadherin and ZEB1 are inversely correlated in a panel of melanoma cell lines.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Constructs and cell lines

Lentiviral vector encoding BRAF^{V600E} (Denoyelle et al., 2006) and retroviral vector encoding a fusion protein consisting of Δ BRAF linked to the T1 form of the human estrogen receptor hormone-binding domain (Pritchard et al., 1995) was generously provided by Dr. Martin McMahon.

Murine HA-ZEB2, murine HA-ZEB1, human SNAIL2, human FLAG-TWIST1, and trans-species shRNA *TWIST1* in pBABE-Puro have been previously described (Ansieau et al., 2008; Morel et al., 2012). The HA-ZEB1 pCMV3.1TOPO expression vector was derived from the HA-ZEB1 pBABE-Puro retroviral construct. To analyze whether miR-200 regulates *ZEB1* and *ZEB2* in MM cells, we generated reporter constructs by cloning 3671-5125 bp and 3618-4935 bp 3'-untranslated sequences respectively of *ZEB1* and *ZEB2* genes (relative to ATG codons) into pMIR-REPORT vector (Invitrogen Carlsbad, CA, USA) vector. To determine *CDH1* promoter activity, we used luciferase reporter vectors containing either a wild-type E-cadherin promoter fragment (-178 - +92 bp) (WT-E-cad), or the same fragment with mutated E-boxes (mutant-E-cad) (Bolos et al., 2003).

Murine shRNA *Zeb1* in pLKO-1 (TRCN0000070819 = NM_011546.1-2785s1c1 and TRCN0000070821 = NM_011546.1-770s1c1) and shRNA *Fra1* (TRCN0000042687 = NM_010235.1-851s1c1 and TRCN0000042683 = NM_010235.1-664s1c1) were purchased from Sigma-Aldrich (St-Louis, MO, USA).

Melan-a cell line (kindly provided by Dorothy Bennett, St. George's Hospital Medical School, London, UK) and melan-a-derivatives were cultured in RPMI medium (Invitrogen) supplemented with 10% FBS (Cambrex), 100 U/ml penicillin-streptomycin (Invitrogen), 2 mM L glutamine (Invitrogen) and 100 nM PMA (Sigma-Aldrich). Δ BRAF-ER was activated by treatment with 50 nM 4-OH-Tamoxifen (Sigma-Aldrich). Primary adult normal human epidermal melanocyte cells (NHEMs) were purchased from Lonza GmbH (Cologne, Germany) and cultured in MBM4 medium supplemented with FBS, CaCl₂, rhFGF-B, PMA, rh-insulin, hydrocortisone, BPE, and endothelin3 (MGM4 bullet kit). WM-266-4, RPMI-7951, SK-MEL-5, and SK-MEL-28 melanoma cell lines were obtained from ATCC. IPC-298 and A375-derived cell lines were obtained from the German Collection of Microorganisms and Cell Cultures

(Leibniz Institut DSMZ, Braunschweig, Germany) and Wellcome Trust Functional Genomics Cell Bank (St. George's, University of London, UK) respectively. Cells were cultured according to the ATCC recommendation and treated with 10 μ M U0126, PD184352 (both purchased from Merck, Darmstadt, Germany), or 10 μ M PLX4720 (SeleckChem, Houston, TX, USA) or with DMSO for the indicated times. UM-UC-3 and RT112 carcinoma cells were cultured in DMEM medium supplemented with 10% FBS (GE Healthcare, Amersham, UK) and non-essential amino acids (Invitrogen).

Retroviral and lentiviral infection

4.10⁶ human embryonic kidney 293T cells were transfected with retroviral or lentiviral expression constructs (10 μ g) in combination with GAG-POL (5 μ g) and ENV expression vectors (10 μ g) using calcium phosphate. Viral stocks were collected 48 h post-transfection, filtered (0.45 μ m) and placed in contact of 2 x 10⁶ melanocytes or melanoma cells for 8 h in the presence of 8 μ g/ml polybrene. 48 h post-infection, cells were selected in presence of puromycin (1.5 μ g/ml), neomycin (100 μ g/ml) or hygromycin (25 μ g/ml) (all from Invitrogen).

siRNA

Transfections of siRNA were performed by electroporation with a single pulse of 250 V and 250 μ Fd by using the Gene Pulser Xcell electroporation system (Bio-Rad, Hercules, CA, USA).

