Supplemental Information

Materials and Methods

Immunoblotting. Cells were solubilized in M-PER® mammalian protein extraction reagent (Pierce Biotechnology, Rockford, IL, USA) containing Protease Inhibitor cocktail (Roche, Mannheim, Germany). Protein concentrations of extracts were measured using the BCA protein assay kit (Pierce). Cell extracts (30 μg/lane) were separated on SDS polyacrylamide gels (Invitrogen). After electrophoresis, proteins were transferred electrophoretically from the gels to Invitrolon PVDF transfer membranes (Invitrogen). The filters were incubated with antibodies to SOX9 (rabbit 1 μg/ml; Chemicon, Temecula, CA, USA), SOX9 ser181 (rabbit 1:500, Abcam, Cambridge, UK), PRAME (rabbit 1 μg/ml; Abcam), p21 (mouse 1:2,000, Cell Signaling, Danvers, MA, USA), MITF (mouse 1:8,000, a generous gift from Dr. Heinz Arnheiter at NIMH), β-actin (mouse, 1:20,000, Abcam) or GAPDH (rabbit 1:10,000, Santa Cruz) for 1 h at 23°C to overnight at 4ºC (depending on the antibody) and were then incubated with appropriate horseradish peroxidase-linked anti-rabbit, anti-mouse or anti-goat whole antibodies (at 1:10,000, GE Healthcare, Buckinghamshire, UK) at room temperature for 1 h. Antigens were detected using ECL-plus Western Blotting Detection System (GE Healthcare). Each experiment was performed at least in triplicate.

Immunocytochemical staining. Melanoma cell were cultured in two well Lab-Tek chamber slides (Nalge Nune International Corp., Naperville, IL, USA) were processed for indirect fluorescence to detect the expression of proteins using primary antibodies to SOX9 (rabbit 1:50, Abcam), MITF (mouse 1:500, generous gift from Dr. Heinz Arnheiter), and p21 (mouse 1:50, DAKO, Glostrup, Denmark). Bound antibodies were visualized with appropriate secondary antibodies, Alexa Fluor® 488 goat anti-rabbit IgG (H+L), Alexa Fluor® 594 mouse anti-rabbit IgG (H+L), Alexa Fluor® 488 goat anti-mouse IgG (H+L) or Alexa Fluor® 594 goat anti-mouse IgG (H+L) (all from Molecular Probes, Eugene, OR, USA) at 37ºC for 30 min at 1: 500 dilution with 5% goat serum. DAPI (Vector, Burlingame, CA; USA) was used as a counter-stain. The green fluorescence produced by Alexa 488®, red produced by Alexa 594®, and blue by DAPI

was observed and captured using a Leica DMR B/D MLD fluorescence microscope (Leica, Wetzlar, Germany) and a Dage-MTI 3CCD 3-chip color video camera (Dage-MTI, Michigan City, IN, USA).

Immunohistochemistry. Melanoma tissue arrays were obtained from BioChain Institute, Inc (Hayward, CA, USA) (lot No: A902061) and from US Biomax (lot # ME1001). Skin specimens obtained from the backs of healthy volunteers were taken after informed consent. The expression of proteins of interest was detected by indirect immunofluorescence using the following as primary antibodies: SOX9 (rabbit 1:50, Abcam), p21 (mouse 1/50; DAKO), S100 (rabbit 1/100; Abcam), MITF (mouse 1:50, generous gift from Dr. Heinz Arnheiter) and MART1 Ab-3 (mouse 1:100; NeoMarkers). Bound antibodies were visualized with appropriate secondary antibodies and fluorescence was observed and analyzed using a fluorescence microscope as detailed above.

Metabolic Labeling. Radioactive ^{35}S metabolic labeling and immunoprecipitation was performed as described previously (1). Where indicated, PGD2 was added from the "pulse" period to the "chase" period. Cell extracts were incubated with normal rabbit serum (Vector) and were then incubated with protein G beads (GE Healthcare). The supernatants were incubated with PRAME (Abcam) or GAPDH (Santa-Cruz) antibodies. The immune-complexes were separated by incubation with beads and were further washed with immunoprecipitation lysis buffer. The pellets were eluted, electrophoresed and visualized by autoradiography.

RT-PCR- For RT-PCR, total cytoplasmic RNA was isolated from cultured cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the supplier's instructions. RT was performed using 500 ng cytoplasmic RNA using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). After denaturation at 95°C for 2 min, PCR was performed for 33 cycles (1 min at 95ºC, 45 s at 63ºC, 45 s at 72ºC) using platinum Taq polymerase (Invitrogen). Transcript levels were compared relative to the GAPDH housekeeping gene using the following primers: P21: Forward (fw): gccccaggcgcagtttgctc 3', reverse (rv) aggggacgggtcctggcaca; PRAME: fw: ctcagcaccgctc

cgggaca, rv: caccccgccccgcaagtcta; GAPDH: fw: accacagtccatgccatcac, rv: ccaccaccctgttgctgta.

Chromatin immunoprecipitation. ChIP assays were performed as described previously (2) using 4 μg of SOX9 antibody (Chemicon) or 4 μg of non-specific IgG (Invitrogen). The DNA recovered was subjected to amplification by PCR before analysis using agarose gel electrophoresis. The primers used for PCR were the human p21 promoter region (5'-TGATGTGCCACAGTTCACAA -3' and 5'- TCCTGCCAGTTTTCCTGTTC -3') and the HSP70 promoter (5'-CCTCCAGTGAATCCCAGAAGACTCT-3' and 5' -TGGGACAACGGGAGTCACTCTC-3').

1. Yasumoto, K., et al. 2004. Epitope mapping of the melanosomal matrix protein gp100 (PMEL17): rapid processing in the endoplasmic reticulum and glycosylation in the early Golgi compartment. *J. Biol. Chem.* **279**:28330–28338.
2. Passeron, T., et al. 2007. SOX9 is a key player in ultraviolet B-induced melanocyte differentiation and pigmentation. *Proc. Natl. Acad. Sci. U. S. A.* **104**:13984–13989.

Supplementary Figure Legends

Suppl. Figure 1

Expression of SOX9, MART1 and p21 in normal skin and in melanomas in vivo. (a) Immunohistochemistry of paraffin-embedded sections stained with SOX9 antibody (red), p21 antibody (green) and DAPI (blue). Colocalization of the red and green signals is shown in yellow on the right. **(b)** Immunohistochemistry of unexposed and UVB-exposed normal human skin. Samples were stained with SOX9 antibody (red), MART1 antibody (green) and DAPI (blue). Colocalization of the red and green signals is shown in yellow in the merged images on the right.

Suppl. Figure 2

PGD2 decreases the expression of PRAME in A375 cells. A375 cells were treated for 72 h with PGD2 and compared to untreated controls. Proteins were extracted and were analyzed using immunoblotting with PRAME and β-actin antibodies.

Suppl. Figure 3

RA increases the expression of SOX9 in B16 but not in A375. B16/F10 and A375 cells, known to be respectively sensitive and resistant to RA, were treated for 72 h with RA at 10^{-7} M and compared to untreated controls. Proteins were extracted and were analyzed using immunoblotting with SOX9 and GAPDH antibodies.