List of siRNAs used for transient transfections:

Target name	siRNA sequence (sense strand)
<i>SNAI2</i>	GGACCACAGUGGCUCAGAA(UU)
<i>ZEB1</i>	GGACUCAAGACAUCUCAGUdTdG
<i>ZEB2</i>	GAACAGACAGGCUUACUUAAdTdT
<i>TWIST1</i>	ON-TARGET plus SMARTpool

siRNA were purchased from Ambion (Austin, TX, USA) or Dharmacon (Lafayette, CO, USA).

Soft-agar colony formation assay

Melan-a cells expressing Δ BRAF-ER or A375 MM cell lines were transduced with cDNA or shRNA retroviral or lentiviral expression vectors and selected with puromycin. Plates were prepared by coating with 0.75% low-melting agarose (Lonza) in growth medium and then overlaid with cell suspension in 0.45% low-melting agarose (2×10^4 cells/well for melan-a or 5×10^3 cells/wells for A375 cells). Melan-a cells were applied in a medium supplemented with 4-OHT (50 nM), PMA (100 nM) and puromycin (1.5 μ g/ml). Plates were incubated for 2-3 weeks at 37°C. Colonies were stained with crystal violet (1 mg/ml Sigma-Aldrich) and counted under microscope.

Transwell invasion assays

Melan-a/ Δ BRAF-ER cells infected with retroviral vectors expressing EMT-TFs, or MM cells with transiently depleted EMT-TFs with siRNAs (48 hours post-transfection) were seeded on matrigel-coated porous membranes (pore size, 8 μ m; BD Biosciences) in transwells. Cells were allowed to invade matrigel towards FBS gradients for 24 hours. Cells that did not migrate were removed using a cotton swab. The membranes were then fixed with methanol and stained with Gurr rapid staining kit (BDH). The number of invaded cells was determined by counting ten random fields using an inverted Nikon TE2000-U microscope.

Immunoblot analysis

Cells were washed twice with phosphate buffered saline (PBS) containing CaCl_2 and then lysed in a 100 mM NaCl, 1% NP40, 0.1% SDS, 50 mM Tris pH 8 RIPA buffer supplemented with a complete protease inhibitor cocktail (Roche, Mannheim, Germany) and phosphatase inhibitors (Sigma-Aldrich). Protein expression was examined by western blot using the anti-HA clone 11 (BabCO), anti-TWIST Twist2C1a (Abcam, Cambridge, MA, USA), anti-P-ERK1/2 (#9106, Cell Signaling

Technology, Danvers, MA, USA), anti-BRAF clone F-7 (sc-5284, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-MITF clone C5 (Thermo Scientific, Huntville, AL, USA), anti-E-cadherin clone 36 (BD Biosciences), anti-ZEB1 H102 (Santa Cruz), anti-ZEB2 (Sayan et al., 2009), anti-ERK1/2 #9102 (Cell Signaling), anti-FRA1 sc-605 (Santa Cruz), anti Phospho-FRA1 #3880 (Cell Signaling), anti-ER HC20 (Santa Cruz) rabbit polyclonal antibodies, and the anti-SNAIL2 G-18 (Santa Cruz) goat polyclonal antibody for primary detection. The anti-SNAIL1 antibody was a gift from Dr Antonio Garcia de Herreros. Loading was controlled using the anti- β -actin clone AC-15 (Sigma-Aldrich), anti-Ku80 clone 7/Ku80 (BD Biosciences) or anti- α -Tubulin (T5168, Sigma-Aldrich) antibodies. Horseradish peroxidase-conjugated rabbit anti-mouse, goat anti-rabbit and donkey anti-goat polyclonal antibodies (Dako, Glostrup, Denmark) were used as secondary antibodies. Western blots were revealed using an ECL detection kit (Amersham) or a western-blotting Luminol reagent (Santa Cruz).

Immunofluorescence analysis

For immunofluorescent staining, cells were transfected with a ZEB1-expressing vector HA-ZEB1 pCMV3.1TOPO or mock-transfected, cultured for 3 days on coverslips and stained according to standard protocols. Briefly, cells were fixed with 4% PFA for 10 min at room temperature, permeabilized with 0.1% Triton X-100 and blocked with 10% FCS in DMEM. Slides were consecutively incubated with the indicated primary and then secondary antibodies. After counter staining with DAPI (Molecular Probes), cells were examined and photographed using a fluorescent Nikon TE2000-U microscope.

Immunohistochemistry

Sections (4 μ m) of formalin-fixed paraffin embedded (FFPE) nevi, melanoma and metastatic melanoma were rehydrated, heated in TE (10 mM Tris, 1 mM EDTA buffer, pH 9.0, or citrate buffer) in a 750W microwave oven at full power for 20 min and allowed to cool down for 20-30 min at room temperature. Sections were incubated in protein blocking solution (Novocastra, Newcastle, UK) and incubated at 4°C overnight in the presence of a primary antibody. Primary antibodies were: rabbit

monoclonal antibodies to human SNAIL2 (clone C19G7 Cell Signaling); rabbit polyclonal antibodies to human ZEB1 sc-H-102 (Santa Cruz Biotech), ZEB2 (Sayan et al., 2009), and TWIST1 sc-R20 (Santa Cruz Biotech); murine monoclonal antibody to human SNAIL1 (Franci et al., 2006), phospho-ERK1/2 #9102 (Cell Signaling) and E-cadherin #610181, (BD Biosciences). Detection was performed using the NovolinkTM Polymer System, purchased from Novocastra. Staining was visualized in 3, 3'-diaminobenzidine (DAB) chromogen in Novolink DAB substrate buffer, for 5 min, counterstained in Mayer's Haematoxylin and mounted. Agarose-embedded cell pellets known to be positive or negative for EMT-TFs were used as controls.

Quantitative analysis of immunostaining

Positive staining was scored via the H-score (Kinsel et al., 1989), and three observers agreed the criteria for levels of positive staining. Four representative dermal fields were assessed; two from the superficial tumor close to the epidermal basal lamina in the papillary dermis and two from the deepest tumor sites within the reticular dermis, and a mean H-Score was calculated for both the superficial and deep sites. Superficial and deep sites of the metastatic lesions were also scored using the same criteria. Inter-observer agreement was measured using 12 randomly selected cases and an intra-class correlation coefficient (ICC) indicated high agreement (ICC = 0.92).

5'-RACE (Rapid amplification of cDNA ends)

ZEB2 and *ZEB1* transcription start sites were identified using RACE kit (Roche) according to the manufacturer's protocol. Total RNA was isolated from A375P cells treated or mock treated with 10 μ M U0126 for 24 h and used for the synthesis of the first strand cDNA using the SP2 primer complementary to the exon E2 of *ZEB2* or *ZEB1*. After purification, terminal deoxynucleotidyl transferase was used to add dA tail to 3'-end of cDNA. cDNA was amplified using the oligo-dT anchor primer and *ZEB2*- or *ZEB1*-specific primer pairs (*ZEB2*, TGTTGTGCCAGGGGTGTTCCAC and ACAATTCAGGAAAGATTTTTGTCATGG; *ZEB1*, GTCTGGTCTGTTGGCAGGTCATCC and TCCTCTGGTACACCTTCACAGTCAGC). Amplified DNA was analyzed in agarose gels. For *ZEB2*, DNA was gel-purified, cloned, and the sequence of 20 recombinant plasmid DNAs was determined.

Chromatin immunoprecipitation

Chromatin purification and precipitation was performed following manufacturer's protocols (Active Motif, Rixensart, Belgium). For each chromatin preparation, cells grown in a 70% confluent 15 cm plate were fixed with 1% formalin for 5 min at room temperature. Fixation was stopped by an excess of glycine; nuclei from collected cells were extracted using a Dounce homogenizer and precipitated by brief centrifugation. Chromatin was sheared using sonication with one quarter setting (25 seconds for 4 times on ice). Sheared chromatin was used for each precipitation or input control. Chromatin immunoprecipitation was performed using R-20 anti-FRA1 N-terminal antibody (sc-605, Santa-Cruz Biotech), anti-pospho-FRA1 peptide antibody (New England Biolabs) or negative or positive control antibodies included in the kit. After extensive washing of chromatin-protein G complex, the samples were eluted, reverse cross-linked and treated with Proteinase-K. Following inactivation of Proteinase-K, the sample was used for PCR amplifications using EMT-TF gene-specific or control primer pairs encompassing the AP-1 binding sequence elements. *GAPDH* control primers were from Active Motif).