Suppl. Figure 4

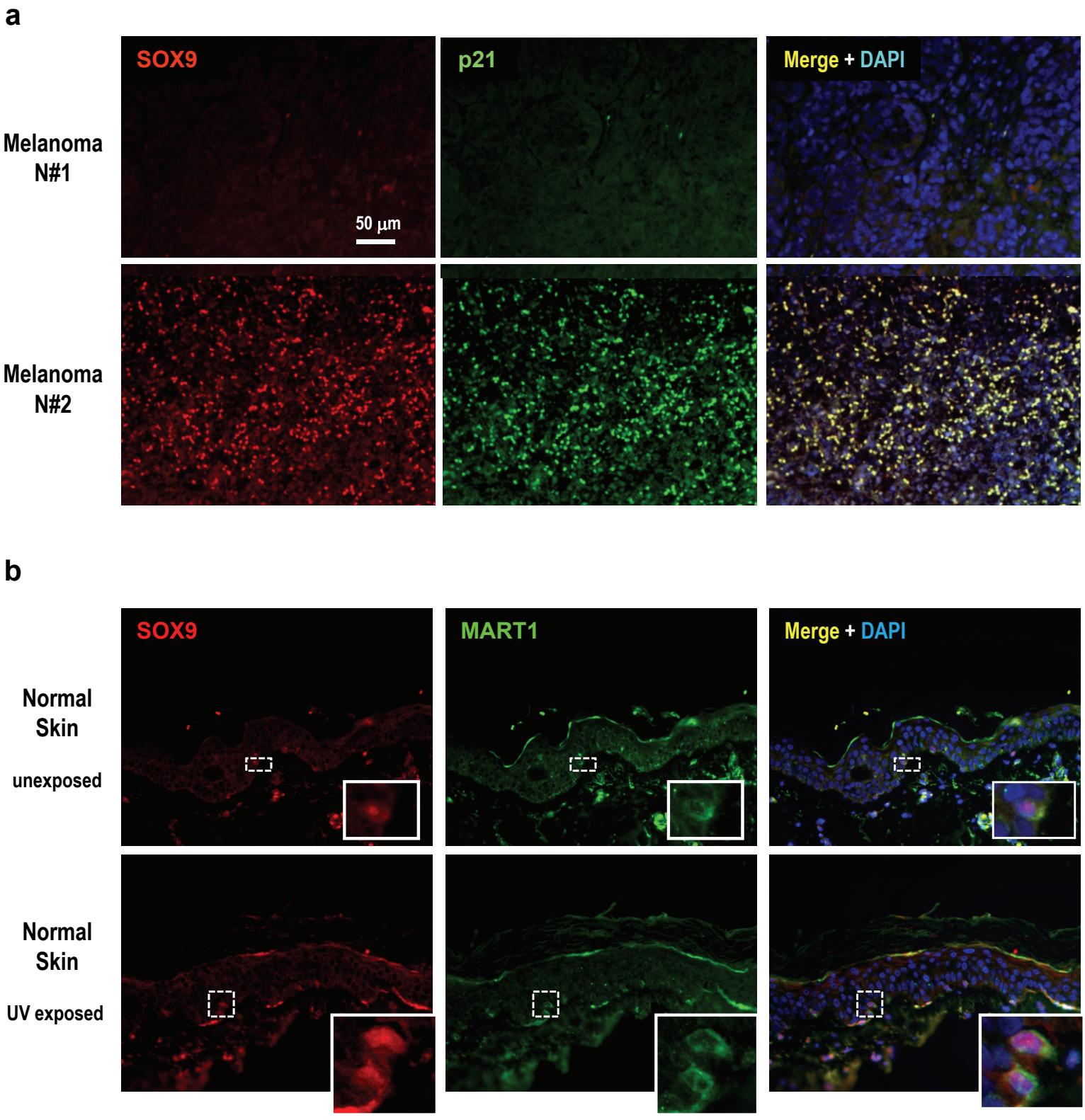
SOX9 over-expression restores MITF expression in A375 melanoma cells in ex vivo model. A375 melanoma cells transduced with SOX9 lentivirus or parental cells as a control were included in a human reconstructed skin model. Immunostaining of the samples was performed with MITF antibody (red), SOX9 antibody (green) and DAPI (blue). Dashed squares focus on melanoma tumors which are shown in magnified insets.

Suppl. Figure 5

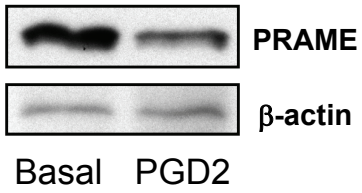
Increased expression of SOX9 and MITF after BW245C with or without RA treatment in melanoma tumors in vivo. Immunohistochemistry of A375 melanoma

cells after injection subcutaneously into nude mice. After 8 d, mice were treated intraperitoneally with BW245C and/or RA 3 times a week for 3 weeks. After 3 weeks, immunostaining of the tumor tissues was performed with SOX9 (red) and MITF antibodies (green), and DAPI (blue). Colocalization of the red and green signals is shown in yellow in the merged images on the right.

Suppl. Figure 1



Suppl. Figure 2



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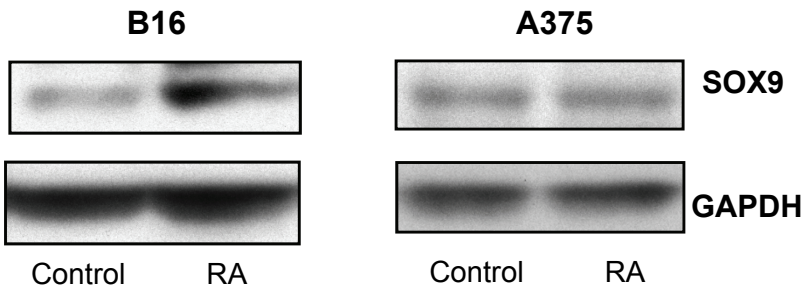
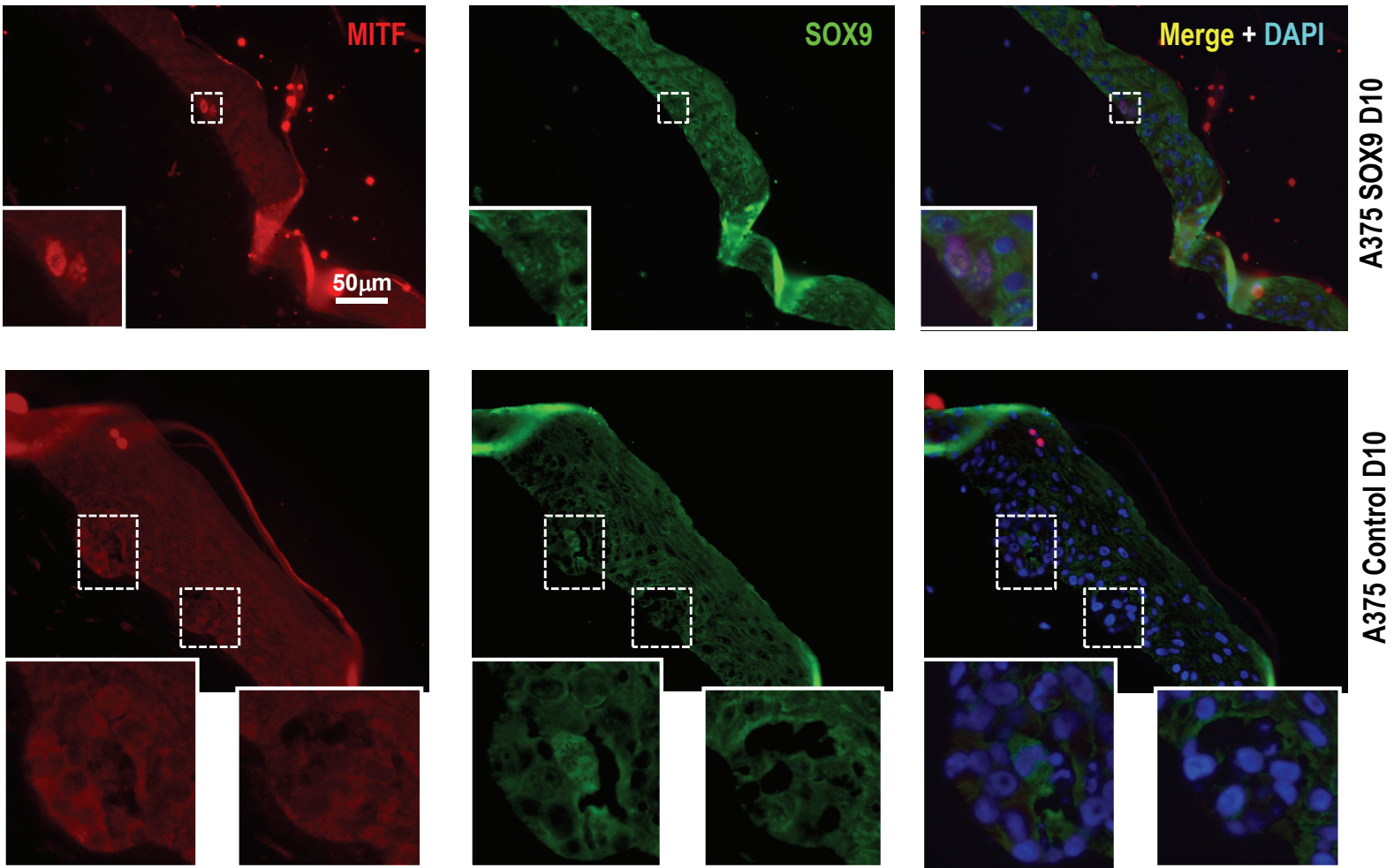


Suppl. Figure 4



Suppl. Figure 5