List of primer pairs used for ChIP

<i>ZEB2</i> , AP-1	F, TGCACGTGAGAAAACCGTTTGGC R, GTGTTGTGACTCAGGAGGTGGGC
<i>ZEB1</i> , AP-1-1	F, ACTCATTCCGCTCTACTAAGGAGGC R, TGACCCGCGCAGCCCGGACTC
<i>ZEB1</i> , AP-1-2	F, CTCCTGGGAGGCTGTATTCTGAAG R, CAGTTCACCGTGGAACAAAGGAG
<i>SNAI2</i> , AP-1-1	F, TCCATCTGAAATTGTGGCAATTG R, GACATCTATTGGTCAGCTTCAGAAGAC
<i>SNAI2</i> , AP-1-2	F, GACGTCATAACATTCCCTTTCTCGC R, GCAATTGCCACAATTTAGATGGAC
<i>TWIST1</i> , AP-1	F, TGCAAACATGCCAAGTTTGCAG R, GGGACTACCTTCTTTGGGAATGC

Luciferase reporter assays

Cells were transiently transfected with luciferase reporter vectors along with the β -galactosidase expression vector, pCMV β -gal (Invitrogen). Forty-eight hours post-

transfection, cells were harvested, lysed; and the luciferase activity was measured with a Lumat LB9501 tube luminometer (Berthold). β -galactosidase activity was determined and used for the normalization of luciferase data.

Microarray processing and analysis

Δ BRAF-ER-melan-a cells expressing TWIST1, ZEB1, SNAIL2, or ZEB2 were treated with 4-OHT for 1 week before isolation of total RNA. Microarray processing and data analysis were performed on the ProfileXpert core facility (Bron, France). Total RNA (100 ng) was amplified and biotin-labeled using Kit GeneChip 3' IVT Express and procedures from Affymetrix (Santa Clara, CA, USA, <http://www.affymetrix.com>). Microarrays analyses were performed using high-density oligonucleotide arrays (Mouse Genome 430 2.0 Array, Affymetrix). Fifteen μ g of biotinylated cRNA were fragmented, and hybridization on chip was performed following Affymetrix protocol. Arrays were washed and stained with streptavidin-phycoerythrin using GeneChip Hybridization and Wash stain kit from Affymetrix in a Fluidics Station 450 according to the manufacturer's instructions. The arrays were scanned with a confocal laser (Genechip scanner 3000, Affymetrix).

CEL files were generated using the Affymetrix GeneChip Command Console (AGCC) software 3.0. The complete set of CEL files is available at the GEO database under accession number Geo39030. The obtained data were normalized with Affymetrix Expression Console software using Robust Multiarray Average (RMA) statistical algorithm. Data were analyzed using tools in Partek Genomic Suite 6.4 software (Partek Inc., St. Louis, MO, USA). The retained genes of interest were listed and classified according to their functions using Ingenuity Pathway Analysis and Ingenuity® iReport™ (Mountain View, CA, USA). Probeset intensities were summarized and normalized using RMA, and significant differential expression was determined by a moderated t-test (Limma) using a fold change cutoff of 2.

Transcriptional expression analysis

Total RNA was isolated using Trizol reagent (Sigma-Aldrich) and reverse transcribed using a high cDNA capacity reverse transcription kit (Invitrogen) following the manufacturer's instructions. Real-time PCR intron-spanning assays were designed using the ProbeFinder software (Roche). All reactions, including no-template controls

and RT controls were performed in triplicate on the 96-well StepOnePlus™ Fast Real Time PCR system (Applied Biosystems, Foster City, CA, USA). Human *HPRT1* or mouse *Rplp0* was used for normalization. List of primer pairs and probes used:

Gene	Species	Probe	Primers
<i>ZEB1</i>	human	57	Applied Biosystems (Hs00232783_m1)
<i>ZEB2</i>	human	68	Applied Biosystems (Hs00207691_m1)
<i>TWIST1</i>	human	88	Applied Biosystems (Hs01675818_s1)
<i>SNAI2</i>	human	86	Applied Biosystems (Hs00950344_m1)
<i>HPRT1</i>	human	73	F, TGACCTTGATTTATTTTGCATACC
			R, CGAGCAAGACGTTCAATCCT
<i>Zeb1</i>	mouse	57	F, GCCAGCGTCATGATGAAAA
			R, TATCACAATACGGGCAGGTG
<i>Bgn</i>	mouse	40	F, CACTTGGACAACAACAAGCTG
			R, TGATGTTGTTGGAGTGCAGATA
<i>Col5a1</i>	mouse	79	F,GGGCAGAAGGGAAGCAAG
			R, GGCCTTGAGGACCGGTAG
<i>Dct</i>	mouse	6	F, GGCTACAATTACGCCGTTG
			R, CACTGAGAGAGTTGTGGACCAA
<i>Fbn1</i>	mouse	40	F, CAGTGGACCGGGAATGAC
			R, TGGGCAAATATCAGGATCTAATG
<i>Grp143</i>	mouse	18	F, GTATACTTGGTGATCAGGAGATCG
			R, CCTCCACACAGAGCAGCAC
<i>Il6</i>	mouse	55	F, ACGGCCTTCCCTACTTCAC
			R, ACAGGTCTGTTGGGAGTGGT
<i>Kit</i>	mouse	15	F, GATCGCTCTGCGTCCTGTT
			R, CTTGCAGATGGCTGAGACG
<i>Lox</i>	mouse	48	F, CAGGCTGCACAATTCACC
			R, CAAACACCAGGTACGGCTTT

<i>Mitf-M</i>	mouse	27	F, CTAAGTGGTCTGCGGTGTCTC
			R, GGTTTTCCAGGTGGGTCTG
<i>Mmp13</i>	mouse	89	F, GCCAGAACTTCCCAACCAT
			R, TCAGAGCCCAGAATTTTCTCC
<i>Mmp1A</i>	mouse	94	F, TGTGTTTCACAACGGAGACC
			R, GCCCAAGTTGTAGTAGTTTTCCA
<i>Mmp3</i>	mouse	7	F, TTGTTCTTTGATGCAGTCAGC
			R, GATTTGCGCCAAAAGTGC
<i>Mreg</i>	mouse	6	F, TTCGTAATCAGCAGACCAAAGA
			R, CTGCCGCAGGGTGTAGATA
<i>Nid2</i>	mouse	10	F, ATGACCAGCACACTTGTATCTTG
			R, AGGTGTGACTGCCATCGAG
<i>Silver</i>	mouse	76	F, AGCTTCCTTCCCGTGCTT
			R, CACCAAGCCAGTCCTGATTC
<i>Slc45a2</i>	mouse	29	F, GAGATGCGGTCGTATCAGC
			R, TGATGCTTATGGCCCAGAT
<i>Sparc</i>	mouse	73	F, GCCCCTCAGCAGACTGAA
			R, GTCTCCTCCACCACGGTTT
<i>Tagln</i>	mouse	13	F, CCTTCCAGTCCACAAACGAC
			R, GTAGGATGGACCCTTGTTGG
<i>Thbs1</i>	mouse	22	F, CACCTCTCCGGGTTACTGAG
			R, GCAACAGGAACAGGACACCTA
<i>Trp1</i>	mouse	67	F, GGTGCGGGATGTAAAGAAGA
			R, TGTAGTTGCAGCGGTAGGC
<i>Tyr</i>	mouse	26	F, TCGTCACCCTGAAAATCCTAA
			R, CTGATCTGCTACAAATGATCTGC
<i>Tyrp1</i>	mouse	104	F, ATGGATATGGGTGCACGAG
			R, CCTTGTAAGTGAAGCCCTCCT
<i>Rplpo</i>	mouse	9	F, ACTGGTCTAGGACCCGAGAAG

			R, TCCCACCTTGTCTCCAGTCT
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To analyze the expression of miRs, quantitative real-time PCR Taqman® MicroRNA assay (Applied Biosystems) was used. MiR-132, miR-345 and miR-191 were identified as the most appropriate endogenous control genes for miR expression. Selection of stable endogenous control genes was carried out using geNorm algorithm-based software.

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